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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIVISION DE NUEVAS CREACIONES
E. S. D.

Re: Expediente No. **09.111.636**
Solicitud de patente a nombre de **CATALYST BIOSCIENCES, INC.**

Yo, SILVANA MARIA LOPEZ DÍAZ, actuando como apoderada sustituta de acuerdo con la sustitución presentada el 30 de agosto de 2013 e identificada como aparece al pie de mi firma, domiciliada en la carrera 11 A No. 86-53, piso 6, del solicitante de **CATALYST BIOSCIENCES, INC.**, de la patente de la referencia, *interpongo recurso de reposición* contra la resolución **No. 62493** emitida el 25 de octubre de 2013 por el Superintendente de Industria y Comercio, por medio de la cual se niega el privilegio de patente para la solicitud contenida en el expediente de la referencia.

1. FUNDAMENTOS DEL RECURSO

Con fundamento en la facultad que confiere el artículo 34 de la Decisión 486 al solicitante para modificar la solicitud de patente en cualquier momento del trámite, presento junto con este memorial un nuevo capítulo reivindicatorio, el cual consta de 31 reivindicaciones y reemplaza completamente el capítulo presentado anteriormente.

En cuanto a estas modificaciones, me permito decir que las mismas no amplían de ninguna manera la materia objeto de la presente solicitud y se realizaron con el ánimo de superar las objeciones planteadas por el examinador en su concepto técnico.

Así las cosas, cabe aclarar que el nuevo capítulo reivindicatorio que consta de 31 reivindicaciones y la materia contenida en dichas reivindicaciones corresponde a **la misma materia de las reivindicaciones allegadas junto con el memorial de respuesta al oficio No. 6804** notificado por fijación en lista el 23 de abril de 2013, con la diferencia que se han cancelados las antiguas reivindicaciones 4 y 12.

En relación con los argumentos presentados por ese despacho en la resolución impugnada **No. 62493** emitida el 25 de octubre de 2013, es necesario señalar que carecen de un enfoque adecuado de conformidad con las normas, jurisprudencia y reglas técnicas que existen para evaluar el nivel inventivo de la invención.

1.1 La indebida interpretación del artículo 36 de la Decisión 486/00.

Al respecto, el solicitante quisiera anotar que el Artículo 36 de la Decisión 486 establece lo siguiente:

"*Artículo 36.- El solicitante* ***podrá, en cualquier momento del trámite****, dividir su solicitud en dos o más fraccionarias, pero* ***ninguna de éstas podrá implicar una ampliación de la protección que corresponda a la divulgación contenida en la solicitud inicial****.*

La oficina nacional competente podrá, en cualquier momento del trámite, requerir al solicitante que divida la solicitud si ella no cumpliera con el requisito de unidad de invención.

Cada solicitud fraccionaria se beneficiará de la fecha de presentación y, en su caso, de la fecha de prioridad de la solicitud inicial (...)." (Subraya y negrilla fuera del texto).

Como se puede observar, la única restricción que, de acuerdo a la norma aplicada en Colombia tienen las solicitudes divisionales, es que no pueden implicar una ampliación de la protección correspondiente a la divulgación contenida en la solicitud inicial. En este sentido, cabe señalar que en ninguna parte del artículo 36 de la citada norma se establece que contener la misma materia que la solicitud inicial sea una causa válida de negación o rechazo de una solicitud divisional, sino que se determina que lo que no se permite es que se trate de una **ampliación** de la materia con respecto a aquella contenida en la solicitud inicial. En otras palabras, que contenta la misma materia a que contenga una ampliación de la misma son dos conceptos absolutamente distintos, y que en ningún momento deben ser confundidos, causando en consecuencia un detrimento a mi cliente, ya que en este caso no se trata de una ampliación de la materia.

El examinador indica que la solicitud divisional N ° 13.225.455, se rechaza según ese despacho las pretensiones de la solicitud divisional coinciden con las reivindicaciones de la presente solicitud.

Teniendo en cuenta lo establecido en el artículo 36, el solicitante debe tener la oportunidad de continuar con el examen de patentabilidad de la solicitud divisional. Tal como se indicó en el recurso presentado el 25 de noviembre de 2013 en contra de la resolución No. 60085 del día 17 de octubre de 2013 para la solicitud divisional N ° 13.225.455, dicha divisional se presentó porque había una aparente falta de unidad de invención.

En aras de superar cualquier posible superposición entre la materia cubierta por las reivindicaciones de la solicitud divisional y las reivindicaciones de la solicitud original, se han cancelado las antiguas reivindicaciones 4 y 12.

Así las cosas, consideramos que la resolución impugnada incumple lo establecido en el artículo 36 de la Decisión 486 de la Comunidad Andina y como se manifestó en el recurso de reposición para dicha solicitud divisional, se debe aceptar la misma y proceder a realizar el estudio técnico correspondiente.

Sumado a lo anterior, es necesario hacer énfasis sobre el hecho que en la presente solicitud, se modificó de tal manera el capítulo reivindicatorio en aras de que todas las reivindicaciones pendientes dependen de una característica técnica común de un polipéptido FVII modificado que incluye una sustitución de aminoácidos seleccionada de entre D196L , D196I , K197E , K197D , K197L , K197M , K197I , K197V , K197Y , K197F , K197W , K199D y K199E , de tal modo que cuando el polipéptido FVII modificado es activado muestra una actividad coagulante dependiente de TF

En clara contraposición, la solicitud divisional cubre polipéptidos FVII 'que comprenden un dominio Gla heterólogo. Por lo tanto, es claro que ninguna de las reivindicaciones de la solicitud divisional recita un reemplazo de aminoácidos seleccionado de entre D196L , D196I , K197E , K197D , K197L , K197M , K197I , K197V , K197Y , K197F , K197W , K199D y K199E y más aún con la cancelación de la reivindicación 12 en la presente solicitud, ninguna de las reivindicaciones hace referencia a un polipéptido FVII que contenga un dominio Gla heterólogo.

De hecho, es importante señalar que las reivindicaciones de la solicitud divisional comparte el concepto inventivo de un polipéptido FVII que comprende un dominio Gla heterólogo, lo cual es un concepto **completamente diferente** de aquel de las reivindicaciones de la presente invención.

Así las cosas, respetuosamente reiteramos que no existe ninguna superposición entre las pretensiones de la presente solicitud y las reivindicaciones de la solicitud divisional.

En consecuencia, teniendo en cuenta los anteriores argumentos, es claro que las reivindicaciones tanto de la solicitud divisional y las de la presente invención cumplen a cabalidad con los requisitos de patentabilidad según la Decisión 486/00, es decir, novedad, nivel inventivo y aplicabilidad industrial.

1.2 La indebida interpretación de los artículos 14 y 18 de la Decisión 486/00.

El señor examinador ha indicado en la resolución impugnada que las reivindicaciones 1-33 siguen siendo rechazados por carecer de actividad inventiva sobre la publicación de patente de EE.UU. N ° US20030096338 (D2)

y en vista de la publicación de patente internacional WO 2004083361 (D3).

En este sentido, el examinador alega que D3 describe polipéptidos FVII modificados con una modificación de aminoácidos en la posición 196 que exhiben una mayor actividad. Además, el examinador alega que el experto en la materia combinaría modificaciones descritas en D2 y D3 con una expectativa razonable de obtener un polipéptido FVII modificado que incluyera una sustitución de aminoácidos seleccionada de entre D196L , D196I , K197E , K197D , K197L , K197M , K197I , K197V , K197Y , K197F , K197W , K199D y K199E y que cuando se active, presente una mayor actividad coagulante dependiente de TF en comparación con el polipéptido FVII no modificado.

En este sentido, es importante argumentar que el señor examinador no indica de qué manera una persona medianamente versada en la materia técnica se vería motivado a hacer específicamente los cambios propuestos en los aminoácidos específicos con alguna expectativa de éxito en el incremento de la actividad coagulante dependiente de TF. En este sentido respetuosamente se solicita que sea retirada la objeción en cuanto a la reivindicación 2.

En efecto, la reivindicación 2 establece que "la modificación de un polipéptido FVII corresponde a la sustitución seleccionada entre K197E , K197D , K197L , K197M , K197I , K197V , K197F , K197W , K199D , y K199E. " Como se puede ver, la reivindicación 2 sólo indica sustituciones de aminoácidos en la posición **197** o **199** y como bien lo ha aceptado el señor examinador en la página 6 tercer párrafo de la resolución impugnada, D3 no enseña o sugiere una modificación en la posición 197 o 199. Por lo tanto, en cuanto a la objeción relacionada con la reivindicación 2, carece de todo sustento técnico dado que dicha anterioridad no motivaría a dicha persona para hacer modificaciones en un punto tan específico como lo es la posición 197 ó 199.

Además, tal como se describe a continuación, con respecto a la reivindicación 1 y las reivindicaciones dependientes, ninguna de las referencias citadas, solas o en combinación, enseñan o sugieren las modificaciones de aminoácidos en particular reivindicados, ni los resultados obtenidos de ese modo.

En efecto, la reivindicación 1 establece:
Un polipéptido factor VII (FVII) modificado que comprende modificaciones en un polipéptido FVII, variantes alélicas y especies variantes de la misma o fragmentos activos de los mismos, en donde la modificación es una sustitución de aminoácido en una posición correspondiente a una o más de las posiciones D196 , K197 y K199 en un polipéptido de FVII que tiene una secuencia de aminoácidos expuesta en la SEC ID Nº: 3 o en los residuos de un polipéptido de FVII correspondiente;
el polipéptido de FVII no modificado comprende una secuencia de aminoácidos

expuesta en cualquiera de las SEC ID NOS : 1-3 y 18-74;
posiciones correspondientes están determinadas por la alineación con el polipéptido de SEQ ID NO : 3 ; y la modificación corresponde a una sustitución seleccionada entre D196L , D196I , K197E , K197D , K197L , K197M , K197I , K197V , K197Y , K197F , K197W , K199D y K199E , por el que el polipéptido FVII modificado, cuando se activa, muestra actividad coagulante dependiente de TF comparada con el polipéptido FVII no modificado.

Las reivindicaciones 2-31 dependen de la reivindicación 1. Las reivindicaciones 2-22 cubren particularidades del polipéptido. Por ejemplo, la reivindicación 2 establece que la modificación de un polipéptido FVII corresponde a la sustitución seleccionada entre K197E , K197D , K197L , K197M , K197I , K197V , K197F , K197W , K199D , y K199E.

Las reivindicaciones 23 y 24, cubren una molécula de ácido nucléico que codifica el polipéptido y un vector que incluye la molécula de ácido nucléico, respectivamente. Las reivindicaciones 25 y 26, protegen la célula modificada que incluye el vector. Las reivindicaciones 27-31 reclaman una composición farmacéutica que incluye el polipéptido, el ácido nucleico, el vector o la célula.

Teniendo en cuenta esto, ahora es importante señalar qué es lo que cada una de las anterioridades enseña para poder evidenciar que ninguna de ellas podría llevar de manera evidente a la persona medianamente versada en la técnica a la materia contenida en las reivindicaciones.

En efecto, como se logró establecer en la respuesta presentada al oficio no. 6804, D2 describe una matriz de variantes de FVII. De hecho, D2 enseña la modificación de FVII mediante la **introducción** y/o **eliminación** de aminoácidos que incluyen un grupo de unión para un parte o porción no polipéptidica, la introducción de un sitio de glicosilación *in vivo* de origen no natural en el FVII, o la **eliminación** de un sitio de glicosilación mediante la sustitución de uno o más aminoácidos (véase, por ejemplo, la página 10, párrafo [0081]).

En ese orden de ideas, es necesario señalar que D2 describe cientos de modificaciones de aminoácidos, pero no conduce de manera evidente a un experto en la técnica a las mutaciones reivindicadas actualmente. D2, en efecto, sólo establece que las posiciones citadas se pueden mutar, pero no selecciona o identifica las mutaciones reclamadas ni describe los resutlados alcanzados con dichas mutaciones. Es claro entonces que, D2 se refiere a la modificación del polipéptido para **insertar** o **eliminar sitios de unión**, tales como sitios de glicosilación. Por lo tanto, las mutaciones particulares descritas en las posiciones 196, 197 y 199 están diseñadas para crear un sitio de glicosilación, que tiene la

secuencia canónica NX (C/S/T), donde X **no es prolina**. Así las cosas, las mutaciones introducen un S o T en una posición, si no están ya presentes, y una N en la otra. En particular, las únicas mutaciones específicas descritas en las posiciones 196 , 197 y 199 son las siguientes modificaciones que introducen un sitio de glicosilación : 1) D196N (en combinación con I198S/T); 2) K197N (en combinación con F195N); 3) K199N (en combinación con W201S/T); 4) K197N/K199S, y 5) K197N/K199T (véase, por ejemplo , la página 12, párrafo [0129]; página 13, párrafo [0135], y la reivindicación 47). Aún más, D2 enseña que cada una de estas mutaciones crea un sitio de N-glicosilación para la unión de una parte de azúcar.

Por consiguiente, D2 no enseña ni sugiere que cualquiera de sus modificaciones aumente la actividad o de la resistencia a los inhibidores, ni habría que esperar dicha actividad, **ya que los residuos modificados crean una glicosilación**. Ninguno de los reemplazos reivindicados en la presente invención a saber: D196L , D196I , K197E , K197D , K197L , K197M , K197I , K197V , K197Y , K197F , K197W , K199D y K199E , crean un sitio de glicosilación. Por lo tanto, la selección de los residuos actualmente reivindicados no puede ser sugerido por la referencia D2, **toda vez que las sustituciones reivindicadas no dan lugar a la creación de un sitio de glicosilación**.

Así las cosas, es claro entonces que D2 no enseña o sugiere la selección de posición 196, 197 o 199 para la modificación tal como se reivindica, ni los mutantes de combinación de las reivindicaciones dependientes. Adicionalmente, D2 no enseña las modificaciones particulares en estos sitios ni otros de los sitios reclamados que incrementan la actividad catalítica o la resistencia a inhibidores, particularmente TFPI. Como se muestra en la solicitud, y como se discute y se reproduce a continuación, las mutaciones D196L , D196I , K197E , K197D , K197L , K197M , K197I , K197V , K197Y , K197F , K197W , K199D y K199E aumentan la resistencia TPFI y/o incrementan la actividad coagulante.

Por tanto, debe quedar claro que D2 no enseña ni sugiere tales características técnicas y tales deficiencias en D2 no pueden ser cubiertas o complementadas por lo expuesto en D3 como se menciona a continuación.

En efecto, D3 enseña variantes de FVII que tienen una modificación en una o más de las posiciones de aminoácidos 196, 237 y 341. Como se señaló anteriormente, y como el mismo examinador notó, D3 no enseña nada acerca de las posiciones 197 y 199. D3 no es de relevancia para las variantes de FVII con mutaciones en las posiciones
197 y 199.

En particular, la anterioridad D3 enseña que las modificaciones se seleccionan de entre D196N , D196K , G237L , G237GXX , G237GXXX , G237GXXXX ,

K341Q y K341N, donde X es **cualquier** aminoácido. Por lo tanto, D3 no enseña cualquiera de las variantes de FVII específicas expuestas en la reivindicación 1, ni ninguna de las variantes del FVII cubiertas por en cualquiera de las reivindicaciones dependientes de la misma. Es más, D3 no enseña ni sugiere un polipéptido de FVII con una modificación seleccionada de entre D196L o D196I ni ninguna de las modificaciones en 197 y 199 como se reivindica.

Sumado a lo anterior, también resulta pertinente señalar que D3 no enseña que las sustituciones de aminoácidos en particular reivindicadas resulten en un polipéptido de FVII que cuando sea activado, presente una mayor resistencia al inhibidor de la ruta del factor tisular (TFPI) y/o aumente la actividad coagulante en comparación con el polipéptido de FVII no modificado.
Así las cosas, no hay ninguna descripción en D3 de cualquier modificación de las posiciones 197 y 199, incluyendo cualquiera que diera lugar a un polipéptido del factor VII con una mayor resistencia a los inhibidores, en particular el TFPI. La única mención del residuo K199 es con respecto al porcentaje de cadena lateral expuesta como así lo puede corroborar el señor examinador en el Ejemplo 1 de D3.

En efecto, ese despacho puede corroborar que K197 está incluido en una lista y también se incluye en una lista de residuos dentro de 10 átomos de la tríada catalítica. Por lo tanto, sólo estos residuos se mencionan en largas listas en el Ejemplo 1, que describe la estructura de FVII, y **no** la modificación de FVII. Por consiguiente, no hay ninguna indicación o sugerencia de sustituir cualquiera K197 o K199, ni los resultados de dicha sustitución.

Con respecto a la posición del aminoácido correspondiente a 196 de FVII, D3 describe reemplazos D196N, los cuales introducen **un sitio de glicosilación** y D196K al igual que en D2. Por consiguiente, la anterioridad D3 **sólo enseña que estas mutaciones disminuyen el tiempo de coagulación** sin datos que muestren que ellos exhiben actividad coagulante incrementada ni resistencia a TFPI. Así las cosas, es claro que no sólo no existen mutaciones dentro del alcance de la presente reivindicación 1 ni mutaciones en D3 que incrementen la actividad coagulante dependiente de TF.

Es más, la actual reivindicación 1 cubre reemplazos particulares en 196; estos reemplazos no incluyen ni 196N ni 196K . Como se muestra en la presente solicitud (véase, por ejemplo, el cuadro 16, reproducido a continuación (el subrayado es nuestro)) los reemplazos reclamados D196L y D196I confieren aumento de la actividad catalítica TF- dependiente; **D196K no lo hace**. Por lo tanto, **no se puede concluir que el tiempo de coagulación disminuido reportado observado para D196K y D196N en D3 se derive del aumento de la actividad catalítica TF- dependiente**.

Mutación (Numeración de FVII maduro)	Mutación (Numeración Quimotripsina)	k_{cat}/K_M ($M^{-1}s^{-1}$)	k_{cat}/K_M (% WT)
WT	WT	3.42×10^7	100
D196K	D60K	**1.10×10^7**	**23**
D196R	D60R	2.25×10^7	46
D196L	D60L	**8.57×10^7**	**176**
D196I	D60I	**7.99×10^7**	**164**

El examinador indica que D3 describe que un polipéptido FVII modificado que incluye modificación de aminoácidos D196K muestra actividad incrementada. Sobre este particular, es necesario hacer énfasis sobre el hecho que D196K **no está incluido** en la lista de modificaciones de aminoácidos cubiertos por la reivindicación 1, ni ninguna otra reivindicación. Como se muestra en los datos anteriores, D196K no aumenta la actividad catalítica de FVII; mientras que D196L y D196I aumentan más de 2 veces. D196K no presenta aumento de la actividad catalítica dependiente de TF, ni está dentro del alcance de las reivindicaciones actualmente presentadas.

Como se describió anteriormente , la reivindicación 1 indica que el polipéptido FVII incluye una sustitución de aminoácidos seleccionada entre (el subrayado es nuestro): **D196L**, **D196I** , K197E , K197D , K197L , K197M , K197I , K197V , K197Y , K197F , K197W , K199D y K199E , de tal suerte que el polipéptido FVII modificado, cuando se activa, exhibe mayor actividad coagulante dependiente de TF en comparación con el polipéptido FVII no modificado.
Por lo tanto, es claro que D3 no enseña ni sugiere que una modificación de aminoácidos seleccionada de entre **D196L** y **D196I** aumente la actividad coagulante dependiente de TF. Es más, la referencia D3 no enseña o sugiere que reemplazando la modificación D196K con D196L o D196I aumentaría la actividad dependiente de TF. En contraste, la solicitud describe y proporciona ejemplos de trabajo que demuestran que cada uno de las modificaciones de aminoácidos expuestas en la reivindicación 1 aumenta la actividad coagulante dependiente de TF.

En efecto, los Ejemplos de trabajo en la memoria descriptiva demuestran tales efectos cuando las modificaciones de aminoácidos indicadas en la reivindicación 1 se combinan con otras modificaciones de aminoácidos, incluyendo modificaciones de aminoácidos establecidas en las reivindicaciones dependientes.

Además, la presente solicitud muestra que la sustitución de aminoácido D196K descrita en D3 **disminuye la actividad dependiente de TF y disminuye la resistencia del TFPI** como así se indica en, por ejemplo, las Tablas 13, 16 y 21, la entrada CB554). Estos resultados no son ni enseñados ni sugeridos por D3 el

cual únicamente, describe sólo que la sustitución de aminoácido D196K disminuye el tiempo de coagulación. D3 no describe ningún resultado que muestre que una modificación de aminoácidos en la posición 196, incluyendo D196K, aumente la actividad dependiente de TF. Así las cosas la presente solicitud es la única que describe modificaciones de aminoácidos en la posición D196 aumenta la actividad dependiente de TF, y la presente solicitud describe que la modificación de aminoácidos **D196L** y **D196I**, pero no D196K, aumentan la actividad dependiente de TF (ver, por ejemplo, la Tabla A a continuación). Como se describió anteriormente, la presente solicitud describe los resultados que muestran que la modificación de aminoácidos D196K **disminuye** la actividad catalítica dependiente de TF y disminuye la resistencia de TFPI (véase, por ejemplo, la Tabla 16, reproducida en la parte de arriba, y la Tabla 21, reproducida en la parte de abajo).

ID	Mutación (numeración de FVII maduro)	Mutación (numeración de quimotripsina)	TFPI resistencia a doblez
CB553	wt	wt	1.0
CB554	**D196K**	D60K	0.6

En este orden de ideas, resulta importante hacer énfasis sobre el hecho que la anterioridad D3 **no describe la actividad de la medición de la variante D196K en un ensayo dependiente de TF**. La presente solicitud muestra que la D196K no incrementa la resistencia al TFPI ni la actividad catalítica dependiente de TF.

Análisis – La Combinación de las enseñanzas de D2 y D3 y las diferencias con las reivindicaciones

Luego de tener claras cuáles son las enseñanzas de los dos documentos citados por ese despacho, y como se describió anteriormente, la anterioridad D2 suministra una **lista de cientos** de modificaciones de aminoácidos. De hecho, D2 **no enseña ninguna de las modificaciones en 196, 197 o 199 de un polipéptido FVII que incremente la actividad catalítica o la resistencia a inhibidores, particularmente el TFPI**.

Es más, las únicas mutaciones en cualquiera entre 196, 197 y 199 descritas en D2 son D196N, K197N, K199N, K197N/K199S y K197N/K199T, y estos remplazos **crean in sitio de glicosilación**. Por lo tanto, D2 no enseña ni sugiere que cualquiera de estas modificaciones incremente la actividad o la resistencia a los inhibidores. Es más, D2 no enseña ni sugiere una modificación que corresponda a un remplazo seleccionado entre D196L, D196I, K197E, K197D,

K197L, K197M, K197I, K197V, K197Y, K197F, K197W, K199D y K199E, ni **ninguna** modificación que incremente la actividad coagulante dependiente de TF en comparación con el polipéptido FVII sin modificar.

En este orden de ideas, si una persona medianamente versada en la materia técnica, se viera motivada de alguna manera por las enseñanzas de D2, no encontraría ninguna enseñanza en D3 que pudiera subsanar las deficiencias de las enseñanzas de D2. En efecto, como se discutió previamente, el documento D3 **no describe ninguna de las mutaciones en 197 o 199**, y solamente enseña las mutaciones específicas D196N y D196K. D3 no describe D196L o D196I ni las propiedades conferidas por tales mutaciones.

En clara contraposición, las presentes reivindicaciones, por lo tanto, recitan un remplazo en el residuo 196 que no se describe ni sugiere en D3 y que tiene propiedades particulares no descritas ni enseñadas por tal anterioridad. De hecho, D3 enseña que remplazar D196 con **N** o **K** altera el ligado del TFPI, pero la presente solicitud muestra que D196K **disminuye** la actividad dependiente de TF.

Adicionalmente, resulta importante señalar que el documento D3 no sugiere ningún otro remplazo que tenga un efecto sobre el ligado del TFPI, ni sugiere que D196L y D196I hagan eso. Por lo tanto, D3 no tiene relevancia para ninguna de las mutaciones en las posiciones 197 y 199 y, como se describió antes, D3 no sugiere las mutaciones reivindicadas ni las propiedades conferidas por estas.

En consecuencia, ni D2 ni D3, individualmente o en combinación, enseñan o sugieren las variantes FVII descritas en la reivindicación 1, ni cualquiera de las variantes FVII descritas en cualquiera de las reivindicaciones dependientes, ni resultados que logren esto. De hecho, **es la presente solicitud la que describe la identificación de las mutaciones que exhiben resistencia incrementada al TFPI y/o exhiben actividad coagulante incrementada por virtud de los cambios en aminoácidos**. Resultados adicionales no enseñados ni sugeridos por D2 o D3 se describieron en la respuesta anterior, y se resumen como sigue.

Datos de ejemplo en la solicitud que muestran resultados no enseñados ni sugeridos por las referencias D2 y/o D3.

Como se describió en la respuesta anterior, y adelante, los datos de la solicitud demuestran que cada una de las modificaciones de aminoácidos seleccionada entre D196L, D196I, K197E, K197D, K197L, K197M, K197I, K197V, K197Y, K197F, K197W, K199D y K199E incrementa la actividad coagulante dependiente de TF del polipéptido FVII modificado. Además, los datos de la solicitud muestran que las propiedades conferidas por una mutación particular son aditivas o retenidas cuando se combinan con otras mutaciones. En particular,

la solicitud suministra ejemplos de trabajo que muestran que polipéptidos con una pluralidad de modificaciones de aminoácidos como se menciona en las reivindicaciones dependientes exhiben actividad coagulante dependiente de TF incrementada y/o resistencia al TFPI incrementada, lo que resulta en una actividad coagulante dependiente de TF incrementada.

Los ejemplos de trabajo de la solicitud muestran que una modificación de aminoácidos seleccionada entre D196L, D196I, K197E, K197D, K197L, K197M, K197I, K197V, K197Y, K197F, K197W, K199D y K199E incrementa la resistencia al TFPI o incrementa la actividad del FVII dependiente del factor de tejido (TF) (ver, por ejemplo, Tabla, abajo). La solicitud enseña que la resistencia al TFPI incrementada o la actividad catalítica dependiente de TF incrementada, incrementa la actividad coagulante dependiente de TF. Por lo tanto, las mutaciones de la reivindicación 1 incrementan la actividad coagulante dependiente de TF. Todas las reivindicaciones dependen de la reivindicación 1. Por lo cual, todas las reivindicaciones dicen que el polipéptido FVII modificado incluye una mutación de aminoácidos que incrementa la actividad coagulante dependiente de TF.

Tabla A. Polipéptidos FVII modificados

• Variante	• Efecto(s) de la variacion	• Referencia(s)
• D196L	• Incrementa la actividad catalítica dependiente de TF	• Página 213, Tabla 16, entrada CB603.
• D196I	Incrementa la actividad catalítica dependiente de TF	• Página 213, Tabla 16, entrada CB604,
• K197E	• Incrementa la resistencia al TFPI; • Incrementa la actividad específica; incrementa la actividad catalítica	• Página 219, Tabla 21, entrada CB558 (según se modificó); • páginas 194 y 195, Tabla 12, entrada CB558; y • página 191, Tabla 11, entrada CB558
• K197D	• Incrementa la resistencia al TFPI; • Incrementa la actividad específica; incrementa la actividad catalítica	• Página 219, Tabla 21, entrada CB557; • página 191, Tabla 11, entrada CB557; y páginas 194 y 195, Tabla 12, entrada CB557
• K197L	• Incrementa la resistencia al TFPI	• Página 219, Tabla 21, entrada CB560
• K197M	Incrementa la actividad catalítica dependiente de TF	• Página 213, Tabla 16, entrada CB599
• K197I	Incrementa la actividad catalítica dependiente de TF	• Página 213, Tabla 16, entrada CB595

• Variante	• Efecto(s) de la variacion	• Referencia(s)
• K197V	Incrementa la actividad catalítica dependiente de TF	• Página 213, Tabla 16, entrada CB596
• K197Y	Incrementa la actividad catalítica dependiente de TF; incrementa la actividad específica; Incrementa la actividad catalítica	• Pàgina 213, Tabla 16, entrada CB561; • página 191, Tabla 11, entrada CB561; y páginas 194 y 195, Tabla 12, entrada CB561
• K197F	Incrementa la actividad catalítica dependiente de TF	• Página 213, Tabla 16, entrada CB597
• K197W	Incrementa la actividad catalítica dependiente de TF	• Página 213, Tabla 16, entrada CB598
• K199D	Incrementa la actividad específica	• Página 191, Tabla 11, entrada CB562
• K199E	Incrementa la actividad específica; Incrementa la actividad catalítica; Incrementa la resistencia al TFPI	• Página 191, Tabla 11, entrada CB563; Página 194, Tabla 12, entrada CB563; Página 219, Tabla 21, entrada CB563

Así, como se puede corroborar de la tabla anterior, los ejemplos de trabajo en la solicitud muestran que una modificación de aminoácidos seleccionada entre D196L, D196I, K197E, K197D, K197L, K197M, K197I, K197V, K197Y, K197F, K197W, K199D y K199E Incrementa la resistencia al TFPI del FVII o Incrementa la actividad dependiente del factor de tejido (TF) del FVII. La solicitud enseña que la resistencia al TFPI incrementada o que la actividad catalítica dependiente de TF incrementada incrementan la actividad coagulante dependiente de TF. Por lo tanto, **una modificación de aminoácidos seleccionada entre D196L, D196I, K197E, K197D, K197L, K197M, K197I, K197V, K197Y, K197F, K197W, K199D y K199E incrementa la actividad coagulante dependiente de TF**. Las mutaciones descritas en la reivindicación 1 confieren el efecto técnico de incrementar la actividad coagulante dependiente de TF del FVII.

Además, como se mencionó en la respuesta previa (ver por ejemplo, Sección IV), los polipéptidos reivindicados comparten rasgos estructurales significativos. Estos son mutantes de FVII y solamente requieren diferir en el uno o dos residuos de aminoácido según se requiera. Cualquiera otra mutaciones pueden ser las mismas. Así, los polipéptidos comparten homología estructural e identidad de secuencia significativas. Como se describió en la respuesta anterior, los mutantes reivindicados comparten rasgos funcionales comunes por virtud de las mutaciones reivindicadas en la estructura.

También, la solicitud suministra ejemplos de trabajo que muestran que un polipéptido FVII modificado que incluye una modificación de aminoácidos seleccionada entre D196L, D196I, K197E, K197D, K197L, K197M, K197I, K197V, K197Y, K197F, K197W, K199D y K199E, en combinación con una o más modificaciones, tales como las modificaciones mencionadas en las reivindicaciones 4 – 13, 16 y 17, exhiben actividad incrementada y/o resistencia al TFPI incrementada. La tabla que sigue (Tabla B) resume los datos de la solicitud que demuestran que los polipéptidos FVII modificados que incluyen una modificación de aminoácidos según la reivindicación 1, en combinación con una o más de otras modificaciones de aminoácidos, exhiben actividad incrementada y/o resistencia al TFPI incrementada (modificaciones de aminoácidos descritas en la reivindicación 1 se dan en negrilla).

Tabla B. Polipéptidos FVII modificados

Variante	Referencia(s)
D196R/**K197E**/**K199E**	Tabla 11, entrada CB586
D196K/**K197E**/**K199E**	Tabla 11, entrada CB587
D196R/**K197E**/**K199E**/R290E	Tabla 11, entrada CB588
D196R/**K197M**/**K199E**	Tablas 11 and 16, entrada CB589
D196R/**K197M**/ **K199E**/R290E	Tablas 11, 13 and 21, entrada CB590
D196L/ **K197L**	Tabla 16, entrada CB611
D196M/**K197L**	Tabla 16, entrada CB613
D196F/**K197E**	Tabla 16, entrada CB615
D196W/**K197E**	Tabla 16, entrada CB616
K197E/K341Q	Tablas 21 and 23, entrada CB637
K197L/K341Q	Tabla 21, entrada CB638
K197E/G237V/K341Q	Tablas 21 and 23, entrada CB671
K197E/**K199E**	Tablas 21 and 23, entrada CB688
K197E/G237V	Tablas 16, 21 and 23, entrada CB689
K197E/G237V/M298Q	Tabla 16, entrada CB690
K197E/**K199E**/K341Q	Tablas 21 and 23, entrada CB691
K197E/G237V/M298Q/K341Q	Tablas 18, 21 and 23, entrada CB692
K197E/**K199E**/G237V/M298Q/K341Q	Tablas 21 and 23, entrada CB693
K199E/K341Q	Tablas 21 and 23, entrada CB694
K197E/M298Q	Tablas 16, 18 and 23, entrada CB902

Todas las reivindicaciones requieren que el polipéptido FVII modificado incluya una modificación de aminoácidos seleccionada entre D196L, D196I, K197E, K197D, K197L, K197M, K197I, K197V, K197Y, K197F, K197W, K199D y K199E, porque todas las reivindicaciones en últimas dependen, e incorporan las limitaciones de la reivindicación 1. LA solicitud demuestra que una modificación seleccionada entre D196L, D196I, K197E, K197D, K197L, K197M, K197I, K197V, K197Y, K197F, K197W, K199D y K199E incrementa la resistencia al TFPI y/o incrementa la actividad individualmente o en combinación con otras mutaciones (ver por ejemplo, Tabla A y Tabla B). Como se describió antes, la solicitud **suministra ejemplos de trabajo que demuestran que cada una de las modificaciones de aminoácidos citadas en la reivindicación 1 incrementan la actividad coagulante dependiente de TF**, y los ejemplos de

trabajo en la solicitud demuestran tales efectos cuando las modificaciones de los aminoácidos se combinan (ver, por ejemplo, Tabla A y Tabla B).

Además de los ejemplos de la solicitud que demuestran polipéptidos FVII modificados que exhiben actividad incrementada y/o resistencia al TFPI incrementada, había disponible considerable conocimiento para la persona versada en la materia con anterioridad a la fecha de prioridad más temprana con respecto a las relaciones estructura/función del FVII y las variantes de aminoácidos de este.

La FVII es una proteína bien caracterizada. Numerosas variantes de aminoácidos de la FVII eran conocidos antes de la prioridad más temprana. La Tabla G en el Anexo 1 suministra citas que describen mutaciones ilustrativas de un polipéptido FVII, incluyendo muchas mutaciones de las recitadas en las reivindicaciones dependientes.

Así, la persona versada en la materia puede combinar las nuevas mutaciones suministradas en esta solicitud y recitadas en la reivindicación 1 con aquellas conocidas en la técnica y recitadas en las reivindicaciones dependientes. Las referencias dadas en la Tabla G suministran evidencia del conocimiento considerable antes de la prioridad más temprana respecto a las relaciones estructura/función de las modificaciones FVII.

Por lo tanto, la persona versada en la técnica era consciente de los efectos de las modificaciones de los aminoácidos y/o podría determinar fácilmente los efectos de las modificaciones de aminoácidos, incluyendo las modificaciones combinadas con una modificación según la reivindicación 1. Por al menos estas razones, y por las razones adicionales descritas enseguida con respecto a las reivindicaciones que mencionan modificaciones de aminoácidos adicionales, la Solicitante, de manera respetuosa, afirma que ni D2 ni D3 ni su combinación, destruye el nivel inventivo de la reivindicación 1, ni de ninguna de las reivindicaciones dependientes de esta.

Además del conocimiento disponible para una persona versada en la técnica con respecto a los efectos de las modificaciones FVII, la solicitud suministra datos que muestran los efectos de las mutaciones específicas dadas en las reivindicaciones 4-13, 16 y 17. Como se describe adelante, se describen modificaciones de aminoácidos mencionadas en las reivindicaciones 4-13, 16 y 17 que se describen en la solicitud y/o eran conocidas para la persona versada en la técnica, como se evidencia por las referencias disponibles antes de la fecha de prioridad más temprana. Para cada reivindicación, se resumen los datos de la solicitud y el conocimiento disponible para la persona versada en la técnica.

1. Reivindicación 4

La reivindicación 4 menciona que el polipéptido FVII modificado incluye una modificación de aminoácido adicional seleccionada entre un remplazo correspondiente a:

D196K, D196R, D196A, D196Y, D196F, D196W, D196L, D196I, K197Y, K197A, K197E, K197D, K197L, K197M, K197I, K197V, K197F, K197W, K199A, K199D, K199E, G237W, G237T, G237I, G237V, T239A, R290A, R290E, R290D, R290N, R290Q, R290K, K341E, K341R, K341N, K341M, K341D y K341Q

La , que sigue, describe los efectos de las modificaciones dadas en la reivindicación 4 sobre la estructura de la FVII y/o su actividad. Los efectos de las modificaciones se describen en una o más de las Tablas 11-13, 16, 21 y 23 de la solicitud y/o en las referencias disponibles antes de la fecha de la prioridad más temprana.

. Polipéptidos FVII variants

Variantes de la reivindicación 4	Effecto(s) de la variación	Referencia(s)
D196K	Disminuye la actividad específica, incrementa la actividad catalítica, Incrementa la inhibición por el TFPI, disminuye la actividad catalítica dependiente de TF (ensayo indirecto), disminuye la resistencia al TFPI	Tablas 11-13, 16 and 21, entrada CB554
D196R	Disminuye la actividad específica, incrementa la actividad catalítica, Incrementa la inhibición por el TFPI, disminuye la actividad catalítica dependiente de TF (ensayo indirecto), disminuye la resistencia al TFPI	Tablas 11-13, 16 and 21, entrada CB555
D196A	Disminuye la actividad específica, incrementa la actividad catalítica, Incrementa la inhibición por el TFPI, disminuye la actividad catalítica dependiente de TF (ensayo indirecto), disminuye la resistencia al TFPI; disminuye la rata de activación del Factor X; no cambia en el ligado al TF	Tablas 11-13, 16 and 21, entrada CB556; Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
D196F	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto), disminuye la resistencia al TFPI	Tablas 16 and 21, entrada CB600
D196Y	Incrementa la actividad catalítica dependiente de TF (ensayo indirecto), disminuye la resistencia al TFPI	Tablas 16 y 21, entrada CB601
D196W	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto), disminuye la resistencia al TFPI	Tablas 16 y 21, entrada CB602
D196L	Incrementa la actividad catalítica dependiente de TF (ensayo indirecto), disminuye la resistencia al TFPI	Tablas 16 y 21, entrada CB603
D196I	Incrementa la actividad catalítica dependiente de TF (ensayo indirecto), disminuye la resistencia al TFPI	Tablas 16 y 21, entrada CB604
K197D	Incrementa la actividad específica, incrementa la actividad catalítica, disminuye la inhibición por el TFPI, disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la resistencia al TFPI	Tablas 11-13, 16 y 21, entrada CB557

Variantes de la reivindicación 4	Effecto(s) de la variación	Referencia(s)
K197E	Incrementa la actividad específica, incrementa la actividad catalítica, disminuye la inhibición por el TFPI, disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la resistencia al TFPI; incrementa la resistencia al TFPI dependiente de TF	Tablas 11-13, 16, 21 y 23, entrada CB558
K197A	Incrementa la actividad específica, incrementa la actividad catalítica, disminuye la inhibición por el TFPI, disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la resistencia al TFPI; incrementa la resistencia al TFPI dependiente de TF	Tablas 11-13, 16 y 21, entrada CB559; Dickinson *Proc. Natl. Acad. Sci USA* (1996) 93(25):14379-84
K197L	Disminuye la actividad específica, disminuye la actividad catalítica, disminuye la inhibición por el TFPI, disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la resistencia al TFPI	Tablas 11-13, 16 y 21, entrada CB560
K197Y	Incrementa la actividad específica, incrementa la actividad catalítica, disminuye la inhibición por el TFPI, incrementa la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la resistencia al TFPI	Tablas 11-13, 16 y 21, entrada CB561
K197I	Incrementa la actividad catalítica dependiente de TF (ensayo indirecto), disminuye la resistencia al TFPI	Tablas 16 y 21, entrada CB595
K197V	Incrementa la actividad catalítica dependiente de TF (ensayo indirecto), disminuye la resistencia al TFPI	Tablas 16 y 21, entrada CB596
K197F	Incrementa la actividad catalítica dependiente de TF (ensayo indirecto), disminuye la resistencia al TFPI	Tablas 16 y 21, entrada CB597
K197W	Incrementa la actividad catalítica dependiente de TF (ensayo indirecto), disminuye la resistencia al TFPI	Tabla 16 y 21, entrada CB598
K197M	Incrementa la actividad catalítica dependiente de TF (ensayo indirecto), disminuye la resistencia al TFPI	Tablas 16 y 21, entrada CB599
K199D	Incrementa la actividad específica, disminuye la actividad catalítica, incrementa la inhibición por el TFPI, disminuye la actividad catalítica dependiente de TF (ensayo indirecto), disminuye la resistencia al TFPI	Tablas 11-13, 16, 21, entrada CB562
K199E	Incrementa la actividad específica, incrementa la actividad catalítica, incrementa la inhibición por el TFPI, disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la resistencia al TFPI, incrementa la resistencia al TFPI dependiente de TF	Tablas 11-13, 16, 21 and 23, entrada CB563
K199A	Incrementa la actividad específica, incrementa la actividad catalítica, incrementa la inhibición por el TFPI, incrementa la actividad catalítica dependiente de TF (ensayo indirecto), disminuye la resistencia al TFPI; disminuye el ligado a TF y la tasa de activación del factor X	Tablas 11-13, 16 y 21, entrada CB564; and Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
G237W	Incrementa la actividad catalítica dependiente de TF (ensayo indirecto), disminuye la resistencia al TFPI	Tablas 16 y 21, entrada CB605
G237T	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto), disminuye la resistencia al TFPI, crea sitio de N-glicosilación (con V235N) en una región de sitio activo o en la cadena de la fisura del sitio de ligado activo	Tablas 16 y 21, entrada CB606; y US20060166874
G237I	n/d	entrada CB607

Variantes de la reivindicación 4	Effecto(s) de la variación	Referencia(s)
G237V	Incrementa la actividad catalítica dependiente de TF (ensayo indirecto), disminuye la resistencia al TFPI, incrementa la resistencia al TFPI dependiente de TF	Tablas 16, 21 y 23, entrada CB608
T239A	Disminuye la actividad específica, disminuye la actividad catalítica, disminuye la inhibición por el TFPI, disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la resistencia al TFPI; disminuye la activación del Factor X; disminuye la inhibición del TFPI, no cambia en el ligado al Factor de Tejildo	Tablas 11-13, 16 y 21, entrada CB565; Dickinson, *Proc. Natl. Acad. Sci U S A.* (1996) 93(25):14379-84; Iakhiaev, (2001) *Thromb. Haemost.* 85(3):458-63
R290D	Incrementa la actividad específica, disminuye la actividad catalítica, disminuye la inhibición por el TFPI, disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la resistencia al TFPI, facilita el enlace del grupo químico	Tablas 11-13, 16 y 21, entrada CB566; US 6806063; WO 2006/134173; Mariani, Thromb Haemost. 2005 93(3):481-7; Furlan, J. Thromb Haemost. 2003 1(11):2455-7
R290E	Incrementa la actividad específica, disminuye la actividad catalítica, disminuye la inhibición por el TFPI, disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la resistencia al TFPI, facilita el enlace del grupo químico	Tablas 11-13, 16 y 21, entrada CB567; US 6806063; WO 2006/134173; Mariani, Thromb. Haemost. 2005 93(3):481-7; Furlan, J. Thromb. Haemost. 2003 1(11):2455-7
R290A	Incrementa la actividad específica, disminuye la actividad catalítica, disminuye la inhibición por el TFPI, disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la resistencia al TFPI, disminuye la actividad; no cambia en el ligado al factor de tejido; incrementa la resistencia a las proteasas	Tablas 11-13, 16 y 21, entrada CB568; Dickinson, *Proc. Natl. Acad. Sci. USA* (1996) 93(25):14379-84; WO 88/10295; US 5,580,560; Furlan, J. Thromb. Haemost. (2003) 1(11):2455-7; Jin, *J. Mol. Biol.* (2001) 307(5):1503-17; Ruf *Biochemistry* (1994) 33(38):11631-6
R290N	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la resistencia al TFPI, incrementa la resistencia al TFPI dependiente de TF, incrementa la glicosilación (con A292S/T) mediante crear un sitio de N-glicosilación	Tablas 16, 21 y 23, entrada CB697; US 6806063
R290Q	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la resistencia al TFPI, incrementa la resistencia al TFPI dependiente de TF	Tablas 16, 21 y 23, entrada CB698
R290K	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto), disminuye la resistencia al TFPI, disminuye la resistencia al TFPI dependiente de TF, facilita el enlace del grupo químico, incrementa la resistencia a las proteasas	Tablas 16, 21 y 23, entrada CB699; US 6806063, US 5580560, WO8810295; Furlan, *J. Thromb Haemost.* 2003 1(11):2455-7
K341R	Incrementa la actividad específica, disminuye la actividad catalítica, disminuye la inhibición por el TFPI, disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la resistencia al TFPI	Tablas 11-13, 16 y 21, entrada CB569
K341Q	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la resistencia al TFPI, incrementa la resistencia al TFPI dependiente de TF, disminuye la inhibición dependiente del inhibidor del camino del factor de tejido, incrementa la resistencia a las proteasas	Tablas 16, 21 y 23, entrada CB609; Bjelke (2007) FEBS Lett. 581(1):71-6; Neuenschwander (1995) *Biochemistry* 34(27):8701-8707; WO2004029091; WO2006114105; US 5580560

Variantes de la reivindicación 4	Effecto(s) de la variación	Referencia(s)
K341N	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la resistencia al TFPI, incrementa la resistencia al TFPI dependiente de TF, incrementa la glicosilación (con D343S/T) mediante crear un sitio de N-glicosilación	Tablas 16, 21 y 23, entrada CB733; US 6806063; US20060166874
K341M	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la resistencia al TFPI, incrementa la resistencia al TFPI dependiente de TF	Tablas 16, 21 y 23, entrada CB734
K341D	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la resistencia al TFPI, incrementa la resistencia al TFPI dependiente de TF	Tablas 16, 17, 21 y 23, entrada CB735
K341E	Incrementa la resistencia a las proteasas	US 5580560; Mariani, *Thromb Haemost.* 2005 93(3):481-7; Neuenschwander (1995) *Biochemistry* 34(27):8701-7

La Tabla D que sigue, hace un listado de polipéptidos FVII variantes descritos en lña solicitud que incluyen una modificación de las mencionadas en la reivindicación 4 en combinación con una o más de otras modificaciones, y el efecto(s) de la variación (la(s) modificación (ones) mencionada(s) en la reivindicación 4 se resaltan en negrita).

Tabla D. Polipéptidos FVII variantes.

Variant that includes an amino acid modification recited in claim 4	Effect of variation	Reference(s)
D196R/R290E	Incrementa la actividad específica, disminuye la actividad catalítica, incrementa la inhibición por el TFPI, disminuye la actividad catalítica dependiente de TF (ensayo indirecto), disminuye la resistencia al TFPI	Tablas 11-13, 16 y 21, entrada CB579
D196K/R290E	Disminuye la actividad catalítica, Incrementa la inhibición por el TFPI, disminuye la actividad catalítica dependiente de TF (ensayo indirecto), disminuye la resistencia al TFPI	Tablas 12, 13, 16 y 21, entrada CB580
D196R/R290D	Incrementa la actividad específica, disminuye la actividad catalítica, incrementa la inhibición por el TFPI, disminuye la actividad catalítica dependiente de TF (ensayo indirecto), disminuye la resistencia al TFPI	Tablas 11-13, 16 and 21, entrada CB581
D196R/K197E/ K199E	Incrementa la actividad específica, disminuye la actividad catalítica, incrementa la inhibición por el TFPI, disminuye la actividad catalítica dependiente de TF (ensayo indirecto), disminuye la resistencia al TFPI	Tablas 11-13, 16 y 21, entrada CB586
D196K/K197E/ K199E	Incrementa la actividad específica, disminuye la actividad catalítica, incrementa la inhibición por el TFPI, disminuye la actividad catalítica dependiente de TF (ensayo indirecto), disminuye la resistencia al TFPI	Tablas 11-13, 16 y 21, entrada CB587

Variant that includes an amino acid modification recited in claim 4	Effect of variation	Reference(s)
D196R/K197E/ K199E/R290E	Incrementa la actividad específica, disminuye la actividad catalítica, incrementa la inhibición por el TFPI, disminuye la actividad catalítica dependiente de TF (ensayo indirecto), disminuye la resistencia al TFPI	Tablas 11-13, 16 y 21, entrada CB588
D196R/K197M/ K199E	Incrementa la actividad específica, disminuye la actividad catalítica, incrementa la inhibición por el TFPI, disminuye la actividad catalítica dependiente de TF (ensayo indirecto), disminuye la resistencia al TFPI	Tablas 11-13, 16 y 21, entrada CB589
D196R/K197M/ K199E/R290E	Incrementa la actividad específica, disminuye la actividad catalítica, incrementa la inhibición por el TFPI, disminuye la actividad catalítica dependiente de TF (ensayo indirecto), disminuye la resistencia al TFPI	Tablas 11-13, 16 y 21, entrada CB590
D196L/ K197L	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto), disminuye la resistencia al TFPI	Tablas 16 y 21, entrada CB611
D196F/ K197L	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto), disminuye la resistencia al TFPI	Tablas 16 y 21, entrada CB612
D196M/**K197L**	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto)	Tabla 16, entrada CB613
D196W/K197L	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto), disminuye la resistencia al TFPI	Tablas 16 y 21, entrada CB614
D196F/K197E	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto), disminuye la resistencia al TFPI	Tablas 16 y 21, entrada CB615
D196W/**K197E**	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto)	Tabla 16, entrada CB616
D196V/**K197E**	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la resistencia al TFPI	Tablas 16 y 21, entrada CB617
K197E/K341Q	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la resistencia al TFPI, incrementa la resistencia al TFPI dependiente de TF	Tablas 16, 21 y 23, entrada CB637
K197L/K341Q	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la resistencia al TFPI	Tablas 16 y 21, entrada CB638
G237V/K341Q	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la resistencia al TFPI, incrementa la resistencia al TFPI dependiente de TF	Tablas 16, 21 y 23, entrada CB670
K197E/G237V/ K341Q	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la resistencia al TFPI, incrementa la resistencia al TFPI dependiente de TF	Tablas 16, 21 y 23, entrada CB671
K197E/K199E	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la resistencia al TFPI, incrementa la resistencia al TFPI dependiente de TF	Tablas 16, 21 y 23, entrada CB688
K197E/G237V	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la resistencia al TFPI, incrementa la resistencia al TFPI dependiente de TF	Tablas 16, 21 y 23, entrada CB689

Variant that includes an amino acid modification recited in claim 4	Effect of variation	Reference(s)
K197E/G237V/ M298Q	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la afinidad por el TF soluble	Tablas 16 y 22, entrada CB690
K197E/K199E/ K341Q	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la resistencia al TFPI, incrementa la afinidad por el TF soluble, incrementa la resistencia al TFPI dependiente de TF	Tablas 16, 21, 22 y 23, entrada CB691
K197E/G237V/ M298Q/**K341Q**	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la resistencia al TFPI, incrementa la afinidad por el TF soluble, incrementa la resistencia al TFPI dependiente de TF	Tablas 16, 18, 21, 22 y 23, entrada CB692
K197E/K199E/G23 7V/M298Q/**K341Q**	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la resistencia al TFPI, incrementa la resistencia al TFPI dependiente de TF	Tablas 16, 21 y 23, entrada CB693
K199E/K341Q	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la resistencia al TFPI, incrementa la resistencia al TFPI dependiente de TF	Tablas 16, 21, y 23, entrada CB694
G237V/M298Q	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la afinidad por el TF soluble, disminuye la resistencia al TFPI dependiente de TF	Tablas 16, 18, 22 y 23, entrada CB695
G237V/M298Q/ **K341Q**	Incrementa la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la afinidad por el TF soluble, incrementa la resistencia al TFPI dependiente de TF	Tablas 16, 22 y 23, entrada CB696
K197E/M298Q	Incrementa la actividad catalítica dependiente de TF (ensayo indirecto), disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la resistencia al TFPI dependiente de TF	Tablas 16, 18 y 23, entrada CB902
M298Q/**K341D**	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la resistencia al TFPI dependiente de TF	Tablas 17 y 23, entrada CB945

2. Reivindicación 5

La reivindicación 5 menciona que el polipéptido FVII modificado incluye una modificación de aminoácidos adicional seleccionada entre:

> G237T238insA, G237T238insS, G237T238insV, G237T238insAS, G237T238insSA, D196K197insK, D196K197insR, D196K197insY, D196K197insA,–D196K197insM, K197I198insE, K197I198insY, K197I198insA and K197I198insS.

La Tabla E que sigue enlista polipéptidos FVII variantes descritos en la solicitud que incluyen una modificación mencionada en la reivindicación 5, y los efectos de la modificación sobre la estructura de la FVII y/o su actividad. Como se muestra en la Tabla E, la solicitud describe los efectos de casi cada modificación descrita en la reivindicación 5.

Tabla E. Polipéptidos FVII variantes.

Variantes descritas en la reivindicación 5	Effecto de la variación	Referencia(s)
G237T238insA	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la resistencia al TFPI	Tablas 16, 17 y 23, entrada CB853
G237T238insS	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la resistencia al TFPI	Tablas 16, 17 y 23, entrada CB854
G237T238insV	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la resistencia al TFPI	Tablas 16, 17 y 23, entrada CB855
G237T238insAS	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la resistencia al TFPI	Tablas 16, 17 y 23, entrada CB856
G237T238insSA	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la resistencia al TFPI	Tablas 17 y 23, entrada CB857
D196K197insK	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto)	Tabla 17, entrada CB858
D196K197insR	Incrementa la actividad catalítica dependiente de TF (ensayo indirecto), disminuye la resistencia al TFPI dependiente de TF	Tablas 16, 17 y 23, entrada CB859
D196K197insY	Incrementa la actividad catalítica dependiente de TF (ensayo indirecto)	Tabla 16, entrada CB860
D196K197insA	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto), disminuye la resistencia al TFPI	Tablas 17 y 23, entrada CB862
D196K197insM	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto), disminuye la resistencia al TFPI	Tablas 17 y 23, entrada CB863
K197I198insE	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la resistencia al TFPI dependiente de TF	Tablas 17 y 23, entrada CB864
K197I198insY	Disminuye la actividad catalítica dependiente de TF (ensayo indirecto), incrementa la resistencia al TFPI dependiente de TF	Tablas 17 y 23, entrada CB865
K197I198insA	Incrementa la actividad catalítica dependiente de TF (ensayo indirecto)	Tabla 16, entrada CB866
K197I198insS	Incrementa la actividad catalítica dependiente de TF (ensayo indirecto), disminuye la resistencia al TFPI dependiente de TF	Tablas 17 y 23, entrada CB867

3. Reivindicación 6

La reivindicación 6 describe que el polipéptido FVII modificado incluye modificaciones de aminoácidos adicionales seleccionadas entre los remplazos correspondientes a:

D196R/K197E/K199E, D196K/K197E/K199E, D196R/K197E/K199E/R290E, D196R/K197M/K199E, D196R/K197M/ K199E/R290E, D196K/K197L, D196F/

K197L, D196L/ K197L, D196M/K197L, D196W/K197L, D196F/K197E, D196W/K197E, K196V/K197E, K197E/K341Q, K197L/K341Q, K197E/G237V/K341Q, K197E/K199E, K197E/G237V, K199E/K341Q y K197E/K199E/K341Q.

Excepto para la combinación D196K/K197L, la solicitud ejemplifica específicamente los efectos de cada una de las modificaciones de los aminoácidos según la reivindicación 6 (ver, por ejemplo, la Tabla D arriba). Además, la solicitud describe los efectos individuales de las modificaciones de los aminoácidos D196K y K197L (Ver, por ejemplo, la , arriba), y muestra que se retienen los efectos cuando está combinada.

4. Reivindicación 7

La reivindicación 7 finalmente depende de la reivindicación 1, y describe que el polipéptido FVII modificado comprende **dos o más** modificaciones correspondientes a dos o más de:

D196K, D196R, D196A, D196Y, D196F, D196M, D196W, D196L, D196I, K197Y, K197A, K197E, K197D, K197L, K197M, K197I, K197V, K197F, K197W, K199A, K199D, K199E, G237W, G237T, G237I, G237V, T239A, R290A, R290E, R290D, R290N, R290Q, R290K, K341E, K341R, K341N, K341M, K341D, K341Q, G237T238insA, G237T238insS, G237T238insV, G237T238insAS, G237T238insSA, D196K197insK, D196K197insR, D196K197insY, D196K197insW, D196K197insA, D196K197insM, K197I198insE, K197I198insY, K197I198insA and K197I198insS.

La reivindicación 7 describe que al menos una modificación es seleccionada entre:

D196L, D196I, K197E, K197D, K197L, K197M, K197I, K197V, K197Y, K197F, K197W, K199D and K199E.

La solicitud describe polipéptidos FVII modificados que tienen dos o más modificaciones de aminoácidos seleccionadas entre aquellas descritas en la reivindicación 7, incluyendo polipéptidos FVII que incluyen mutaciones D196R/K197E/K199E, D196K/K197E/K199E, D196R/K197E/K199E/R290E, D196R/K197M/K199E, D196R/K197M/K199E/R290E, D196K/K197L, D196L/K197L, D196F/ K197L, D196M/K197L, D196W/K197L, D196F/K197E, D196W/K197E, K197E/K341Q, K197L/K341Q, K197E/G237V/K341Q, K197E/K199E, K197E/G237V, K197E/G237V/M298Q, K197E/K199E/K341Q, K197E/G237V/M298Q/K341Q y K199E/K341Q (ver, por ejemplo, la Tabla D arriba).

5. Reivindicación 8

La reivindicación 8 depende de las reivindicaciones 1 a 7 y describe que "el

polipéptido FVII contiene 2, 3, 4, 5, 6, o 7 modificaciones." La solicitud describe varios polipéptidos FVII modificados que tienen 2, 3, 4, 5, 6, o 7 modificaciones de aminoácidos y incluye una modificación en un polipéptido FVII que corresponde a un remplazo seleccionado entre D196L, D196I, K197E, K197D, K197L, K197M, K197I, K197V, K197Y, K197F, K197W, K199D y K199E (*ver, por ejemplo*, Tabla B y Tabla D, mutantes D196R/K197E/K199E, D196K/K197E/K199E, D196R/K197E/K199E/R290E, D196R/K197M/K199E, D196R/K197M/ K199E/R290E, D196K/K197L, D196F/ K197L, D196L/ K197L, D196M/K197L, D196W/K197L, D196F/K197E, D196W/K197E, K197E/K341Q, K197L/K341Q, K197E/K199E, K197E/G237V, K199E/K341Q, K197E/G237V/K341Q, K197E/G237V/M298Q, K197E/G237V/M298Q/K341Q, K197E/K199E/G237V/M298Q/K341Q, K197E/M298Q y K197E/K199E/K341Q).

6. Reivindicación 9

La reivindicación 9 depende de la reivindicación 7 y de la 8 y describe que el polipéptido FVII modificado comprende las modificaciones seleccionadas entre:

> D196R/ K197E/ K199E, D196K/ K197E/ K199E, D196R/ K197E/ K199E/R290E, D196R/ K197M/ K199E, D196R/ K197M/ K199E/R290E, D196K/K197L, D196F/ K197L, D196L/ K197L, D196M/K197L, D196W/ K197L, D196F/K197E, D196W/K197E, D196V/K197E, K197E/K341Q, K197L/K341Q, K197E/G237V/K341Q, K197E/K199E, K197E/G237V, K199E/K341Q, K197E/K199E/K341Q y K197E/G237V/M298Q

Con la excepción para D196K/K197L, la solicitud describe los efectos de cada una de las modificaciones de aminoácidos descritas en la reivindicación 9 (ver, por ejemplo, la Tabla D anterior). Además, la solicitud describe los efectos de las modificaciones de aminoácidos D196K y K197L (Ver, por ejemplo, la anterior) y muestra que los efectos son retenidos cuando las mutaciones en estas posiciones son combinadas (Ver, por ejemplo, la Tabla B, entradas CB587, CB611, CB613 y CB638; y la Tabla B, entradas CB580, CB611, CB612, CB613, CB614 and CB638).

7. Reivindicación 10

La reivindicación 10 depende de las reivindicaciones 1 a 9 y describe que el polipéptido FVII modificado "exhibe una resistencia al inhibidor del camino del factor de tejido incrementada (TFPI) en comparación con el polipéptido FVII no modificado," LA solicitud suministra ejemplos de trabajo que muestran que las siguientes variantes de FVII incrementan la resistencia al TFPI:

> K197D, K197L, K199E, T239A, R290D, R290E, R290A, K341R, K341Q, K197E/K341Q, K197L/K341Q , G237V/K341Q, K197V/G237V/K341Q, K197E/K199E, K197E/G237V, K197E/K199E/K341Q, K197E/G237V/M298Q/K341Q, K197E/K199E/G237V/M298Q/K341Q,

K199E/K341Q, G237V/M298Q/K341Q, R290N, R290Q, R290V, K341N, K341M y K341D.

La solicitud también suministra ejemplos de trabajo que muestran que las siguientes variantes de FVII incrementan la resistencia al TFPI dependiente de TF:

K197A, K197E, M298Q, G237V, K341Q, K197E/K341Q, G237V/K341Q, K197V/G237V/K341Q, K197E/K199E, K197E/G237V, K197E/K199E/K341Q, K197E/G237V/M298Q/K341Q, K197E/K199E/G237V/M298Q/K341Q, K199E/K341Q, G237V/M298Q/K341Q, R290N, R290Q, R290M, R290V, K341N, K341M, K341D, G237T238insA, G237T238insS, G237T238insV, G237T238insAS, G237T238insSA, K197I198insE, K197I198insY, K197E/M298Q y M298Q/K341D.

(Ver, por ejemplo, las Tablas 21 y 23, enseguida (énfasis agregado para los mutantes que exhiben resistencia al TFPI incrementada y/o resistencia incrementada al TFPI dependiente de TF)):

Tabla 21. Inhibición de las variantes de FVIIa por el TFPI

ID	Mutación (numeración de FVII maduro)	Mutación (numeración de la quimiotripsina)	Número de resistencia al TFPI
CB553	p	P	1.0
CB554	D196K	D60K	0.6
CB555	D196R	D60R	0.5
CB556	D196A	D60A	<0.3
CB557	**K197D**	**K60aD**	**3.0**
CB558	K197E	K60aE	1.3
CB559	K197A	K60aA	0.7
CB560	**K197L**	**K60aL**	**16.0**
CB561	K197Y	K60aY	1.5
CB562	K199D	K60cD	0.9
CB563	**K199E**	**K60cE**	**1.3**
CB564	K199A	K60cA	0.9
CB565	**T239A**	**T99A**	**4.5**
CB566	**R290D**	**R147D**	**3.3**
CB567	**R290E**	**R147E**	**6.1**
CB568	**R290A**	**R147A**	**2.8**
CB569	**K341R**	**K192R**	**4.1**
CB579	D196R/R290E	D60R/R147E	0.4
CB580	D196K/R290E	D60K/R147E	0.7
CB581	D196R/R290D	D60R/R147D	0.4
CB586	D196R/ K197E/ K199E	D60R/K60aE/K60cE	<0.3
CB587	D196K/ K197E/ K199E	D60K/K60aE/K60cE	0.3
CB588	D196R/ K197E/ K199E/R290E	D60R/K60aE/K60cE/R147E	0.4
CB589	D196R/ K197M/ K199E	D60R/K60aM/K60cE	0.6
CB590	D196R/ K197M/ K199E/R290E	D60R/K60aM/K60cE/R147E	0.9
CB591	L305V/S314E/L337A/F374Y	L163V/S170bE/K188A/F225Y	0.6
CB592	M298Q	M156Q	0.5
CB593	V158D/E296V/M298Q	V21D/E154V/M156Q	0.8

ID	Mutación (numeración de FVII maduro)	Mutación (numeración de la quimiotripsina)	Número de resistencia al TFPI
CB594	V158D/E296V/M298Q/K337A	V21D/E154V/M156Q/K188A	0.3
CB595	K197I	K60aI	0.8
CB596	K197V	K60aV	0.6
CB597	K197F	K60aF	<0.3
CB598	K197W	K60aW	<0.3
CB599	K197M	K60aM	0.3
CB600	D196F	D60F	<0.3
CB601	D196Y	D60Y	<0.3
CB602	D196W	D60W	<0.3
CB603	D196L	D60L	<0.3
CB604	D196I	D60I	<0.3
CB605	G237W	G97W	<0.3
CB606	G237T	G97T	0.3
CB608	G237V	G97V	0.8
CB609	K341Q	K192Q	12.2
CB611	K197L/D196L	K60aL/D60L	<0.3
CB612	K197L/D196F	K60aL/D60F	<0.3
CB614	K197L/D196W	K60aL/D60W	<0.3
CB615	K197E/D196F	K60aE/D60F	<0.3
CB617	K197E/D196V	K60aE/D60V	<0.3
CB637	K197E/K341Q	K60aE/K192Q	80.7
CB638	K197L/K341Q	K60aL/K192Q	39.0
CB670	G237V/K341Q	G97V/K192Q	14.1
CB671	K197V/G237V/K341Q	K60aE/G97V/K192Q	18.9
CB688	K197E/K199E	K60aE/K60cE	2.1
CB689	K197E/G237V	K60aE/G97V	1.7
CB691	K197E/K199E/K341Q	K60aE/K60cE/K192Q	34.3
CB692	K197E/G237V/M298Q/K341Q	K60aE/G97V/M156Q/K192Q	15.2
CB693	K197E/K199E/G237V/M298Q/K341Q	K60aE/K60cE/G97V/M156Q/K192Q	22.1
CB694	K199E/K341Q	K60cE/K192Q	19.1
CB696	G237V/M298Q/K341Q	G97V/M156Q/K192Q	3.2
CB697	R290N	R147N	1.5
CB698	R290Q	R147Q	1.2
CB699	R290K	R147K	0.6
CB700	R290M	R147M	1.0
CB701	R290V	R147V	2.5
CB733	K341N	K192N	10.7
CB734	K341M	K192M	4.4
CB735	K341D	K192D	148.0

Tabla 23. Resistencia de las variantes FVIIa a la inhibición por el TFPI

ID	Mutación (Numeración del FVIImaduro)	Mutación (Numeración de la quimiotripsina)	Ensayo de la resistencia al TFPI dependiente de TF			
			Células 293-F		Células BHK-21	
			Ligado (p/ variante)	Disoc-iación (p/ variante)	Ligado (p/ variante)	Disoc-iación (p/ variante)
CB553	P	P	1.0	1.0	1.0	1.0
CB558	K197A	K60aE	1.4	1.3		
CB563	K197E	K60cE	1.4	1.4		
CB592	M298Q	M156Q	1.9	1.8		
CB593	V158D/E296V/M298Q	V21D/E154V/M156Q	0.8	0.8	1.0	1.0
CB608	G237V	G97V	2.2	1.8		
CB609	K341Q	K192Q	13.0	19.7		
CB637	K197E/K341Q	K60aE/K192Q	67.2	171.1		
CB670	G237V/K341Q	K192Q/G97V	12.8	12.4		
CB671	K197V/G237V/K341Q	K60aE/K192Q/G97V	18.9	21.8		
CB688	K197E/K199E	K60aE/K60cE	2.2	2.1		
CB689	K197E/G237V	K60aE/G97V	1.8	1:6		
CB691	K197E/K199E/K341Q	K60aE/K60cE/K192Q	33.9	68.1		
CB692	K197E/G237V/M298Q/K341Q	K60aE/G97V/M156Q/K192Q	15.9	21.5		
CB693	K197E/K199E/G237V/M298Q/K341Q	K60aE/K60cE/G97V/M156Q/K192Q	23.4	43.5		
CB694	K199E/K341Q	K60cE/K192Q	18.8	30.7		
CB695	G237V/M298Q	G97V/M156Q	0.9	0.8		
CB696	G237V/M298Q/K341Q	G97V/M156Q/K192Q	4.5	6.0		
CB697	R290N	R147N	1.6	1.5		
CB698	R290Q	R147Q	1.3	1.2		
CB699	R290K	R147K	0.7	0.7		
CB700	R290M	R147M	1.1	1.1		
CB701	R290V	R147V	2.6	2.7		
CB733	K341N	K192N	11.3	63.8		
CB734	K341M	K192M	4.9	5.4		
CB735	K341D	K192D	185.6	380.7		
CB853	G237T238insA	G97T98insA	-	-	6.5	18.2
CB854	G237T238insS	G97T98insS	-	-	5.2	13.0
CB855	G237T238insV	G97T98insV	-	-	7.3	16.8
CB856	G237T238insAS	G97T98insAS	-	-	22.8	154.5
CB857	G237T238insSA	G97T98insSA	-	-	21.5	146.5
CB858	D196K197insK	D60K60ainsK			1.0	0.8
CB859	D196K197insR	D60K60ainsR			0.5	0.5
CB862	D196K197insA	D60K60ainsA			0.5	0.4
CB863	D196K197insM	D60K60ainsM			0.4	0.4
CB864	K197I198insE	K60aI60binsE	-	-	1.3	1.1
CB865	K197I198insY	K60aI60binsY	-	-	1.1	1.0
CB867	K197I198insS	K60aI60binsS			0.7	0.6
CB902	K197E/M298Q	K60aE/M156Q	2.6	2.3		
CB945	M298Q/K341D	M156Q/K192D	146.8	196.5		

8. Reivindicaciones 11 y 12

La reivindicación 11 depende de las reivindicaciones 1 a 10 y describe que el polipéptido FVII modificado... comprende una o más modificaciones de

aminoácidos adicionales en una posición correspondiente a la posición Q176, M298 o E296 en un polipéptido FVII que tiene una secuencia de aminoácidos establecida en la SEQ ID NO:3 o en residuos correspondientes en un polipéptido FVII.

La reivindicación 12 depende de la reivindicación 11 y describe que:

La una o más modificaciones de aminoácidos en una posición correspondiente a Q176, M298 o E296 se seleccionan entre Q176A, M298Q, E296V y E296A.

Los polipéptidos FVII variantes que incluyen una modificación de aminoácidos en la posición Q176, M298 o E296, y en particular, una modificación de aminoácidos seleccionada entre Q176A, M298Q, E296V y E296A, fueron descritos en referencias disponibles con anterioridad a la fecha de prioridad más temprana y/o en los ejemplos de trabajo de la solicitud. Por ejemplo, la Tabla G del apéndice lista las referencias que describen los polipéptidos FVII variantes que incluyen las modificaciones de aminoácidos **Q176A, E296K, E296A, E296R, E296L, E296V, E296I, E296T, E296D, E296M, E296F, E296W, E296P, E296G, E296S, E296C, E296Y, E296N, E296H, E296Q, M298L, M298F, M298W, M298P, M298G, M298T, M298Y, M298H, M298E, M298D, M298S, M298V, M298I, M298Q, M298K, M298A, M298R, M298N, M298C and M298T**, y sus efectos en la actividad y/o estructura del FVII.

La solicitud también suministra ejemplos de trabajo de los polipéptidos FVII K197E/G237V/**M298Q**, K197E/G237V/**M298Q**/K341Q, K197E/K199E/G237V/**M298Q**/K341Q, G237V/**M298Q**, G237V/**M298Q**/K341Q, **M298Q**/K341D, K197E/**M298Q**, V158D/**E296V**/**M298Q**, **M298Q** y V158D/**E296V**/**M298Q**/K337A (Ver, *por ejemplo*, Tabla B y Tabla D anteriores; Tablas 21 and 23, entradas CB592 y CB593; Tabla 21, entrada CB594; y Tabla 23, entrada CB696).

9. Reivindicación 13

La reivindicación 13 depende de la reivindicación 12 y describe que el polipéptido FVII modificado incluye modificaciones de aminoácidos seleccionados entre:

K197E/G237V/M298Q, K197E/G237V/M298Q/K341Q,
K197E/K199E/G237V/M298Q/K341Q, y K197E/M298Q.

Como se describió antes, la solicitud describe ejemplos de trabajo de los polipéptidos FVII variantes K197E/G237V/M298Q, K197E/G237V/M298Q/K341Q, K197E/K199E/G237V/M298Q/K341Q and K197E/M298Q (*ver, por ejemplo*, Tabla b y Tabla D anteriores).

10. Reivindicaciones 16 y 17

La reivindicación 16 y la reivindicación 17 describen que el polipéptido modificado FVII comprende (reivindicación 16) y consiste de (reivindicación 17), una secuencia de aminoácidos establecidas en:

Cualquiera de las SEQ ID NOS: 21, 23-26, 28, 29, 39-43, 128-133, 139-146, 229, 230, 232-235, 242-244, 248 y 250.

Cada uno de los polipéptido FVII modificados cuya secuencia se establece en las SEQ ID NOS: 21, 23-26, 28, 29, 39-43, 128-133, 139-146, 229, 230, 232-235, 242-244, 248 y 250 se lista en la Tabla F abajo. La solicitud describe los efectos de casi cada mutación que corresponda a una secuencia de las descritas en las reivindicaciones 16 y 17. Casi cada polipéptido FVII modificado se describe en la o la Tabla D anteriores. Más aún, como se mencionó antes, la solicitud describe los efectos de las modificaciones de aminoácidos D196K y K197L (ver, por ejemplo, la anterior) y muestra que los efectos son retenidos cuando las mutaciones en estas posiciones son combinadas.

Tabla F. Polipéptidos FVII variantes

SEQ ID NO:	Mutación	Referencia(s)
18	D196K	TABLA C
19	D196R	TABLA C
20	D196A	TABLA C
21	K197Y	TABLA C
22	K197A	TABLA C
23	K197E	TABLA C
24	K197D	TABLA C
25	K197L	TABLA C
26	K197M	TABLA C
27	K199A	TABLA C
28	K199D	TABLA C
29	K199E	TABLA C
30	T239A	TABLA C
31	R290A	TABLA C
32	R290E	TABLA C
33	R290D	TABLA C
34	K341E	TABLA C
35	K341R	TABLA C
36	D196R/R290E	TABLA C
37	D196K/R290E	TABLA C
38	D196R/R290D	TABLA C
39	D196R/K197E/K199E	TABLA C
40	D196K/K197E/K199E	TABLA C
41	D196R/K197E/K199E/R290E	TABLA C
42	D196R/K197M/K199E	TABLA C
43	D196R/K197M/ K199E/R290E	TABLA C
125	D196Y	TABLA C
126	D196F	TABLA C
127	D196W	TABLA C
128	D196L	TABLA C
129	D196I	TABLA C
130	K197I	TABLA C
131	K197V	TABLA C
132	K197F	TABLA C
133	K197W	TABLA C
134	G237W	TABLA C
135	G237T	TABLA C

SEQ ID NO:	Mutación	Referencia(s)
136	G237I	n/a
137	G237V	TABLA C
138	K341Q	TABLA C
139	D196K/K197L	n/a
140	D196F/ K197L	TABLA C
141	D196L/ K197L	TABLA C
142	D196M/K197L	TABLA C
143	D196W/K197L	TABLA C
144	D196F/K197E	TABLA C
145	D196W/K197E	TABLA C
146	D196V/K197E	TABLA C
229	K197E/K341Q	TABLA C
230	K197L/K341Q	TABLA C
232	K197E/K199E	TABLA C
233	K197E/G237V	TABLA C
234	K199E/K341Q	TABLA C
235	K197E/G237V/K341Q	TABLA C
242	K197E/G237V/M298Q	TABLA C
243	K197E/G237V/M298Q/K341Q	TABLA C
244	K197E/K199E/G237V/M298Q/K341Q	TABLA C
248	K197E/M298Q	TABLA C
250	K197E/K199E/K341Q	TABLA C

Teniendo en cuenta toda la información anterior se puede llegar a la conclusión de que la solicitud describe y suministra **ejemplos de trabajo que demuestran que cada uno de los aminoácidos descritos en la reivindicación 1 incrementan la actividad coagulante dependiente del TF**. Es más, los ejemplos de trabajo de la solicitud demuestran tales efectos cuando las modificaciones de los aminoácidos descritas en la reivindicación 1 son combinadas con otras modificaciones aminoácidos, incluyendo las modificaciones de aminoácidos descritas en las reivindicaciones dependientes.

Sumado a lo anterior es necesario hacer énfasis sobre el hecho que había disponible considerable conocimiento con respecto a los efectos de las modificaciones de los aminoácidos en la estructura y/o actividad del FVII. Por lo tanto, el Solicitante de manera respetuosa afirma que las reivindicaciones 1 a 31 comparten efectos técnicos novedosos e inventivos de un polipéptido FVII modificado que incluye una modificación de aminoácidos seleccionada entre D196L, D196I, K197E, K197D, K197L, K197M, K197I, K197V, K197Y, K197F, K197W, K199D y K199E y que exhibe una actividad coagulante dependiente del TF incrementada.

Por otro lado, respecto a la reivindicación 1 rechazada nos permitimos manifestar que dicha reivindicación 1 requiere que el polipéptido FVII modificado incluya al menos una modificación de aminoácidos seleccionada entre D196L, D196I, K197E, K197D, K197L, K197M, K197I, K197V, K197Y, K197F, K197W, K199D y K199E.

En este sentido, es necesario llamar la atención del señor examinador sobre el hecho que esta es una lista de mutaciones es muy específica y cada una confiere la

propiedad requerida. Todas las reivindicaciones son dependientes de la reivindicación 1 y por lo tanto, todas las reivindicaciones están limitadas a los polipéptidos FVII con estas mutaciones específicas. Por lo tanto la reivindicación es muy clara y tiene nivel inventivo sobre la técnica anterior, dado que no habría manera de que una persona medianamente versada en la materia llegara a escoger de manera evidente dichas mutaciones específicas.

Como se describió antes (ver, por ejemplo, la Sección I), la solicitud suministra datos que muestran que cada una de las modificaciones de aminoácidos descritas en la reivindicación 1 incrementa la resistencia al TFPI y/o incrementan la actividad catalítica del FVII individualmente.

La solicitud suministra datos que muestran que las modificaciones de aminoácidos descritas en la reivindicación 1 incrementan la resistencia al TFPI y/o incrementan la actividad catalítica del FVII individualmente y también en combinación con otras modificaciones de aminoácidos. La solicitud también proporciona ejemplos de trabajo que muestran que las modificaciones múltiples de aminoácidos ilustradas descritas en las reivindicaciones dependientes exhiben una actividad coagulante dependiente del TF incrementada y/o una resistencia al TFPI incrementada.

La solicitud en estudio describe ejemplos de trabajo **de más de 80 variantes de polipéptidos FVII** que incluyen un remplazo de un aminoácido o una inserción seleccionada entre D196L, D196I, K197E, K197D, K197L, K197M, K197I, K197V, K197Y, K197F, K197W, K199D y K199E (*ver, por ejemplo*, Tabla B a Tabla D, anteriores).

La solicitud suministra polipéptidos con varias mutaciones. Mutaciones del FVII adicionales descritas en las referencias disponibles con anterioridad a la fecha de prioridad más temprana se listan en la Tabla suministrada en la respuesta anterior. La Respuesta anterior incluyó una tabla que lista cientos de mutaciones de ejemplo de un polipéptido FVII, y sus efectos sobre la estructura y/o actividad del FVII. Estas mutaciones, y mutaciones adicionales, están descritas en la Tabla G adjunta. La técnica, según se ilustra por lo citado en la Tabla G adjunta, y las referencias citadas en la Tabla, informan de muchas mutaciones en FVII y sus efectos sobre su función. La reivindicación 9 modificada limita el número de remplazos de aminoácidos en el polipéptido a 7.

Como se indicó en la Tabla G, había considerable conocimiento de las relaciones estructura/función de los polipéptidos FVII. Entre la técnica y la solicitud los efectos de más de **1100 mutaciones** distribuidas en más de las ¾ partes de las posiciones eran conocidas, incluyendo mutaciones de ocurrencia natural que habían sido mapeadas (ver, por ejemplo, McVey *et al.* (2001) *Hum. Mutat.* 17(1):3-17). Así, había un conocimiento extensivo de los polipéptidos FVII que incluyen una o más modificaciones de aminoácidos, y de los efectos de las mismas sobre la

estructura y/o actividad del FVII.

Adicionalmente, como se estableció en la respuesta al oficio de examen de fondo, (ver, por ejemplo, la Sección IV), los polipéptidos comparten una homología estructural e identidad secuencial significativa. Como se describió en la solicitud, tal como en el Ejemplo 1, los mutantes reivindicados comparten rasgos funcionales comunes por virtud de las mutaciones reivindicadas en la estructura. Como se estableció en la solicitud, las mutaciones fueron diseñadas para tener:

a) contactos electroestáticos complementarios remplazados entre el FVII y el TFPI-1 con contactos carga-carga repulsivos y/o
b) contactos electroestáticos positivos negados mediante el remplazo de los residuos cargados sobre el FVII con un residuo neutro y/o
c) causar impedimentos estéricos mediante el remplazo de un residuo que contenga una cadena lateral pequeña con residuos que contienen cadenas laterales más grandes.

Así, los polipéptidos comparten homología de secuencia de aminoácidos significativa y también son modificados para tener cambios estructurales similares para lograr la resistencia al TFPI incrementada. Por tanto, la actividad común que estos comparten es una resistencia incrementada a los efectos inhibitorios del inhibidor del camino del factor de tejido con base en características estructurales similares.

Así las cosas, respetuosamente nos permitimos decir que el solicitante tiene el derecho a las reivindicaciones conmensuradas en alcance, no solamente con lo que la Solicitante ha ejemplificado, sino conmensuradas en alcance con lo que una persona versada en la materia podría obtener en virtud de lo divulgado por el Solicitante.

De hecho , una persona versada en la materia, luego de leer la presente solicitud, podría reconocer que las enseñanzas de la especificación pueden ser aplicadas para preparar polipéptidos FVII modificados que incluyan al menos una modificación de aminoácidos seleccionada entre D196L, D196I, K197E, K197D, K197L, K197M, K197I, K197V, K197Y, K197F, K197W, K199D y K199E. Después de leer la memoria descriptiva, la persona versada en la materia no requeriría más que el conocimiento de los métodos de rutina, tales como la clonación molecular, para preparar los polipéptidos FVII modificados tal como se reivindican. Así, la persona versada en la técnica estaría muy familiarizada con polipéptidos FVII modificados, y las reivindicaciones describen los requerimientos de secuencias específicas.

Es importante señalar que los mutantes reivindicados también comparten características funcionales comunes en virtud de las mutaciones de la estructura

reivindicadas. Por lo tanto, a la luz del conocimiento que tiene la persona versada en la técnica, y la divulgación de la especificación, la reivindicación 1 es clara.

Finalmente, nos permitimos decir que los argumentos presentados en la resolución impugnada corresponden a un análisis en retrospectiva, dado que como se pudo demostrar a lo largo de la argumentación arriba indicada, las anterioridades citadas no podrían llevar de manera evidente a una persona con conocimientos promedio en el arte correspondiente a la presente invención.

En este sentido, es necesario recalcar que el estudio de la altura inventiva requiere que el examinador haga un esfuerzo intelectual por colocarse en la situación que dicha persona medianamente versada en la materia habría tenido que enfrentar justo antes del conocimiento de la solución proporcionada por la presente invención y no partir de dicha solución para relacionar documentos y enseñanzas que sin una motivación real no logran afectar el nivel inventivo de la presente solicitud como quedó plenamente demostrado a lo largo de este escrito.

Teniendo en cuenta lo dicho anteriormente, solicito a usted lo siguiente:

a) Que de conformidad con lo previsto por los artículos 3 (inciso 3); 35 (inciso 2) y 59 (inciso 2) del Código Contencioso Administrativo se consideren y se resuelvan **TODOS** los argumentos y **TODAS** las cuestiones planteadas así como las que aparezcan con motivo de este recurso.

b) Que se revoque la resolución No. 62493 emitida el 25 de octubre de 2013 expedida por el Superintendente de Industria y Comercio y se proceda a conceder el privilegio de patente de invención a la solicitud de mi mandante

Señor Superintendente,

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ANEXO 1

Tabla G que muestra citas que describen mutaciones ilustrativas de un polipéptido FVII, incluyendo muchas mutaciones de las recitadas en las reivindicaciones dependientes.

Tabla G. Mutaciones FVII

P del Residuo	Variante(s)	Efecto de la variación	Referencia
F4	Insert Y	Increases binding to phospholipids	Harvey, (2003) *J. Biol. Chem.* 278(10):8363-9; WO2003093465
	Y	Increased Activity	US7220837B1
	S/T	Increases glycosylation by creating an N-glycosylation site	US 6806063
E6	Q/D/N	Remove Gla residue	WO2005075635
E7	Q/D/N	Remove Gla residue	WO2005075635
P10	Q	Increases Tissue Factor-dependent activity, coagulant activity, and binding to phospholipids	Harvey, (2003) *J. Biol. Chem.* 278(10):8363-9; Shah *et al.*, (1998) *Proc. Natl. Acad. Sci. U.S.A.* 95:4229-4234; Taboureau *Eur. Biophys. J.* (2007) 36(2):133-44; Henderson, *Thromb Haemost.* (2002) 88(1):98-103; Nelsestuen (2001) *J. Biol. Chem.* 276(43):39825-31; Stone (2005) *Biochemistry* 44(16):6321-30; WO200183725; WO2006035057; WO2005123916; WO2006114105; US 6911323; US20050113565; US20030211094
	E	Increases phospholipid membrane binding affinity and/or tissue factor independent activity	WO2006114105
	D	Increased phospholipid membrane binding affinity and/or tissue factor independent activity	WO2006114105
	N	Increases glycosylation by creating an N-glycosylation site; Increases activity	US 6806063; US 6911323; WO200183725; WO2006114105
S12	C	Facilitates chemical group linkage	WO2006/134173
L13	Q	FVII:C assay (compound heterozygous) = 2	Giansily-Blaizot M. Eur J Hum Genet. 2001 Feb;9(2):105-12.
E14	Q/D/N	Remove Gla residue	WO2005075635
E16	Q/D/N	Remove Gla residue	WO2005075635
K18	E/D/A/F/C	Decreases Tissue Factor binding	US20060205036
E19	Q/D/N	Remove Gla residue	WO2005075635
E20	Q/D/N	Remove Gla residue	WO2005075635
Q21	N	Increases glycosylation by creating an N-glycosylation site	US 6806063
C22	R	Factor VII deficiency; mild bleeding phenotype	Fromovich-Amit, J Thromb Haemost. 2004 2(10):1774-81
S23	C	Facilitates chemical group linkage	WO2006/134173
E25	Q/D/N	Remove Gla residue	WO2005075635
E26	Q/D/N	Remove Gla residue	WO2005075635
R28	E/F	Increased phospholipid membrane binding affinity and/or tissue factor independent activity	WO2006114105
E29	Q/D/N	Remove Gla residue	WO2005075635
I30	C/D/E	Facilitates chemical group linkage	US 6806063

P del Residuo	Variante(s)	Efecto de la variación	Referencia
	N (with K32S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	C	Facilitates chemical group linkage	WO2006/134173
F31	N (with D33S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
K32	E	Increases binding to phospholipids and coagulant activity; Facilitates chemical group linkage;	Cheung (1995) *Thromb. Res.* 80(5):419-27; Harvey, (2003) *J. Biol. Chem.* 278(10):8363-9; Henderson, *Thromb Haemost.* (2002) 88(1):98-103; Nelsestuen (2001) *J. Biol. Chem.* 276(43):39825-31; Shah *et al.*, (1998) *Proc. Natl. Acad. Sci. U.S.A.* 95:4229-4234; Stone (2005) *Biochemistry* 44(16):6321-30; Taboureau (2007) *Eur. Biophys. J.* 36(2):133-44; WO2006/035057; WO2005123916; WO2006114105; US 6806063; US 6911323; US20030211094; US20050113565
	Q	Increases resistance to proteases	US 5580560
	H	Increases resistance to proteases	US 5580560; US 6806063
	A	Increases resistance to proteases	Iakhiaev, (2001) *Thromb. Haemost.* 85(3):458-63; US 5580560
	G	Increases resistance to proteases	US 5580560
	C	Facilitates chemical group linkage	US 6806063; WO 2006134173
	D	Facilitates chemical group linkage; Improved clotting activity	US 6806063; WO 2006114105
	S/T	Increases glycosylation by creating an N-glycosylation site and/or an O-glycosylation site; increases resistance to proteases	US 6806063; US 5580560
	S/T (with I30N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	N (with A34S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
D33	E	Increases binding to phospholipids; increased activity	Harvey et al. (2003) *J. Biol. Chem.* 278(10):8363-9; WO 2006/114105
	F	Increases binding to phospholipids; increased activity	Harvey et al. (2003) *J. Biol. Chem.* 278(10):8363-9; WO 2006/114105; Taboureau and Olsen (2007) *Eur. Biophys. J.* 36(2):133-44
	K	Increases membrane affinity, autoactivation and activity	US2003/0100506
	C	Facilitates chemical group linkage	US 6806063; WO 2006/134173
	S/T (with F31N)	Increases glycosylation by creating an N-glycosylation site	US 6806063

P del Residuo	Variante(s)	Efecto de la variación	Referencia
A34	E	Increases binding to phospholipids; facilitates chemical group linkage	Harvey et al. (2003) *J. Biol. Chem.* 278(10):8363-9; US 6806063; WO 2006/114105
	M	Increases affinity for phospholipids	WO2004111242; WO2006114105
	W/Y	Increases affinity for phospholipids	WO2004111242; WO2006114105
	V	Increases affinity for phospholipids	WO2004111242
	I	Increases affinity for phospholipids	WO2004111242; WO2006114105
	D	Facilitates chemical group linkage; increases affinity for phospholipids	US 6806063; WO 2006114105
	F	Increases affinity for phospholipids	WO2004111242
	L	Increases affinity for phospholipids	WO2004111242
	C	Facilitates chemical group linkage	US 6806063; WO2006134173
	S/T (with K32N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	N (with R36S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
E35	Q/D/N	Remove Gla residue	WO2005075635
R36	E	Increases Tissue Factor-dependent activity; increases affinity for phospholipids	WO2004111242; WO2006114105
	E/D/K/A /F/N/C	Decreases Tissue Factor binding	US20060205036
	D	Increases affinity for phospholipids	WO 2006114105
	S/T (with A34N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	N (with K38S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
T37	D/E	Facilitates chemical group linkage	US 6806063
	C	Facilitates chemical group linkage	US 6806063; WO 2006/134173
	N (with L39S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
K38	E	Increases Tissue Factor-dependent activity	WO2004111242
	C	Facilitates chemical group linkage	US 6806063; WO 2006/134173
	H	Increases resistance to proteases	US 5580560; US 6806063
	N	Increases resistance to proteases	US 5580560; US 6806063
	A	Increases resistance to proteases	US 5580560; US 6806063; WO2005051289
	T	Increases resistance to proteases	Cheung (1995) *Thromb Res.* 80(5):419-27; US 5580560; US 6806063
	D/L/G/S	Increases resistance to proteases	US 5580560; US 6806063
	D/E	facilitates chemical group linkage; increased affinity for phospholipids	US 6806063; WO 2006/114105
	N (with F40S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	S/T (with R36N)	Increases glycosylation by creating an N-glycosylation site	US 6806063

P del Residuo	Variante(s)	Efecto de la variación	Referencia
L39	S/T (with T37N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	E (with K32E, D33N, A34T and K38T)	Creates a binding site for A-7 antibody; can be activated by FXIa	Cheung (1995) *Thromb Res.* 80(5):419-27
	A	Increases TF binding; slight decrease in Factor X activation	Dickinson, (1996) *Proc. Natl. Acad. Sci. U.S.A.* 93(25):14379-84;
	E/Q/H	Improves clotting activity; Increases Tissue Factor binding affinity	WO2006114105; US20060111282
	N (with W41S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
F40	S/T (with K38N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	N (with I42S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
W41	D/E	Facilitates chemical group linkage	US 6806063;
	C	Facilitates chemical group linkage	US 6806063; WO 2006/134173
	N	Increases glycosylation by creating an N-glycosylation site	US 6806063;
	S/T (with L39N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
I42	Q	Increases resistance to proteases	US 5580560
	A	Increases resistance to proteases; contact residue with Tissue Factor, but mutation has little effect on Tissue Factor binding	Dickinson, (1996) *Proc. Natl. Acad. Sci. U.S.A.* 93(25):14379-84; US 5580560
	N	Increases resistance to proteases	US 5580560; US 6806063
	K	Facilitates chemical group linkage; Increases Tissue Factor binding affinity	US 6806063; US20060111282
	R	Improved clotting activity; Increases Tissue Factor binding affinity	WO 2006/114105; US20060111282
	S	Increases glycosylation by creating an O-glycosylation site; increases resistance to proteases	US 6806063; US 5580560
	N (with Y44S/T), S/T (with F40N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	N	Increases glycosylation by creating an N-glycosylation site	US 6806063
	K/D/E	facilitates chemical group linkage	US 6806063
	C	Facilitates chemical group linkage	US 6806063; WO 2006/134173
	N (with D46S/T), S/T (with I42N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
S43	H	Increases Tissue Factor binding affinity	WO2005023308; US20070054366; US20060111282; US20060270002; US20060270001; US20060270000; US20060276377; WO2003093465

P del Residuo	Variante(s)	Efecto de la variación	Referencia
	Q	Increases Tissue Factor binding affinity	WO2005023308; WO2006114105; WO2007022784; US20070054366; US20060166874; US20060111282; US20060270002; US20060270001; US20060270000; US20060276377; WO2003093465
	E/R/A/F/ N/C	Decreases Tissue Factor binding	US20060205036
	C	Facilitates chemical group linkage	WO2006134173; WO2006134174; US 6806063; US20060205036; WO2005024006
	N	Increases glycosylation by creating an N-glycosylation site	US20070243588; US 6806063; US20060205036; WO2005024006
	D/K	Facilitates chemical group linkage; Decreases Tissue Factor binding	US20070243588; US 6806063; US20060205036
	E	Facilitates chemical group linkage	US20070243588; US 6806063;
Y44	S/T	Increases glycosylation by creating an O-glycosylation site	US 6806063; US5580560; WO1988010295 EP370036
	S/T (with I42N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	N (with D46S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	K/D/E	Facilitates chemical group linkage	US20070243588; US 6806063;
	C	Facilitates chemical group linkage	US 6806063; US20070243588; WO2006134173
S45	C	Facilitates chemical group linkage	US 6806063; WO2006134173
	D/E	Facilitates chemical group linkage	US 6806063;
D46	C	Facilitates chemical group linkage	US 6806063; WO2006134173
	S/T (with Y44N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	N (with D48S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
G47	N (with Q49S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
D48	S/T (with D46N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
Q49	S/T (with G47N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
A51	N	Increases glycosylation by creating an N-glycosylation site	WO2004029091; WO2006114105; US 6806063

P del Residuo	Variante(s)	Efecto de la variación	Referencia
S52	A	Same or increased proteolytic activity	WO2005075635; Lino et al., Arch. Biochem. Biophys. 352: 182-192, 1998
S53	N	Decreases clotting activity and Decreases binding to tissue factor	WO2004108763; Williamson (2005) *J. Thromb. Haemost.* 3(6):1250-6.
	C	Facilitates chemical group linkage	WO2006/134173
N57	D	Decreases binding to tissue factor due to misfolding to the FVII EGF1 domain	Williamson et al. (2005) J. Thromb. Haemost. 3(6):1250-1256
G58	N	Increases glycosylation by creating an N-glycosylation site	US 6806063; WO2004029091; WO2006114105
G59	S/T	Increases glycosylation by creating an N-glycosylation site	US 6806063
S60	P	FVII:C assay (compound heterozygous) = 2-9	Wulff K. Hum Mutat. 2000;15(6):489-96.
	A	Same or increased proteolytic activity	WO2005075635; Lino et al., Arch. Biochem. Biophys. 352: 182-192, 1998
K62	D/T	Increases coagulant activity	WO2004108763
	N/Q	Increases coagulant activity	WO2004108763
	A	Decreases Tissue Factor binding; slightly decreases rate of Factor X activation	US20060205036; Dickinson, (1996) *Proc. Natl. Acad. Sci. U.S.A.* 93(25):14379-84
	C/F	Decreases Tissue Factor binding	US20060205036
	D	Decreases Tissue Factor binding	US20060205036
	E	Increases coagulant activity and binding to tissue factor; Increases Tissue Factor binding affinity	Williamson et al. (2005) *J. Thromb. Haemost.* 3(6):1250-6; WO2004108763; WO2006/114105; US20060111282
	R	Improved clotting activity; Increases Tissue Factor binding affinity	WO 2006/114105; US20060111282
Q64	C/E/D/K/R/F/N	Decreases Tissue Factor binding	US20060205036
	A	Decreases Tissue Factor binding; slightly decreases rate of Factor X activation	US20060205036; Dickinson, (1996) *Proc. Natl. Acad. Sci. U.S.A.* 93(25):14379-84
L65	A	Decreases binding to tissue factor	US20060205036
	C/E/K/R/F	Decreases Tissue Factor binding	US20060205036
	Q	Improved clotting activity; Increases Tissue Factor binding affinity	WO 2006/114105; US20060111282
	S	Improved clotting activity; Increases Tissue Factor binding affinity	WO 2006/114105; US20060111282
	N	Increases glycosylation by creating an N-glycosylation site	US 6806063;
S67	C	Facilitates chemical group linkage	WO2006/134173
	A	No change in TF binding or rate of Factor X activation	Dickinson, (1996) *Proc. Natl. Acad. Sci. U.S.A.* 93(25):14379-84
Y68	C	FVII:C assay (compound heterozygous) = 1	Wulff K. Hum Mutat. 2000;15(6):489-96.
I69	C/D/E/F/G/H/K/L/M/N/P/Q/R/S/T/V/W/Y	Decreases Tissue Factor binding	US20060205036

P del Residuo	Variante(s)	Efecto de la variación	Referencia
	A	Decreases Tissue Factor binding; decreases rate of Factor X activation	US20060205036; Dickinson, (1996) *Proc. Natl. Acad. Sci. U.S.A.* 93(25):14379-84
F71	D/Y/E/Q /N	Improved clotting activity; Increases Tissue Factor binding affinity	WO 2006/114105; US20060111282
	A	Decreases Tissue Factor binding; no change in rate of Factor X activation	US20060205036; Dickinson, (1996) *Proc. Natl. Acad. Sci. U.S.A.* 93(25):14379-84
	C	Decreases Tissue Factor binding	US20060205036
L73	C	Decreases Tissue Factor binding	US20060205036
	A	No change in TF binding or rate of Factor X activation	Dickinson, (1996) *Proc. Natl. Acad. Sci. U.S.A.* 93(25):14379-84
P74	A	no change in binding to tissue factor	WO2004108763; Williamson *et al.* (2005) *J. Thromb Haemost.* 3(6):1250-6
	C	Decreases Tissue Factor binding	US20060205036
	S	Increases Tissue Factor-dependent activity	WO 2004/111242; WO2004108763
A75	D	Increases coagulant activity and binding to tissue factor	WO2004108763; Williamson *et al.* (2005) *J. Thromb Haemost.* 3(6):1250-6
	E	Increases coagulant activity and binding to tissue factor	WO2004108763
E77	C	Decreases Tissue Factor binding	US20060205036
	A	No change in affinity for phospholipid reconstituted Tissue Factor or rate of FXa formation	Dickinson, *Proc. Natl. Acad. Sci. U S A.* 1996 93(25):14379-84; WO 2006/114105
G78	C	Decreases Tissue Factor binding	US20060205036
R79	C/A	Decreases Tissue Factor binding; No change in rate of Factor X activation	US20060205036; Dickinson, *Proc. Natl. Acad. Sci. U S A.* 1996 93(25):14379-84;
	W	Asymptomatic phenotype	McVey JH. Hum Mutat. 2001;17(1):3-17
	Q	Asymptomatic phenotype; decreased binding to tissue factor	McVey JH. Hum Mutat. 2001;17(1):3-17; Williamson et al. (2005) J. Thromb. Haemost. 3(6):1250-1256
E82	Q/N	Improved clotting activity; Increases Tissue Factor binding affinity	WO 2006/114105; US20060111282
	K	Increases Tissue Factor binding affinity	US20060111282
	S/T	Increases glycosylation by creating an N-glycosylation site	US 6806063;
T83	C	Facilitates chemical group linkage	WO2006/134173
	K	Increases binding to tissue factor	WO2004108763; Williamson (2005) *Thromb Haemost.* 3(6):1250-6
K85	C	Decreases Tissue Factor binding	US20060205036
Q88	C	Decreases Tissue Factor binding	US20060205036
	A	No change in TF binding or rate of Factor X activation	Dickinson, (1996) *Proc. Natl. Acad. Sci. U.S.A.* 93(25):14379-84
V92	A	No change in TF binding or Factor X activation	Dickinson, *Proc. Natl. Acad. Sci. U S A.* 1996 93(25):14379-84
N93	A	No change in TF binding or Factor X activation	Dickinson, *Proc. Natl. Acad. Sci. U S A.* 1996 93(25):14379-84
	C	Decreases Tissue Factor binding	US20060205036
E94	A	No change in TF binding or Factor X activation	Dickinson, *Proc. Natl. Acad. Sci. U S A.* 1996 93(25):14379-84

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P del Residuo	Variante(s)	Efecto de la variación	Referencia
	L	Asymptomatic phenotype	Mariani et al. (2005) Thromb Haemost 93:481-487
	K	FVII:C assay (homozygous) = 13	Wulff K. Hum Mutat. 2000;15(6):489-96.
N95	S/T	Increases glycosylation by creating an N-glycosylation site	US 6806063;
G96	S	FVII:C assay (compound heterozygous) = 2	Giansily-Blaizot M. Eur J Hum Genet. 2001 Feb;9(2):105-12.
G97	S	Increases glycosylation by creating an N-glycosylation site; asymptomatic phenotype; FVII:C assay (compound heterozygote)= 14-40	US 6806063; McVey (2001) *Hum. Mutat.* 17(1):3-17; Wulff (200) *Hum. Mutat.* 15(6):489-96.;
	C	Mild clinical severity	McVey (2001) *Hum. Mutat.* 17(1):3-17;
	T	Increases glycosylation by creating an N-glycosylation site	US 6806063
Q100	R	Severe clinical severity a major secretion defect and markedly impaired function of the small amount of FVII that is released from cells; FVII:C assay (heterozygous) = 35	McVey (2001) *Hum. Mutat.* 17(1):3-17; Mariani et al. (2005) Thromb Haemost 93:481-487; Hunault et al. (1999) Blood 93(4):1237-1244; D'Andrea, Haematologica. 2004 89(8):979-84
Y101	N	Increases glycosylation by creating an N-glycosylation site	US 6806063
C102	Y	FVII:C assay (heterozygous) = 2	Giansily-Blaizot M. Eur J Hum Genet. 2001 Feb;9(2):105-12.
S103	C	Facilitates chemical group linkage	WO2006/134173
D104	N	Increases glycosylation by creating an N-glycosylation site	US 6806063
T106	D		WO2006114105
	N (alone or with S60T)	Increases glycosylation by creating an N-glycosylation site	US 6806063; US20050113565; US2006/0194289; US 6,806,063; WO2004029091, WO2005123916; WO2006134173; WO2006114105
	C	Facilitates chemical group linkage	WO2006/134173
T108	C	Facilitates chemical group linkage	WO2006/134173
K109	N	Increases glycosylation by creating an N-glycosylation site	US 6806063; WO2006114105
R110	S	Reduces expression	D'Andrea, Haematologica. 2004 89(8):979-84
	C	Reduced expression and reduced activity	D'Andrea, Haematologica. 2004 89(8):979-84
	C (with D123Y)	Reduced functional and antigenic FVII plasma levels	D'Andrea, Haematologica. 2004 89(8):979-84
S111	C	Facilitates chemical group linkage	US 6806063; WO2006/134173
H115	A	No change in TF binding or Factor X activation	Dickinson, *Proc. Natl. Acad. Sci. U S A.* 1996 93(25):14379-84
E116	D		
G117	N	Increases glycosylation by creating an N-glycosylation site	US 6806063
S119	C	facilitates chemical group linkage	US 6806063; WO2006/134173
D123	Y	Reduced expression and reduced activity	D'Andrea, Haematologica. 2004 89(8):979-84

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P del Residuo	Variante(s)	Efecto de la variación	Referencia
	Y (with R110C)	Reduced functional and antigenic FVII plasma levels	D'Andrea, Haematologica. 2004 89(8):979-84
G124	N	Increases glycosylation by creating an N-glycosylation site	WO2004029091; US 6806063
S126	N	Increases glycosylation by creating an N-glycosylation site	US 6806063
	C	Facilitates chemical group linkage	WO2006/134173
T128	N	Increases glycosylation by creating an N-glycosylation site	US 6806063
	C	Facilitates chemical group linkage	WO2006/134173
T130	C	Facilitates chemical group linkage	WO2006/134173
P134	T	Asymptomatic phenotype	McVey (2001) *Hum. Mutat.* 17(1):3-17;
C135	R	FVII:C assay (homozygous) = 1-4	Wulff K. Hum Mutat. 2000;15(6):489-96.
K137	N (with P139S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	E	Asymptomatic phenotype	McVey (2001) *Hum. Mutat.* 17(1):3-17;
I140	N (with E142S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
L141	D	Facilitates chemical group linkage	US 6806063
	E	Facilitates chemical group linkage	US 6806063
	C	Facilitates chemical group linkage	US 6806063; WO2006134173
	N (with K143S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
E142	D	Facilitates chemical group linkage	US 6806063
	S/T (with I140N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	C	Facilitates chemical group linkage	US 6806063; WO2006134173
	N (with R144S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
K143	C/D/E	Facilitates chemical group linkage	US 6806063
	N (with N145S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	S/T	Increases glycosylation by creating an O-glycosylation site	US 6806063
	S/T (with L141N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
R144	D/E	Facilitates chemical group linkage	US 6806063
	C	Facilitates chemical group linkage	US 6806063; WO2006/134173
	N (with A146S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	S/T (with E142N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
N145	Y/G/F/M/I/L/V/P/K/H/Q/E/R/W/C	Decreases glycosylation by disrupting an N-glycosylation site	WO 2005/123916
	D	Decreases glycosylation by disrupting an N-glycosylation site	WO2005123916; US20050113565
	S	Decreases glycosylation by disrupting an N-glycosylation site	WO2005123916; US6806063

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P del Residuo	Variante(s)	Efecto de la variación	Referencia
	T	Decreases glycosylation by disrupting an N-glycosylation site	US 6806063; US20050113565; US2006/0194289; WO2005123916; WO2006114105; WO2006114448; WO2006067230
	Q	Increases activity	WO2005123916
A146	P	Decreases glycosylation by disrupting an N-glycosylation site	WO2005123916
	N with K148S/T	Increases glycosylation by creating an N-glycosylation site	US 6806063
	S/T (with E142N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
S147	N (with P149S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	C	Facilitates chemical group linkage	US 6806063
K148	S/T (with A146N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	P	Decreases glycosylation	WO2005123916
P149	S/T (with S147N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
R152	Q	Severe clinical severity; stable zymogen form that is not cleaved; FVII:C assay (heterozygous) = 1	McVey JH. Hum Mutat. 2001;17(1):3-17; Toso et al. (2003) Biochem J. 369(Pt 3):563-71; Wulff K. Hum Mutat. 2000;15(6):489-96.
I153	N (with G155S/T)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
V154	G, A	Reduces activity	Toso et al. (2003) Biochem J. 369(Pt 3):563-71.
G155	S/T (with I153N)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
G156	D	FVII:C assay (compound heterozygous) = 15	Wulff K. Hum Mutat. 2000;15(6):489-96.
K157	L/I/M/F/ W/P/G/Y /N/ S/T/C/E/ D/V/Q	Increases coagulant activity	WO2002022776
	Q/N	Increases coagulant activity; decreases conjugation to non polypeptide moieties	WO2002022776; US 6806063
	A	No change in activity or binding to tissue factor	Dickinson, *Proc. Natl. Acad. Sci. U S A.* 1996 93(25):14379-84
	R/H	Decreases conjugation to non polypeptide moieties	US 6806063
V158	L/I/M/F/ W/P/G/Y /R/K/H/	Increases Activity	WO2002022776
	C	Increases Activity	WO2002022776; WO2006134173
	T	Increases Activity	WO2002022776; US 6,911,323; US20050113565; US2006/0194289; WO2006114448; WO2006067230

P del Residuo	Variante(s)	Efecto de la variación	Referencia
	S	Increases Activity	WO2002022776; US 6,911,323
	N/Q	Increases Activity	WO2002022776; US 6,911,323
	D	Increases Activity; increases inhibition by AT-III	WO2002022776; Persson, Biochem J. 2004 379(Pt 2):497-503; Allen (2007) *Arterioscler Thromb Vasc Biol.* 27(3):683-9; Bjelke et al. (2007) *FEBS Lett.* 581(1):71-76; Pipe (2005) *J. Thromb. Haemost.* 3(8):1692-701; Persson et al. (2002) *Eur. J. Biochem.* 269(23):5950-5; Soejima et al. (2002) *J. Biol. Chem.* 277(50):49027-35; Petrovan et al. (2002) *Biochemistry* 41(30):9302-9; Persson et al. (2001) *PNAS* 98(24):13583-13588; Persson et al. (2004) *Semin. Hematol.* 41(1 Suppl 1):89-92; WO2006035057; WO2005123916; US 6911323; US20050113565; US20060019894; US2006/0194289; WO 2006/114105; WO 2006/114448; WO 2006/067230;
	A	No change in binding to TF; decreases Factor X activation rate	Dickinson, *Proc. Natl. Acad. Sci. U S A.* 1996 93(25):14379-84
	E	Increases Activity	WO2002022776; Bjelke et al. (2007) *FEBS Lett.* 581(1):71-6
	N	Increases Activity	US 6911323; WO2002022776; Petrovan et al. (2002) *Biochemistry.* 41(30):9302-9
P160	A	No change in activity or binding to tissue factor	Dickinson, *Proc. Natl. Acad. Sci. U S A.* 1996 93(25):14379-84
K161	A	No change in TF binding; Decreases Factor X activation	Dickinson, *Proc. Natl. Acad. Sci. U S A.* 1996 93(25):14379-84
E163	A	No change in activity or binding to tissue factor	Dickinson, *Proc. Natl. Acad. Sci. U S A.* 1996 93(25):14379-84
C164	V (with V299C)	Decreases amidolytic activity in the presence or absense of Tissue Factor; minor increase in proteolytic activity in the absense of Tissue Factor	Olsen et al. (2004) Biochemistry 43:14096-14103
Q167	N (with L169S/T)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
V168	N (with L170S/T/)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
L169	S/T (with Q167N)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874

P del Residuo	Variante(s)	Efecto de la variación	Referencia
	N (with L171S/T/)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
L170	N (with V172S/T)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
	S/T (with V168N)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
L171	N (with N173S/T)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
	A	No change in activity or binding to tissue factor	Dickinson, *Proc. Natl. Acad. Sci. U S A.* 1996 93(25):14379-84
	S/T (with L169N)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
V172	S/T (with L170N)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
	A	No change in activity or binding to tissue factor	Dickinson, *Proc. Natl. Acad. Sci. U S A.* 1996 93(25):14379-84
N173	S/T (with L171N)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
	A	Increases binding to TF; no change in rate of activation of Factor X	Dickinson, *Proc. Natl. Acad. Sci. U S A.* 1996 93(25):14379-84
A175	S/T	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874; WO2006114105; WO2003093465
	N (with L177S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
Q176	A	Decreases TFPI inhibition, inactive proteolytic function	Iakhiaev, (2001) *Thromb. Haemost.* 85(3):458-63; Dickinson, *Proc. Natl. Acad. Sci. U S A.* 1996 93(25):14379-84
L177	S/T/ (with A175N)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
	A	Inactivates proteolytic function	Dickinson, *Proc. Natl. Acad. Sci. U S A.* 1996 93(25):14379-84
	N (with G179S/T)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
C178	Y	Asymptomatic phenotype	McVey JH. Hum Mutat. 2001;17(1):3-17
G179	N	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874; US 6806063
	S/T (with L177N)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
G180	N (with L182S/T)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
T181	N (with I183S/T)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874

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P del Residuo	Variante(s)	Efecto de la variación	Referencia
L182	S/T (with G180N)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
I183	S/T (with T181N)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
T185	C	Facilitates chemical group linkage	WO2006/134173
I186	S/T	Increases glycosylation by creating an N-glycosylation site	US 6806063
V188	N	Increases glycosylation by creating an N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
V189	N (with A191S/T)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
S190	N (with A192S/T)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
A191	S/T (with V189N)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
	N (with H193S/T)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
A192	S/T/ (with S190N)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
H193	S/T (with A191N)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
	N (with F195S/T)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
C194	Y	FVII:C assay (compound heterozygous) < 1	Wulff K. Hum Mutat. 2000;15(6):489-96.
F195	S/T (with H193N)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
	N (with K197S/T)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
D196	N (with I198S/T)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
	A	Decreases rate of activation of Factor X; no change in binding to TF	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
K197	N (with K199S/T)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
	A	Decreases catalytic activity	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
I198	S/T (with D196N)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
	A	No change in activity or binding to tissue factor	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
	N (with N200S/T)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874

P del Residuo	Variante(s)	Efecto de la variación	Referencia
K199	N (with W201S/T)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
	A	Decreases TF binding and rate of activation of Factor X	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
	S/T (with K197N)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
N200	A	Increases TF binding; no change to rate of Factor X activation	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
W201	S/T (with K199N)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
	N (with N203S/T)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
R202	A	No change in binding to TF; decreases rate of activation of Factor X	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
	S/T	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
N203	S/T (with W201N)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
	A	No change in binding to TF; slightly increased rate of activation of Factor X	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
I205	S/T	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
	A	increases TF binding; decreases rate of activation of Factor X	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
A206	T	FVII:C assay (compound heterozygous) = 15	Wulff K. Hum Mutat. 2000;15(6):489-96.
V207	A	No change in TF binding; decreases Factor X activation	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
E210	A	Decreased catalytic activity	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
D212	N	Increases glycosylation by creating an N-glycosylation site	WO2004029091, US 6806063; Mariani et al. (2005) *Thromb Haemost.* 93(3):481-7
	A	decreases rate of acivation of Factor X; no change to binding to TF	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
L213	A	decreased proteolytic function	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
S214	C	Facilitates chemical group linkage	WO2006/134173
	A	decreases rate of acivation of Factor X; no change to binding to TF	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
E215	A	Decreases binding to TF; Decreases Factor X activation	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
H216	A	No change in binding to TF or Factor X activation	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
D217	A	No change in binding to TF or Factor X activation	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
D219	A	Increased TF binding; decreases Factor X activation	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
E220	N	Increases glycosylation by creating an N-glycosylation site	US 6806063

P del Residuo	Variante(s)	Efecto de la variación	Referencia
	A	Decreased catalytic activity; no change to binding to TF	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
Q221	A	No change in activity or binding to tissue factor	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
S222	C	Facilitates chemical group linkage	WO2006/134173
R223	A	No change in activity or binding to tissue factor	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
	W	Asymptomatic phenotype	McVey JH. Hum Mutat. 2001;17(1):3-17.
R224	A	No change in activity or binding to tissue factor	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
Q227	A	No change in activity or binding to tissue factor	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
V228	N (with I230S/T)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
I229	N (with P231S/T)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
I230	N	Increases glycosylation by creating an N-glycosylation site	US 6806063
	N	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
	S/T (with V228N)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
P231	C	Facilitates chemical group linkage	WO2006134173
	N	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
	S/T (with I229N)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
S232	C	Facilitates chemical group linkage	WO2006/134173
	A	No change in activity or binding to tissue factor	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
	N (with Y234S/T)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
T233	C	Facilitates chemical group linkage	WO2006/134173
	N (with V235S/T)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
Y234	N (with P236S/T)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
	S/T (with S232N)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
V235	N (with G237S/T)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
	A	No change in activity or binding to tissue factor	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
P236	N	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874

P del Residuo	Variante(s)	Efecto de la variación	Referencia
G237	N	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
	S/T (with V235N)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
T238	C	Facilitates chemical group linkage	WO2006/134173
	A	No change in activity or binding to tissue factor	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
	N (with N240S/T)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
T239	N (with H241S/T)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	WO2004029091; US 6806063; WO 2006/114105; US20060166874
	A	Decreased activitation of Factor X, decreased TFPI inhibition; no change in binding to TF	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84; Iakhiaev, (2001) *Thromb. Haemost.* 85(3):458-63;
	C	Facilitates chemical group linkage	WO2006/134173
N240	S/T (with T238N)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
	A	Decreased activitation of Factor Xl; no change in binding to TF	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
H241	S/T (with T239N)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
	N (with I243S/T)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
D242	S/T	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
	H	Severe bleeding tendency; FVII:C assay (compound heterozygous) = 2-4	McVey Hum Mutat. 2001;17(1):3-17; Wulff K. Hum Mutat. 2000;15(6):489-96
I243	S/T (with H241N)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
	N (with L245S/T)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
A244	N (with L246S/T)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
	V	Asymptomatic; FVII:C assay (homozygous) = 3	McVey Hum Mutat. 2001;17(1):3-17.; Mariani et al. (2005) Thromb Haemost 93:481-487; Wulff K. Hum Mutat. 2000;15(6):489-96
L245	N (with R247S/T)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
L246	S/T (with A244N)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874

P del Residuo	Variante(s)	Efecto de la variación	Referencia
	N (with L248S/T)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
R247	S/T (with L245N)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
	H	Asymptomatic	McVey Hum Mutat. 2001;17(1):3-17.
	C	FVII:C assay (compound heterozygous) = 10	Wulff K. Hum Mutat. 2000;15(6):489-96.
	A	slightly increased binding to TF; slightly decreased rate of activation of Factor X	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
L248	S/T (with L246N)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
H249	A	No change in activity or binding to tissue factor	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
Q250	C	Facilitates chemical group linkage	WO2006/134173
	A	No change in activity or binding to tissue factor	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
V252	M	FVII:C assay (compound heterozygous) = 9	Wulff K. Hum Mutat. 2000;15(6):489-96.
V253	N	Increases glycosylation by creating an N-glycosylation site; Increases activity	US 6806063; WO2004029090
T255	C	Facilitates chemical group linkage	WO2006/134173
D256	A	No change in activity or binding to tissue factor	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
E265	N	Increases glycosylation by creating an N-glycosylation site	US 6806063
	K	Asymptomatic; FVII:C assay (heterozygous) = 44	McVey Hum Mutat. 2001;17(1):3-17; Wulff K. Hum Mutat. 2000;15(6):489-96.
R266	A	No change in activity or binding to tissue factor	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
T267	C	Facilitates chemical group linkage	WO2006/134173
	N	Increases glycosylation by creating an N-glycosylation site	US 6806063
E270	N	Increases glycosylation by creating an N-glycosylation site	US 6806063
	A	No change in activity or binding to tissue factor	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
R271	A	No change in activity or binding to tissue factor	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
T272	A	No change in activity or binding to tissue factor	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
A274	D/V/I/F/W/P/G/T/C/Y/E/H/S/Q	Increases activity	WO2004029090
	M/L/K/R/	Increases activity	WO200183725; WO2003027147; WO2004029090; US 6911323
F275	H	Improved clotting activity; Increases Tissue Factor binding affinity	WO 2006/114105; US20060111282
	C	Decreases Tissue Factor binding	US20060205036
	A	No change in activity or binding to tissue factor	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84

P del Residuo	Variante(s)	Efecto de la variación	Referencia
R277	N	Increased glycosylation by creating an N-glycosylation site	US 6806063
	A	Reduced affinity for tissue factor	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
	C	Decreases Tissue Factor binding; Mild to severe hemophilia	US20060205036; Mariani et al. (2005) Thromb Haemost 93:481-487
F278	A	Decreases activity; no change in binding to tissue factor	Dickinson et al. (1996) *Proc Natl Acad Sci USA.* 93(25):14379-84
	S/A/G/N/Q	Increases resistance to proteases	US 5580560
S279	C (with V302C)	Increases activity by shifting equilibrium in favor of disulfide locked active conformation	Maun *et al.* (2005) Prot Sci 14:1171-1180
L280	N	Increased glycosylation by creating an N-glycosylation site	US 6806063
	I (with V299M)	minor increase in amidolytic activity in the absense of Tissue Factor; minor decrease in amidolytic activity in the presence of Tissue Factor; minor increase in proteolytic activity in the absense of Tissue Factor	Olsen et al. (2004) Biochemistry 43:14096-14103
	C (with N301C)	Increases activity by shifting equilibrium in favor of disulfide locked active conformation	Maun *et al.* (2005) Prot Sci 14:1171-1180
	K/C/D/E	Facilitates chemical group linkage	US 6806063
V281	C (with V302C)	Increases activity by shifting equilibrium in favor of disulfide locked active conformation	Maun *et al.* (2005) Prot Sci 14:1171-1180
	F	FVII:C assay (compound heterozygous) < 1	Wulff K. Hum Mutat. 2000;15(6):489-96.
	N (with G283S/T)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
S282	C	Facilitates chemical group linkage	US 6806063; WO2006134173
	C (with V299C)	Increases activity by shifting equilibrium in favor of disulfide locked active conformation	Maun *et al.* (2005) Prot Sci 14:1171-1180
	N (with W284S/T)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
G283	S/T (with V281N)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
	S	severe hemophilia	Mariani et al. (2005) Thromb Haemost 93:481-487
	N (with G285S/T)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
W284	S/T (with S282N)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
	N (with Q286S/T)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
G285	S/T (with G283N)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874

P del Residuo	Variante(s)	Efecto de la variación	Referencia
	N (with L287S/T)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
Q286	N (with L288S/T)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
	A	No change in activity or binding to tissue factor	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
L287	N (with D289S)	Increased glycosylation by creating an N-glycosylation site	US 6806063
	A	decreases activity; Decreased amidolytic activity in the presence of Tissue Factor; increased binding to Tissue Factor	Dickinson, Proc Natl Acad Sci U S A. 1996 93(25):14379-84; Jin et al. (2001) J Mol Biol. 307(5):1503-17
	S/T (with G285N)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
L288	K/C/D/E	Facilitates chemical group linkage	US 6806063
	N (with R290S/T)	Increased glycosylation by creating an N-glycosylation site	US 6806063
	S/T (with Q286N)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
	A	No change in activity or binding to tissue factor	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
	C	Facilitates chemical group linkage	WO2006/134173
D289	C	Facilitates chemical group linkage	US 6806063; WO2006134173
	A	No change in activity or binding to tissue factor	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
	N (with G291S/T)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US20060166874
	K	Facilitates chemical group linkage	US 6806063
R290	S/T	Increases resistance to proteases; Increases glycosylation by creating an O-glycosylation site;	WO88/10295; US 5580560; US 6806063
	S/T (with L288N)	Increased glycosylation by creating an N-glycosylation site	US 6806063
	G	Increases resistance to proteases	WO88/10295; US 5580560
	C/D/E	Facilitates chemical group linkage	US 6806063; WO 2006/134173; Mariani, Thromb Haemost. 2005 93(3):481-7; Furlan, J Thromb Haemost. 2003 1(11):2455-7
	A	Decreases activity; no change in binding to tissue factor; Increases resistance to proteases	Dickinson, Proc Natl Acad Sci U S A. 1996 93(25):14379-84; WO 88/10295; 5,580,560; Furlan, J Thromb Haemost. 2003 1(11):2455-7; Jin J .J Mol Biol. 2001 Apr 13;307(5):1503-17; Ruf W. Biochemistry. 1994 Sep 27;33(38):11631-6.

P del Residuo	Variante(s)	Efecto de la variación	Referencia
	K	facilitates chemical group linkage; Increases resistance to proteases	US 6806063; US 5580560); WO8810295; Furlan, J Thromb Haemost. 2003 1(11):2455-7
	N (with A292S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
G291	N	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
	S/T (with R289N)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
	C	Facilitates chemical group linkage	US 6806063; WO2006134173
	K/D/E	Facilitates chemical group linkage	US 6806063
A292	N (with L294S/T)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
	C	Facilitates chemical group linkage	WO2006/134173
	S/T (with R290N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	K/D/E	Facilitates chemical group linkage	US 6806063
T293	K	Facilitates chemical group linkage	US 6806063
	N (with L295S/T)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
	C	Facilitates chemical group linkage	WO2006/134173
	A	decreased proteolytic function	Dickinson, Proc Natl Acad Sci U S A. 1996 93(25):14379-84
A294	S/T (with A292N)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
	V (with C310F/R304Q)	patient experienced gum bleeding	Etro et al. (2005) J. Hematology 88(12):1434-1436
	V	Moderate bleeding tendency; Asymptomatic to severe hemophilia; FVII:C assay (homozygous) = 6	McVey Hum Mutat. 2001;17(1):3-17; Mariani et al. (2005) Thromb Haemost 93:481-487; Wulff K. Hum Mutat. 2000;15(6):489-96.
L295	S/T (with L293N)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
	A	decreases rate of activation of Factor X; no change in binding to TF	Dickinson, Proc Natl Acad Sci U S A. 1996 93(25):14379-84
E296	K	Increases Activity	WO2002022776; US 6911323
	A	decreased TFPI inhibition; decreased activity	Iakhiaev, (2001) *Thromb. Haemost.* 85(3):458-63; Dickinson, Proc Natl Acad Sci U S A. 1996 93(25):14379-84
	R	Increases Activity	WO2002022776; Persson et al. (2001) *PNAS* 98(24):13583-13588; US 6911323
	L	Increases Activity	WO2002022776; Petrovan et al. (2002) *Biochemistry* 41(30):9302-9; US 6911323

P del Residuo	Variante(s)	Efecto de la variación	Referencia
	V	Increases Activity; increases inhibition by AT-III	Allen (2007) *Arterioscler Thromb Vasc Biol.* 27(3):683-9; Persson et al. (2004) *Biochem J.* 379(Pt 2):497-503; Persson et al. (2002) *Eur J Biochem.* 269(23):5950-5.; Petrovan et al. (2002) *Biochemistry.* 41(30):9302-9; Pipe et al. (2005) J. Thromb. Haemost. 3(8):1692-701; Soejima et al. (2002) *J. Biol. Chem.* 277(50):49027-35.; Persson et al. (2001) *PNAS* 98(24):13583-13588; Persson et al. (2004) *Semin Hematol.* 41(1 Suppl 1):89-92; WO2002022776; WO2006035057; WO2005123916; US 6911323; US20050113565; US20060019894
	V (with V158D/M298Q)	increased procoagulant activity; significantly greater platelet contractile force and clot elastic modulus, and shorter force onset time values	Brophy et al. (2007) Haemophilia. 13(5):533-41
	I	Increases Activity	WO2002022776; Bjelke JR,.FEBS Lett. 2007 Jan 9;581(1):71-6.; Petrovan RJ. Biochemistry. 2002 Jul 30;41(30):9302-9.; WO 2006/035057; WO 2005/123916; US2006-0019894; US 6,911,323; US20050113565
	T	Increases Activity	WO2002022776; WO2006035057; WO2005123916; US20060019894; US20050113565)
	D	Increases Activity	WO2006114105
	M/F/W/P/G/S/C/Y/N/ /H/Q	Increases Activity	WO2002022776
M298	L/F/W/P/G/T/Y/H/E	Increases Activity	WO2002022776
	E	Reduces proteolytic activity	WO2002038162
	D/S	Reduces proteolytic activity	Petrovan (2001) *J. Biol. Chem.* 276(9):6616-20; WO2002038162
	V	Reduced proteolytic activity; Asymptomatic phenotype	McVey JH. Hum Mutat. 2001;17(1):3-17.; Petrovan (2001) *J. Biol. Chem.* 276(9):6616-20; WO2002/038162

P del Residuo	Variante(s)	Efecto de la variación	Referencia
	I	Reduced coagulant activity; mild clinical severity; FVII:C assay (heterozygous) = 41	Bernardi F. Hum Mutat. 1996;8(2):108-15; McVey JH. Hum Mutat. 2001;17(1):3-17.; Wulff (2000) Hum. Mutat. 15(6):489-96.; Mariani et al. Blood Coagul Fibrinolysis. 1998, 9 Suppl 1:S83-8;
	Q	Increased in vitro proteolytic activity; increases inhibition by AT-III	Persson E. PNAS 2001 98(24):13583-13588; Persson E. Semin Hematol. 2004 Jan;41(1 Suppl 1):89-92; WO2002022776; WO2006035057; WO2005123916; US20060019894; US 6911323; US20050113565; US20060194289; WO2006114105; WO2006114448; WO2006067230
	Q (with V158D/E2 96V)	increased procoagulant activity; significantly greater platelet contractile force and clot elastic modulus, and shorter force onset time values	Brophy et al. (2007) Haemophilia. 13(5):533-41
	K	Reduced proteolytic activity	Persson E. PNAS 2001 98(24):13583-13588; Petrovan RJ. J Biol Chem. 2001 Mar 2;276(9):6616-20.; WO 2006/035057; WO 2005/123916; WO 2006/114448; WO 2006/067230; WO2002/038162; US 6911323; US2006-0019894; US20050113565; US2006/0194289
	A	decreases activity	Dickinson, Proc Natl Acad Sci U S A. 1996 93(25):14379-84
	R	Increased coagulant activity	WO2002022776; US 6911323
	N	Reduced proteolytic activity	US 6,911,323; Petrovan RJ. J Biol Chem. 2001 Mar 2;276(9):6616-20.; WO2002/038162
	C	facilitates chemical group linkage	WO 2006/134173
	T	Increases glycosylation by creating an N-glycosylation site	US 6806063; Peyvandi F. Thromb Haemost. 2000 Aug;84(2):250-7.
V299	C (with S282C)	Increases activity by shifting equilibrium in favor of disulfide locked active conformation	Maun *et al.* (2005) Prot Sci 14:1171-1180
	C (with C164V)	decreased amidolytic activity in the presence or absense of Tissue Factor; minor increase in proteolytic activity in the absense of Tissue Factor	Olsen et al. (2004) Biochemistry 43:14096-14103

P del Residuo	Variante(s)	Efecto de la variación	Referencia
	M	minor decrease in amidolytic activity in the absense or presence of Tissue Factor; minor increase in proteolytic activity in the absense of Tissue Factor	Olsen et al. (2004) Biochemistry 43:14096-14103
	M (with L280I)	minor increase in amidolytic activity in the absense of Tissue Factor; minor decrease in amidolytic activity in the presence of Tissue Factor; minor increase in proteolytic activity in the absense of Tissue Factor	Olsen et al. (2004) Biochemistry 43:14096-14103
L300	P	Moderate clinical severity	McVey JH. Hum Mutat. 2001;17(1):3-17
N301	C (with L280C)	Increases activity by shifting equilibrium in favor of disulfide locked active conformation	Maun *et al.* (2005) Prot Sci 14:1171-1180
	A	No change in activity or binding to tissue factor	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
V302	C (with S279C)	Increases activity by shifting equilibrium in favor of disulfide locked active conformation	Maun *et al.* (2005) Prot Sci 14:1171-1180
P303	A	Reduced affinity for tissue factor	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
	R	FVII:C assay (compound heterozygous) = 15	Wulff K. Hum Mutat. 2000;15(6):489-96.
	S/T	Increases glycosylation by creating an N-glycosylation site	US 6806063
R304	Y/F/L/M	Increases activity	WO200183725; US 6911323
	A	Reduced activity and reduced affinity for tissue factor	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
	W	asymptomatic clinical phenotype	McVey JH. Hum Mutat. 2001;17(1):3-17.;
	Q	asymptomatic to moderate clinical phenotype	McVey JH. Hum Mutat. 2001;17(1):3-17.; Mariani et al. (2005) Thromb Haemost 93:481-487
	Q (with C310F/A294V)	patient experienced gum bleeding	Etro et al. (2005) J. Hematology 88(12):1434-1436
	G/T/A/S/N	Increases resistance to proteases	US 5580560; Dickinson et al. (1996) *Proc Natl Acad Sci U S A.* 93(25):14379-84

P del Residuo	Variante(s)	Efecto de la variación	Referencia
L305	V	stabilizes helical structure; Increases activity; increases inhibition by AT-III	Persson, (2004) Biochem J. 379(Pt 2):497-503; Persson *et al.* (2001) PNAS 98:13583-13588; Soejima K. J Biol Chem. 2002 Dec 13;277(50):49027-35.; Persson E. PNAS 2001 98(24):13583-13588; Persson E. Semin Hematol. 2004 Jan;41(1 Suppl 1):89-92; WO200183725; WO2003027147, WO2004029091; WO2006035057); WO2005123916; WO 2006/114105; WO 2006/114448; WO 2006/067230; US 6911323; US2006-0019894); US20050113565
	I	Stabilizes helical structure; increases activity	Persson, Biochem J. 2004 379(Pt 2):497-503; Persson *et al.* (2001) PNAS 98:13583-13588; WO200183725; WO2003027147, WO2004029091; WO200183725; WO 2006/035057); WO 2005/123916; US2006-0019894); US 6,911,323; US20050113565
	Y	Stabilizes helical structure; increases activity	Persson, Biochem J. 2004 379(Pt 2):497-503; Persson *et al.* (2001) PNAS 98:13583-13588; WO200183725; WO2003027147, WO2004029091; WO200183725; US 6,911,323
	T	Stabilizes helical structure; increases activity	WO 2006/035057); WO 2005/123916; US2006-0019894); US20050113565; WO 2006/114448; WO 2006/067230; Persson, Biochem J. 2004 379(Pt 2):497-503; WO200183725
	C	Stabilizes helical structure; increases activity	WO200183725; WO 2006/134173
	F/M/W/P /G/S/N/E /K/R/H/ D/Q	Stabilizes helical structure; increases activity	WO200183725

P del Residuo	Variante(s)	Efecto de la variación	Referencia
	A	Decreases Tissue Factor binding and proteolytic function	WO200183725; Dickinson et al., Proc Natl Acad Sci U S A. 1996 93(25):14379-84; Persson, Biochem J. 2004 379(Pt 2):497-503; Dickinson et al., Proc Natl Acad Sci U S A. 1996 93(25):14379-84
	N	Stabilizes helical structure; increases activity; Increases glycosylation by creating an N-glycosylation site	WO200183725; US 6806063
M306	A	Reduced affinity for tissue factor	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
	D	increases activity	WO200183725; Persson et al. (2001) Biochemistry. 40(11):3251-6; WO 2006/114448; WO 2006/067230; WO 2006/035057 WO 2005/123916 US 6911323; US20060019894; US20050113565; US20060194289; WO2006114448; WO2006067230
	N	increases activity	Persson (2001) *Biochemistry.* 40(11):3251-6; WO200183725; US 6911323
	C	Decreases Tissue Factor binding	US20060205036
T307	C	Facilitates chemical group linkage	WO2006/134173
	A	Decreases rate of activation of Factor X; no change in binding to TF	Dickinson, Proc Natl Acad Sci U S A. 1996 93(25):14379-84
Q308	C	Decreases Tissue Factor binding	US20060205036
	A	No change in activity or binding to tissue factor	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
D309	S	increases activity	WO200183725; WO2006/035057; WO2005/123916 WO 2006/114448 WO 2006/067230; US6,911,323; US2006-0019894; US20050113565; US2006/0194289
	A	Reduced affinity for tissue factor	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
	T	increases activity	WO200183725; US 6,911,323
	C	Decreases Tissue Factor binding	US20060205036
C310	F	Asymptomatic to severe hemophilia; FVII:C assay (heterozygous) = 30	McVey JH. Hum Mutat. 2001;17(1):3-17.; Chafa, J. Thromb Haemost. 2005 3(1):175-7; Menegatti, Am J Hematol. 2004 77(1):90-1; Mariani et al. (2005) Thromb Haemost 93:481-487; Wulff K. Hum Mutat. 2000;15(6):489-96

P del Residuo	Variante(s)	Efecto de la variación	Referencia
	F (with A294V/R304Q)	patient experienced gum bleeding	Etro et al. (2005) J. Hematology 88(12):1434-1436
	F (with G331S)	Asymptomatic	Etro et al. (2005) J. Hematology 88(12):1434-1436
L311	A	Decreases TF binding; slight decrease in Factor X activation	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
Q312	N	Increases glycosylation by creating an N-glycosylation site	US 6806063
	A	No change in activity or binding to tissue factor	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
Q313	K, D, E	facilitates chemical group linkage	US 6806063
	N (with R315S/T)	Increases glycosylation by creating an N-glycosylation site	WO2004029091, US 6806063
	A	slightly decreases Factor X activation rate	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
S314	E	Increases catalytic activity; facilitates chemical group linkage	Persson, Biochem J. 2004 379(Pt 2):497-503; WO2004029090; Persson et al. (2004) *Semin Hematol.* 41(1 Suppl 1):89-92.; WO2006/035057; WO2005/123916; WO 2006/114448; WO 2006/067230; US 6806063; US 6911323; US20050113565; US2006-0019894
	M	Increases catalytic activity	Persson (2004) *Biochem J.* 379(Pt 2):497-503; WO2004029090
	A	Decreases activity; no change in binding to tissue factor	Dickinson (1996) *Proc. Natl. Acad. Sci. U.S.A.* 93(25):14379-84; WO2004029090
	C	facilitates chemical group linkage	US 6806063; WO 2006134173; WO2004029090
	D	facilitates chemical group linkage	US 6806063; WO2004029090
	N (with K316S/T)	Increases glycosylation by creating an N-glycosylation site	WO2004029091; US6806063;
	K	facilitates chemical group linkage; increased activity	US6806063; US 6911323; WO2004029090
	V/I/L/F/W/P/G/T/L/Y/N/R/H/Q	increases activity	WO2004029090
R315	A	No change in activity or binding to tissue factor	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
	G/A/Q	increases resistance to proteases	US 5580560
	C	facilitates chemical group linkage	US 6806063; WO 2006/134173
	K	facilitates chemical group linkage; slightly decreases activity	Furlan, Haematologica. 2004 89(12):1504-9; US 6806063
	K/D/E	facilitates chemical group linkage	US 6806063
	N (with V317S/T)	Increases glycosylation by creating an N-glycosylation site	WO2004029091

P del Residuo	Variante(s)	Efecto de la variación	Referencia
	S/T	Increases glycosylation by creating an O-glycosylation site; increases resistance to proteases	US 5580560; US 6806063
	S/T (with Q313N)	Increases glycosylation by creating an N-glycosylation site	US 6806063; WO2004029091
K316	S/T (with S314N)	Increases glycosylation by creating an N-glycosylation site	WO2004029091
	D, E	Facilitates chemical group linkage	US 6806063
	A	Increases binding to TF; decreases activation of Factor X	Dickinson, Proc Natl Acad Sci U S A. 1996 93(25):14379-84
	C	Facilitates chemical group linkage	US 6806063; WO 2006/134173
	N (with G318S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
V317	K, C, D, E	facilitates chemical group linkage	US 6806063
	N (with D319S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	A	No change in activity or binding to tissue factor	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
	S/T (with S315N)	Increases glycosylation by creating an N-glycosylation site	WO2004029091
G318	N	Increases glycosylation by creating an N-glycosylation site	US 6806063; WO2006114105
	S/T (with K316N),	Increases glycosylation by creating an N-glycosylation site	US 6806063
D319	A	No change in activity or binding to tissue factor	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
P321	N (with I323S/T)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
N322	Y/G/F/M/S/I/L/T/V/P/K/H/Q/E/R/W/C	Decreases glycosylation by disrupting an N-glycosylation site	WO2005123916
	Q	Increases activity	WO2005123916
I323	S/T	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
T324	N with (Y326S/T)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
E325	A	No change in activity or binding to tissue factor	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
Y326	S/T (with T324N)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
M327	I	FVII:C assay (homozygous) < 1	Giansily-Blaizot M. Eur J Hum Genet. 2001 Feb;9(2):105-12.
F328	N (with A330S/T)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
	S	severe clinical phenotype	McVey JH. Hum Mutat. 2001;17(1):3-17.;
A330	S/T (with F328N)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
G331	N	Increases glycosylation by creating an N-glycosylation site	US 6806063

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P del Residuo	Variante(s)	Efecto de la variación	Referencia
	D	Asymptomatic phenotype	McVey JH. Hum Mutat. 2001;17(1):3-17.;
	S	asymptomatic to moderate hemophilia	Mariani et al. (2005) Thromb Haemost 93:481-487; Etro et al. (2005) J. Hematology 88(12):1434-1436
	S (with C310F)	Asymptomatic	Etro et al. (2005) J. Hematology 88(12):1434-1436
Y332	A	Decreases activity; no change in binding to tissue factor	Dickinson, Proc Natl Acad Sci U S A. 1996 93(25):14379-84
	S/A/G/N/Q	Increases resistance to proteases	US 5580560
	C	Facilitates chemical group linkage	US 6806063
S333	A	No change to TF binding; decreases Factor X activation rate	Dickinson, Proc Natl Acad Sci U S A. 1996 93(25):14379-84
D334	N	Increases glycosylation by creating an N-glycosylation site	WO2004029091; US 6806063; WO2006114105
	V/I/M/F/W/P/L/T/Y/K/R/H/S/Q	Increases activity	WO2004029090
	C	Facilitates chemical group linkage; increases activity	WO2006134173; WO2004029090
	A	Decreases activity; no change in binding to tissue factor	Dickinson et al. (1996) *Proc Natl Acad Sci U S A.* 93(25):14379-84; Jin et al. (2001) Mol Biol. 307(5):1503-17; Petrovan et al. (2000) *J. Biochemistry.* 39(47):14457-63
	G/E	Increases activity	WO2004029090; US6911323
S336	A	Decreases activity; no change in binding to tissue factor	Dickinson (1996) *Proc. Natl. Acad. Sci. USA* 93(25):14379-84
	C	Facilitates chemical group linkage	US 6806063; WO2006134173
	G	Decreases *in vitro* proteolytic activity	US 6911323; US20050113565; US20060019894; US20060194289; WO2002022776; WO2006035057; WO2005123916; WO 2006/114448; WO 2006/067230
	E	Increases activity	US 6911323; WO2002022776; WO2004029090
	/V/I/M/F/W/P/L/T/C/Y/N/K/R/H/D/Q,	Increases activity	WO2004029090
K337	V/S/T/D/E	Increases activity	US6911323; WO2002022776
	R/Q/H	Increases activity	US6,806,063; WO2002022776

P del Residuo	Variante(s)	Efecto de la variación	Referencia
	A	Increases activity; increases inhibition by AT-III; no effect on TF binding	Persson E. PNAS 2001 98(24):13583-13588; Dickinson (1996) *Proc. Natl. Acad. Sci. USA* 93(25):14379-84; WO2002022776, WO2004029091; Jin et al. (2001) J Mol Biol. 307(5):1503-17
	C	Increases activity	WO2006/134173; WO2002022776
	G	Increases activity	WO 2006/114448; WO 2006/067230; WO2002022776
	L/I/M/F/ W/P/Y	Increases activity	WO2002022776
	N	Increases glycosylation by creating an N-glycosylation site	US 6806063
S339	N (with K341S/T)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
K341	Q;	Decreases Tissue Factor Pathway Inhibitor-dependent inhibition	Bjelke (2007) *FEBS Lett.* 581(1):71-6; Neuenschwander (1995) *Biochemistry.* 34(27):8701-8707; WO2004029091; WO2006114105
	E	Increases resistance to proteases	US 5580560; Mariani, Thromb Haemost. 2005 93(3):481-7; Neuenschwander PF. Biochemistry. 1995 Jul 11;34(27):8701-7.
	Q/G/T/A /S	Increases resistance to proteases	US 5580560
	S/T (with S339N)	Increases glycosylation by creating an N-glycosylation site	US 6806063; US20060166874
	N (with G343S/T)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
	A	Decreases activity; no change in binding to tissue factor	Dickinson (1996) Proc. Natl. Acad. Sci. U.S.A. 93(25):14379-84
	S/T (with D343N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
G342	N	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
	R	Asymptomatic phenotype	McVey JH. Hum Mutat. 2001;17(1):3-17.;
D343	S/T (with G341N)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
	H	inactive; FVII:C assay (compound heterozygous) = 18	Toso et al. (2003) Biochem J. 369(Pt 3):563-71.; Wulff K. Hum Mutat. 2000;15(6):489-96.
	N (with G345S/T)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
S344	S/T (with G342N)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874

P del Residuo	Variante(s)	Efecto de la variación	Referencia
	A	Decreases proteolytic activity	Dickinson (1996) Proc. Natl. Acad. Sci. U.S.A. 93(25):14379-84; Toso et al. (2003) Biochem J. 369(Pt 3):563-71
	N (with G346S/T)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
G345	N (with P347S/T)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
	S	reduced catalytic activity	Lee et al. (2008) Blood Coagul Fibrinolysis 19(1):92-4.
	S/T (with D343N)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
G346	S/T (with S344N)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
P347	N (with A349S/T)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; Reiner AP,. J Thromb Haemost. 2007 Jan;5(1):42-9.; US 6806063; US20060166874
	S/T (with G345N)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
H348	N	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
	Q	moderate to severe hemophilia	Mariani et al. (2005) Thromb Haemost 93:481-487
	R	Mild clinical bleeding	McVey JH. Hum Mutat. 2001;17(1):3-17.;
A349	S/T (with P347N)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
H351	A	No change in activity or binding to tissue factor	Dickinson (1996) Proc. Natl. Acad. Sci. U.S.A. 93(25):14379-84
R353	N	Increases glycosylation by creating an N-glycosylation site	US 6806063
	Q	Factor VII deficiency; approximately 25% reduction in FVII coagulant activity in heterozygous carriers and 50% reduction in homozygous carriers	Hahn, Genetics. 2004 167(2):867-77; Shikata et al. (2004) 103(7):2630-2635
	P with (C22R)	increased prothrombin time, reduced FVII activity	Zhidong and Xiaojun (2007) Thromb Haemost 98:687-688
	A	No change in activity or binding to tissue factor	Dickinson (1996) Proc. Natl. Acad. Sci. U.S.A. 93(25):14379-84
W356	A	Increases TF binding; no change to Factor X activation	Dickinson (1996) Proc. Natl. Acad. Sci. U.S.A. 93(25):14379-84
Y357	N	Increases glycosylation by creating an N-glycosylation site	US 6806063
L358	N (with G360S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
T359	N (with I361S/T)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874

P del Residuo	Variante(s)	Efecto de la variación	Referencia
	M	Mild to severe hemophilia; FVII:C assay (compound heterozygous) = 2-4	McVey JH. Hum Mutat. 2001;17(1):3-17.; Mariani et al. (2005) Thromb Haemost 93:481-487; Wulff K. Hum Mutat. 2000;15(6):489-96.
G360	S/T (with L358N)	Creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
	N (with V362S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
I361	N	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
	S/T (with T359N)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
V362	N (with W364S/T)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
	A	Decreases proteolytic activity	Dickinson (1996) Proc. Natl. Acad. Sci. U.S.A. 93(25):14379-84
	S/T (with G360N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
S363	I	moderate hemophilia	Mariani et al. (2005) Thromb Haemost 93:481-487
	N (with G365S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
W364	S/T (with V362 N)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
	C	severe hemophilia	Mariani et al. (2005) Thromb Haemost 93:481-487
	A	Inactive proteolytic function	Dickinson (1996) Proc. Natl. Acad. Sci. U.S.A. 93(25):14379-84
	N (with Q366S/T)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
G365	N (with G367S/T)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
	S/T (with S363N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
Q366	S/T (with W364 N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	A	reduced proteolytic function	Dickinson (1996) Proc. Natl. Acad. Sci. U.S.A. 93(25):14379-84
G367	S/T (with G365N)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
T370	N (with G372S/T)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
	A	Increases TF binding; no change to Factor X activation	Dickinson, Proc Natl Acad Sci U S A. 1996 93(25):14379-84
V371	A	Decreases activity; no change in binding to tissue factor	Dickinson, Proc Natl Acad Sci U S A. 1996 93(25):14379-84
G372	S/T (with T370N)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874

P del Residuo	Variante(s)	Efecto de la variación	Referencia
F374	Y	Increases catalytic activity	Persson, (2004) *Biochem J.* 379(Pt 2):497-503; WO2004029090; WO200183725; WO 2006/035057; WO 2005/123916; US2006-0019894; US20050113565;
	C	Increases catalytic activity	US 6,911,323; WO 2006/134173) WO200183725; WO 2006/035057; WO 2005/123916; US2006-0019894
	A/V/I/L/ M/W/G/S /T/N/E/K /R/H/D/ Q	Stablizes alpha helix; increases activity	WO200183725; WO 2006/035057; WO 2005/123916; US 6911323; US2006-0019894
	P	Stablizes alpha helix; increases activity	WO200183725; WO 2006/035057; WO 2005/123916; US 2006-0019894; US 20050113565; US 2006/0194289; WO 2006/114448; WO 2006/067230
V376	N	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
Y377	N (with R379S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
T378	N (with V380S/T)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
R379	A	Reduced affinity for tissue factor	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
	N	Increases glycosylation by creating an N-glycosylation site	US 6806063
	S/T (with Y377N)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
	C	Decreases Tissue Factor binding	US20060205036
V380	S/T (with T378N)	creates N-glycosylation site in active site region or at the ridge of the active site binding cleft	US 6806063; US20060166874
	N (with Q382S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
Q382	A	No change in activity or binding to tissue factor	Dickinson Proc. Natl. Acad. Sci U S A. 1996 93(25):14379-84
	N (with I384S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	S/T (with V380N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
Y383	N (with E385S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
I384	S/T (with Q382N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
E385	A	No change in activity or binding to tissue factor	Dickinson, Proc Natl Acad Sci U S A. 1996 93(25):14379-84

P del Residuo	Variante(s)	Efecto de la variación	Referencia
	S/T	Increases glycosylation by creating an N-glycosylation site	US 6806063
W386	N (with Q388S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
L387	N (with K389S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
Q388	S/T (with W386N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
K389	N (with M391S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	S/T (with L387N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
L390	K/D/E	Facilitates chemical group linkage	US 6806063
	C	Facilitates chemical group linkage	US 6806063; WO 2006/134173
	N (withR392S /T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
M391	K/D/E	Facilitates chemical group linkage	US 6806063
	C	Facilitates chemical group linkage	US 6806063; WO 2006/134173
	N	Increases glycosylation by creating an N-glycosylation site	US 6806063
	S/T (with K389N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
R392	C/D/E	Facilitates chemical group linkage	US 6806063
	A	No change in activity or binding to tissue factor	Dickinson, Proc Natl Acad Sci U S A. 1996 93(25):14379-84
	N	Increases glycosylation by creating an N-glycosylation site	US 6806063
	S, T (with L390N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
S393	K/D/E	Facilitates chemical group linkage	US 6806063
	N (with P395S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	C	Facilitates chemical group linkage	US 6806063; WO 2006/134173
E394	S/T (with R392N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	A	No change in activity or binding to tissue factor	Dickinson, Proc Natl Acad Sci U S A. 1996 93(25):14379-84
	N with R396S/T	Increases glycosylation by creating an N-glycosylation site	US 6806063
	K	Facilitates chemical group linkage	US6,806,063
	C	Facilitates chemical group linkage	US6,806,063; WO 2006/134173
P395	K/D/E	Facilitates chemical group linkage	US6,806,063
	N (with P397S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	S/T (with S393N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	C	Facilitates chemical group linkage	US6,806,063; WO 2006/134173
R396	K/D/E	Facilitates chemical group linkage	US6,806,063
	N (with G398S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	A	No change in activity or binding to tissue factor	Dickinson, Proc Natl Acad Sci U S A. 1996 93(25):14379-84

P del Residuo	Variante(s)	Efecto de la variación	Referencia
	S/T (with E394N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	C	Facilitates chemical group linkage	US6,806,063; WO 2006/134173
P397	K/D/E	Facilitates chemical group linkage	US6,806,063
	S/T (with P395N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	N (with V399S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	C	Facilitates chemical group linkage	US6,806,063; WO 2006/134173
G398	K/D/E	Facilitates chemical group linkage	US6,806,063
	S/T (with R396N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	N (with L400S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	C	Facilitates chemical group linkage	US6,806,063; WO 2006/134173
V399	K/D/E	Facilitates chemical group linkage	US6,806,063
	A	No change in activity or binding to tissue factor	Dickinson, Proc Natl Acad Sci U S A. 1996 93(25):14379-84
	S/T (with P397N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	N (with L401S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	C	Facilitates chemical group linkage	US6,806,063; WO 2006/134173
L400	K	Facilitates chemical group linkage	US 6806063
	A	No change in activity or binding to tissue factor	Dickinson, Proc Natl Acad Sci U S A. 1996 93(25):14379-84
	N (with R402S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	S/T (with G398N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
L401	K/D/E	Facilitates chemical group linkage	US6,806,063
	S/T (with V399N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	N (with A403S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	C	Facilitates chemical group linkage	US6,806,063; WO 2006/134173
R402	K/D/E	Facilitates chemical group linkage	US6,806,063
	S/T (with L400N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	N (with P404S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	C	Facilitates chemical group linkage	US6,806,063; WO 2006/134173
A403	K/D/E	Facilitates chemical group linkage	US6,806,063
	S/T (with L401N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	N with F405S/T	Increases glycosylation by creating an N-glycosylation site	US 6806063
	C	Facilitates chemical group linkage	US6,806,063; WO 2006/134173
P404	K/D/E	Facilitates chemical group linkage	US6,806,063
	S/T (with R402N)	Increases glycosylation by creating an N-glycosylation site	US 6806063

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P del Residuo	Variante(s)	Efecto de la variación	Referencia
	N (with P406S/T)	Increases glycosylation by creating an N-glycosylation site	US 6806063
	C	Facilitates chemical group linkage	US6,806,063; WO2006134173
F405	K	Facilitates chemical group linkage	US 6806063
	S/T (with A403N)	Increases glycosylation by creating an N-glycosylation site	US 6806063
P406	C	Facilitates chemical group linkage	WO2006134173
	S/T (with P404N)	Increases glycosylation by creating an N-glycosylation site	US 6806063

Industria y Comercio
SUPERINTENDENCIA

Espacio reservado para el adhesivo de radicación

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DIRECCIÓN DE NUEVAS CREACIONES
MODIFICACIONES Y/O CORRECCIONES

1. Identificación del Tramite

[ ] **Error material** [X] **Modificación a una Solicitud en trámite**

[X] Patente de Invención
[ ] Patente de Modelo de Utilidad
[ ] Registro de Diseño Industrial
[ ] Esquema de Trazado de Circuitos Integrados

2. Datos del solicitante

Persona Natural [ ] Persona Jurídica [X]

CATALYST BIOSCIENCES, INC.
Nombre o denominación / Nombre o razón social completa

Nombre representante legal
Tipo de empresa Micro [ ] Pequeña [ ] Mediana [ ] Otra [ ]

Documento de identificación: [ ] C.E. [ ] NIT [ ] Otro [ ] hero [ ]

Nacionalidad/País de constitución	Dirección y domicilio del titular	Ciudad
Estados Unidos de América	260 LittleField Avenue South San Francisco, California 94080	California
Dirección electrónica	**No. Fax**	**Número telefónico**

[ ] **Autorizo expresamente a la Superintendencia de Industria y Comercio para que todas las comunicaciones sean enviadas a través de la dirección de correo electrónico**

3. Datos del apoderado:

SILVANA MARIA LÓPEZ DÍAZ	C.C. 22.468.787	147463 CSJ
Apellidos y Nombre	**Documento de identificación**	**Tarjeta profesional**

Dirección y domicilio		Ciudad
Carrera 11 No. 86-53, Piso 6, Bogotá D.C., Colombia		Bogotá D.C.
Dirección electrónica	**No. Fax**	**Número telefónico**
notificaciones@clarkemodet.com.co	6350824	6181088

En caso de haber presentado poder general para asuntos que se adelanten ante la Delegatura de Propiedad Industrial, sírvase indicar el número de su radicación o protocolo de poder general _____

4. Datos del trámite

Corrección:

Expediente No. 09-111.636

Aparece _____

_____

Debe ser _____

_____

Modificación:

☐ Presentación Solicitud Divisional.

_____

Modificación al capítulo descriptivo.
Presento junto con este recurso nuevo capítulo reivindicatorio, el cual consta de 31 reivindicaciones y reemplaza completamente el capítulo anteriormente presentado.

■ _____

_____

Modificación al capítulo Reivindicatorio

☐ _____

_____

Modificación al resumen.

☐ _____

_____

Modificación a los dibujos.

5. Anexos

■ Comprobante de pago de la tasa para la presentación de la corrección y/o modificación

☐ Poder, si fuere el caso.

■ Documento que contiene las modificaciones o correcciones.

☐ Copia de la solicitud y sus anexos en formato magnético.

6. Firma	
SILVANA MARIA LÓPEZ DÍAZ	
Nombre y apellidos	Firma
C.C. 22.468.787	Tarjeta Profesional T.P. 147463 CSJ

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
NIT : 800.176.089-2
- / -

Industria y Comercio
SUPERINTENDENCIA

RECIBO DE CAJA

No. 13 - 112340

Bogotá D.C., Noviembre 05 de 2013 - 16:52:33

RECIBIDO DE : CLARKE MODET NI 830.008.643

*** Soporte del Pago ***

TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PAGO	VR.PAGO
CONSIGNACION	BANCO DE BOGOTA	062754387	406756884	05/11/2013	72.360.000.00

*** Conceptos Pagados ***

CANT.	RENTISTICO	CONCEPTO	Vr.UNDITARIO	Vr.CONCEPTO
1	50005-01-03 CAMBIO DE MODALIDAD Y MODIFICACIONES	SOLICITUD MODIFICACIONES CAMBIO SOLICITANTE POR CESION	125.000.00	125.000.00
				$125.000.00

SON: **CIENTO VEINTICINCO MIL PESOS MONEDA CORRIENTE***

Responsable: _____

Recibo de Caja Aplicado al Expediente No. _____

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
No. 09-111636- -00015-0000
Fecha: 2013-12-13 14:57:54 Dep. 2020 DIR.NUEVASCR
Tra. 11 PATENTEIYII Eve: 379 FASENACIONALII
Act. 412 REPOSPRESRECU Folios: 82

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Industria y Comercio
SUPERINTENDENCIA

PLANILLA DE RADICACIÓN - DIGITALIZACIÓN DIARIA
RELACIÓN DE ANEXOS

71

Dependencia: 2020	Fecha: 2013 / 12 / 13 (AAAA / MM / DD)	Recorrido:
Tipo de Radicación: Entrada: ☒ Salida: ☐ Traslado: ☐ Convenio: ☐		
Radicador:		Planilla:

ID	No. Radicación	Consecutivo	Item
1	09-111636-	15	
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			
17			

Item	Unidad de Conservación
1	Bolsa
2	Caja
3	Libro
4	Sobre
5	Tomo
6	Otro
Item	**Soporte Documental**
7	CD
8	Disquete
9	DVD
10	Folleto
11	Foto
12	Periódico
13	Plano
14	Publicación Periódica
15	USB
16	Otro

Observaciones:

Folio 71 CD

Patty

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ANEXO 3

Nuevo pliego de reivindicaciones.

P-2623
Rvs modificadas
Recurso 13-12-13

Reivindicaciones

1. Un polipéptido de factor VII (FVII) modificado, que comprende una modificación en un polipéptido de FVII, variante alélica y de especie del mismo o fragmentos activos del mismo, en donde

una modificación es un reemplazo de aminoácido en una posición que corresponde a una o más de las posiciones D196, K197 y K199 en un polipéptido FVII que tiene una secuencia de aminoácidos dispuesta en la SEQ ID NO:3 o en los residuos correspondientes en un polipéptido FVII;

el polipéptido FVII sin modificar comprende una secuencia de aminoácidos como se establece en cualquiera de SEQ ID NOS:1-3 y 18-74;

la posiciones correspondientes están determinadas por alineaciones con el polipéptido de SEQ ID NO: 3; y

la modificación corresponde a un reemplazo seleccionado de entre D196L, D196I, K197E, K197D, K197L, K197M, K197I, K197V, K197F, K197W, K197D y K197E, por lo que el polipéptido FVII modificado, cuando se activa, exhibe actividad coagulante dependiente de TF comparado con el polipéptido FVII sin modificar.

2. El polipéptido FVII modificado de la reivindicación 1, en donde la modificación en un polipéptido FVII corresponde a un reemplazo que se selecciona entre K197E, K197D, K197L, K197M, K197I, K197V, K197F, K197W, K199D, y K199E.

3. El polipéptido FVII modificado de la reivindicación 1, en donde la modificación en un polipéptido FVII corresponde al reemplazo del aminoácido en 197 con Y.

4.

4. (modificada) El polipéptido FVII modificado de cualquiera de las reivindicaciones 1-3, en donde el polipéptido FVII comprende una modificación del aminoácido adicional que se selecciona entre un reemplazo que corresponde a D196K, D196R, D196A, D196Y, D196F, D196W, D196L, D196I, K197Y, K197A, K197E, K197D, K197L, K197M, K197I, K197V, K197F, K197W, K199A, K199D, K199E, G237W, G237T, G237I, G237V, T239A, R290A, R290E, R290D, R290N, R290Q, R290K, K341E, K341R, K341N, K341M, K341D y K341Q.

5. (modificada) El polipéptido FVII modificado de cualquiera de las reivindicaciones 1-3, en donde el polipéptido FVII comprende una modificación adicional que es una inserción del aminoácido seleccionada entre G237T238insA, G237T238insS, G237T238insV, G237T238insAS, G237T238insSA, D196K197insK, D196K197insR, D196K197insY, D196K197insW, D196K197insA, D196K197insM, K197I198insE, K197I198insY, K197I198insA y K197I198insS.

6. El polipéptido FVII modificado de la reivindicación 4 o 5, que comprende las modificaciones seleccionadas entre los reemplazos correspondientes a D196R/K197E/K199E, D196K/K197E/K199E, D196R/K197E/K199E/R290E, D196R/K197M/K199E, D196R/K197M/K199E/R290E, D196K/K197L, D196F/K197L, D196L/K197L, D196M/K197L, D196W/K197L, D196F/K197E, D196W/K197E, K196V/K197E, K197E/K341Q, K197L/K341Q, K197E/G237V/K341Q, K197E/K199E, K197E/G237V, K199E/K341Q y K197E/K199E/K341Q.

7. El polipéptido modificado del factor VII (FVII) de cualquiera de las reivindicaciones 1 - 6, que comprende dos o más modificaciones en un polipéptido de FVII, o fragmentos activos del mismo, en donde:

dos o más modificaciones de aminoácido se seleccionan entre las modificaciones de aminoácido que corresponden a D196K, D196R, D196A, D196Y, D196F, D196M, D196W, D196L, D196I, K197Y, K197A, K197E, K197D, K197L, K197M, K197I, K197V, K197F, K197W, K199A, K199D, K199E, G237W, G237T, G237I, G237V, T239A, R290A, R290E, R290D, R290N, R290Q, R290K, K341E, K341R, K341N, K341M, K341D, K341Q, G237T238insA, G237T238insS, G237T238insV, G237T238insAS, G237T238insSA, D196K197insK, D196K197insR, D196K197insY, D196K197insW, D196K197insA, D196K197insM, K197I198insE, K197I198insY, K197I198insA o K197I198insS en un polipéptido de FVII que tiene una secuencia de aminoácidos dispuesta en la SEQ ID NO:3 o en residuos correspondientes en un polipéptido de FVII; y

al menos una modificación se selecciona de entre D196L, D196I, K197E, K197D, K197L, K197M, K197I, K197V, K197Y, K197F, K197W, K199D y K199E, y la segunda modificación es diferente de la primera modificación.

8. El polipéptido FVII modificado de cualquiera de las reivindicaciones 1 - 7, en donde el polipéptido de FVII contiene hasta 2, 3, 4, 5, 6 o 7 modificaciones.

9. El polipéptido FVII modificado de la reivindicación 7 o de la reivindicación 8, que comprende las modificaciones seleccionadas entre D196R/K197E/K199E, D196K/K197E/K199E, D196R/K197E/K199E/R290E, D196R/K197M/K199E, D196R/K197M/K199E/R290E, D196K/K197L, D196F/K197L, D196L/K197L, D196M/K197L, D196W/K197L, D196F/K197E, D196W/K197E, D196V/K197E, K197E/K341Q, K197L/K341Q, G237V/K341Q, K197E/G237V/K341Q, K197E/K199E, K197E/G237V, K199E/K341Q, K197E/K199E/K341Q y K197E/G237V/M298Q.

10. El polipéptido FVII modificado de cualquiera de las reivindicaciones 1 - 9 que exhibe resistencia aumentada al inhibidor de vía del factor de tejido (TFPI) en comparación con el polipéptido FVII no modificado.

11.

11. El polipéptido FVII modificado de cualquiera de las reivindicaciones 1 a 10, que comprende una o más modificaciones adicionales de aminoácido en la posición Q176, M298 o E296 en un polipéptido de FVII que tiene una secuencia de aminoácidos establecida en la SEQ ID NO:3 o en residuos correspondientes en un polipéptido de FVII.

12. El polipéptido FVII modificado de la reivindicación 11, en donde la una o más modificaciones del aminoácido en la posición Q176, M298 o E296 se seleccionan entre Q176A, M298Q, E296V y E296A.

13. El polipéptido FVII modificado de la reivindicación 12, que comprende las modificaciones del aminoácido seleccionadas entre K197E/G237V/M298Q, K197E/G237V/M298Q/K341Q, K197E/K199E/G237V/M298Q/K341Q, y K197E/M298Q y M298Q/K341D.

14. El polipéptido FVII modificado de cualquiera de las reivindicaciones 1 – 13, en donde el polipéptido FVII sin modificar comprende una secuencia de aminoácidos como se establece en cualquiera de las SEQ ID NOS: 1 – 3.

15. El polipéptido FVII modificado de cualquiera de las reivindicaciones 1 a 14, en donde el polipéptido FVII no modificado tiene una secuencia de aminoácidos dispuesta en la SEQ ID NO:3.

16. El polipéptido FVII modificado de cualquiera de las reivindicaciones 1 – 15, que comprende una secuencia de aminoácidos dispuesta en cualquiera de las SEQ ID NO: 21, 23-26, 28, 29, 39-43, 128-133, 139-146, 229, 230, 232-235, 242-244, 248 y 250.

17. El polipéptido FVII modificado de la reivindicación 16, que consiste de una secuencia de aminoácidos como se establece en cualquiera de SED ID NOS: 21, 23-26, 28, 29, 39-43, 128-133, 139-146, 229, 230, 232-235, 242-244, 248 y 250.

18. El polipéptido FVII modificado de cualquiera de las reivindicaciones 1 – 17, que es un polipéptido del Factor VII activado por dos cadenas (FVIIa) que esta partido entre los residuos que corresponden a la arginina en la posición 152 y la isoleucina en la posición 153 de la SEQ ID NO. 3.

19. El polipéptido FVII modificado de cualquiera de las reivindicaciones 1 - 18, en donde el polipéptido de FVII es glicosilatado,

carboxilatado, hidroxilado, sulfatado, fosforilatado, albuminatado, o conjugado a una porción del polietilenglicol (PEG).

20. El polipéptido FVII modificado de cualquiera de las reivindicaciones 1 a 19 que es un polipéptido de cadena simple.

21. El polipéptido FVII modificado de cualquiera de las reivindicaciones 1 a 19 que es un polipéptido de cadena doble.

22. El polipéptido FVII modificado de cualquiera de las reivindicaciones 1 a 19 que es activo o activado.

23. Una molécula del ácido nucleico, que comprende una secuencia de nucleótidos que codifican un polipéptido FVII modificado de cualquiera de las reivindicaciones 1 a 22, en donde:
la secuencia de la molécula de ácido nucléico sin modificar comprende la secuencia de nucleótidos establecida en SEQ ID NO: 108 o SEQ ID NO: 109 o degenerados de las mismas o una porción de las mismas que codifiquen el polipéptido FVII de la SEQ ID NO. 3.

24. Un vector, que comprende la molécula del ácido nucleico de la reivindicación 23, en donde el vector se selecciona de entre pCMV, pQE 32, pQE 30, pQE 31, pET 11a, pET 12a-c, pET 15b y pET 19b.

25. Una célula, que comprende el vector de la reivindicación 24, en donde la célula se selecciona de entre células de riñón de hámster bebe (BHK-21), células 293, células CHO, células de *Pichia sp.* y células de *Pichia pastoris.*

26. Una célula que comprende el vector de la reivindicación 24, en donde la célula es seleccionada de células de E. coli, *Saccharomyces cerevisiae*, *Drosophila*, *lepidopteran*, *Schizosaccharomyces pombe*, *Yarrowia lipolytica*, *Kluyveromyces lactis, Pichia sp.*, *Pichia pastoris*, Sf9, *Pseudaletia unipuncta* (A7S), *Danaus plexippus* (DpN1), Schnieder 2 (S2), Kc, C7, Balb/3T3, HeLa, MT2, célula NS0 (que no secretan) de ratón, Sp2/0, COS, NIH3T3, 2B8, HKB y células EBNA-1 libres de suero.

27. Una composición farmacéutica, que comprende una concentración o una cantidad terapéuticamente efectiva de un polipéptido FVII modificado de cualquiera de reivindicaciones 1 a 22, o una molécula del ácido nucleico de la reivindicación 23 o un vector de la reivindicación 24 o una célula de la reivindicación 25 o la reivindicación 26, en un vehículo farmacéuticamente aceptable.

28. La composición farmacéutica de la reivindicación 27 que se formula para administración local, sistémica, o tópica.

29. La composición farmacéutica de la reivindicación 27 o de la reivindicación 30 que se formula para administración oral, nasal, bucal pulmonar, transdérmica, subcutánea, intraduodenal, enteral, parenteral, intravenosa, o intramuscular.

30. La composición farmacéutica de cualquiera de las reivindicaciones 27-29 que se formula para liberación controlada.

31. La composición farmacéutica de cualquiera de las reivindicaciones 27-29 que se formula para administración de dosis sencilla.

P-2623
Rvs modificadas
Recurso 13-12-13

Reivindicaciones

1. Un polipéptido de factor VII (FVII) modificado, que comprende una modificación en un polipéptido de FVII, variante alélica y de especie del mismo o fragmentos activos del mismo, caracterizado porque:

una modificación es un reemplazo de aminoácido en una posición que corresponde a una o más de las posiciones D196, K197 y K199 en un polipéptido FVII que tiene una secuencia de aminoácidos dispuesta en la SEQ ID NO:3 o en los residuos correspondientes en un polipéptido FVII;

el polipéptido FVII sin modificar comprende una secuencia de aminoácidos como se establece en cualquiera de SEQ ID NOS:1-3 y 18-74;

la posiciones correspondientes están determinadas por alineaciones con el polipéptido de SEQ ID NO: 3; y

la modificación corresponde a un reemplazo seleccionado de entre D196L, D196I, K197E, K197D, K197L, K197M, K197I, K197V, K197F, K197W, K197D y K197E, por lo que el polipéptido FVII modificado, cuando se activa, exhibe actividad coagulante dependiente de TF comparado con el polipéptido FVII sin modificar.

2. El polipéptido FVII modificado de la reivindicación 1, en donde la modificación en un polipéptido FVII corresponde a un reemplazo que se selecciona entre K197E, K197D, K197L, K197M, K197I, K197V, K197F, K197W, K199D, y K199E.

3. El polipéptido FVII modificado de la reivindicación 1, en donde la modificación en un polipéptido FVII corresponde al reemplazo del aminoácido en 197 con Y.

4. El polipéptido FVII modificado de cualquiera de las reivindicaciones 1-3, en donde el polipéptido FVII comprende una modificación del aminoácido adicional que se selecciona entre un reemplazo que corresponde a D196K, D196R, D196A, D196Y, D196F, D196W, D196L, D196I, K197Y, K197A, K197E, K197D, K197L, K197M, K197I, K197V, K197F, K197W, K199A, K199D, K199E, G237W, G237T, G237I, G237V, T239A, R290A, R290E, R290D, R290N, R290Q, R290K, K341E, K341R, K341N, K341M, K341D y K341Q.

5. El polipéptido FVII modificado de cualquiera de las reivindicaciones 1-3, en donde el polipéptido FVII comprende una modificación adicional que es una inserción del aminoácido seleccionada entre G237T238insA, G237T238insS, G237T238insV, G237T238insAS, G237T238insSA, D196K197insK, D196K197insR, D196K197insY, D196K197insW, D196K197insA, D196K197insM, K197I198insE, K197I198insY, K197I198insA y K197I198insS.

6. El polipéptido FVII modificado de la reivindicación 4 o 5, que comprende las modificaciones seleccionadas entre los reemplazos

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correspondientes a D196R/K197E/K199E, D196K/K197E/K199E, D196R/K197E/K199E/R290E, D196R/K197M/K199E, D196R/K197M/K199E/R290E, D196K/K197L, D196F/K197L, D196L/K197L, D196M/K197L, D196W/K197L, D196F/K197E, D196W/K197E, K196V/K197E, K197E/K341Q, K197L/K341Q, K197E/G237V/K341Q, K197E/K199E, K197E/G237V, K199E/K341Q y K197E/K199E/K341Q.

7. El polipéptido modificado del factor VII (FVII) de cualquiera de las reivindicaciones 1 - 6, que comprende dos o más modificaciones en un polipéptido de FVII, o fragmentos activos del mismo, caracterizado porque:

dos o más modificaciones de aminoácido se seleccionan entre las modificaciones de aminoácido que corresponden a D196K, D196R, D196A, D196Y, D196F, D196M, D196W, D196L, D196I, K197Y, K197A, K197E, K197D, K197L, K197M, K197I, K197V, K197F, K197W, K199A, K199D, K199E, G237W, G237T, G237I, G237V, T239A, R290A, R290E, R290D, R290N, R290Q, R290K, K341E, K341R, K341N, K341M, K341D, K341Q, G237T238insA, G237T238insS, G237T238insV, G237T238insAS, G237T238insSA, D196K197insK, D196K197insR, D196K197insY, D196K197insW, D196K197insA, D196K197insM, K197I198insE, K197I198insY, K197I198insA o K197I198insS en un polipéptido de FVII que tiene una secuencia de aminoácidos dispuesta en la SEQ ID NO:3 o en residuos correspondientes en un polipéptido de FVII; y

al menos una modificación se selecciona de entre D196L, D196I, K197E, K197D, K197L, K197M, K197I, K197V, K197Y, K197F, K197W, K199D y K199E, y la segunda modificación es diferente de la primera modificación.

8. El polipéptido FVII modificado de cualquiera de las reivindicaciones 1 - 7, caracterizado porque el polipéptido de FVII contiene hasta 2, 3, 4, 5, 6 o 7 modificaciones.

9. El polipéptido FVII modificado de la reivindicación 7 o de la reivindicación 8, que comprende las modificaciones seleccionadas entre D196R/K197E/K199E, D196K/K197E/K199E, D196R/K197E/K199E/R290E, D196R/K197M/K199E, D196R/K197M/K199E/R290E, D196K/K197L, D196F/K197L, D196L/K197L, D196M/K197L, D196W/K197L, D196F/K197E, D196W/K197E, D196V/K197E, K197E/K341Q, K197L/K341Q, G237V/K341Q, K197E/G237V/K341Q, K197E/K199E, K197E/G237V, K199E/K341Q, K197E/K199E/K341Q y K197E/G237V/M298Q.

10. El polipéptido FVII modificado de cualquiera de las reivindicaciones 1 - 9 que exhibe resistencia aumentada al inhibidor de vía del factor de tejido (TFPI) en comparación con el polipéptido FVII no modificado.

11. El polipéptido FVII modificado de cualquiera de las reivindicaciones 1 a 10, que comprende una o más modificaciones adicionales de

aminoácido en la posición Q176, M298 o E296 en un polipéptido de FVII que tiene una secuencia de aminoácidos establecida en la SEQ ID NO:3 o en residuos correspondientes en un polipéptido de FVII.

12. El polipéptido FVII modificado de la reivindicación 11, en donde la una o más modificaciones del aminoácido en la posición Q176, M298 o E296 se seleccionan entre Q176A, M298Q, E296V y E296A.

13. El polipéptido FVII modificado de la reivindicación 12, que comprende las modificaciones del aminoácido seleccionadas entre K197E/G237V/M298Q, K197E/G237V/M298Q/K341Q, K197E/K199E/G237V/M298Q/K341Q, y K197E/M298Q.

14. El polipéptido FVII modificado de cualquiera de las reivindicaciones 1 – 13, en donde el polipéptido FVII sin modificar comprende una secuencia de aminoácidos como se establece en cualquiera de las SEQ ID NOS: 1 – 3.

15. El polipéptido FVII modificado de cualquiera de las reivindicaciones 1 a 14, en donde el polipéptido FVII no modificado tiene una secuencia de aminoácidos dispuesta en la SEQ ID NO:3.

16. El polipéptido FVII modificado de cualquiera de las reivindicaciones 1 – 15, que comprende una secuencia de aminoácidos dispuesta en cualquiera de las SEQ ID NO: 21, 23-26, 28, 29, 39-43, 128-133, 139-146, 229, 230, 232-235, 242-244, 248 y 250.

17. El polipéptido FVII modificado de la reivindicación 16, que consiste de una secuencia de aminoácidos como se establece en cualquiera de SED ID NOS: 21, 23-26, 28, 29, 39-43, 128-133, 139-146, 229, 230, 232-235, 242-244, 248 y 250.

18. El polipéptido FVII modificado de cualquiera de las reivindicaciones 1 – 17, que es un polipéptido del Factor VII activado por dos cadenas (FVIIa) que esta partido entre los residuos que corresponden a la arginina en la posición 152 y la isoleucina en la posición 153 de la SEQ ID NO. 3.

19. El polipéptido FVII modificado de cualquiera de las reivindicaciones 1 - 18, en donde el polipéptido de FVII es glicosilatado, carboxilatado, hidroxilado, sulfatado, fosforilatado, albuminatado, o conjugado a una porción del polietilenglicol (PEG).

20. El polipéptido FVII modificado de cualquiera de las reivindicaciones 1 a 19 que es un polipéptido de cadena simple.

21. El polipéptido FVII modificado de cualquiera de las reivindicaciones 1 a 19 que es un polipéptido de cadena doble.

22. El polipéptido FVII modificado de cualquiera de las reivindicaciones 1 a 19 que es activo o activado.

23. Una molécula del ácido nucleico, que comprende una secuencia de nucleótidos que codifican un polipéptido FVII modificado de cualquiera de las reivindicaciones 1 a 22, caracterizado porque
la secuencia de la molécula de ácido nucléico sin modificar comprende la secuencia de nucleótidos establecida en SEQ ID NO: 108 o SEQ ID NO: 109 o degenerados de las mismas o una porción de las mismas que codifiquen el polipéptido FVII de la SEQ ID NO. 3.

24. Un vector, que comprende la molécula del ácido nucleico de la reivindicación 23, en donde el vector se selecciona de entre pCMV, pQE 32, pQE 30, pQE 31, pET 11a, pET 12a-c, pET 15b y pET 19b.

25. Una célula, que comprende el vector de la reivindicación 24, en donde la célula se selecciona de entre células de riñón de hámster bebe (BHK-21), células 293, células CHO, células de *Pichia sp.* y células de *Pichia pastoris.*

26. Una célula que comprende el vector de la reivindicación 24, en donde la célula es seleccionada de células de E. coli, *Saccharomyces cerevisiae*, *Drosophila*, *lepidopteran*, *Schizosaccharomyces pombe*, *Yarrowia lipolytica*, *Kluyveromyces lactis, Pichia sp.*, *Pichia pastoris*, Sf9, *Pseudaletia unipuncta* (A7S), *Danaus plexippus* (DpN1), Schnieder 2 (S2), Kc, C7, Balb/3T3, HeLa, MT2, célula NS0 (que no secretan) de ratón, Sp2/0, COS, NIH3T3, 2B8, HKB y células EBNA-1 libres de suero.

27. Una composición farmacéutica, que comprende una concentración o una cantidad terapéuticamente efectiva de un polipéptido FVII modificado de cualquiera de reivindicaciones 1 a 22, o una molécula del ácido nucleico de la reivindicación 23 o un vector de la reivindicación 24 o una célula de la reivindicación 25 o la reivindicación 26, en un vehículo farmacéuticamente aceptable.

28. La composición farmacéutica de la reivindicación 27 que se formula para administración local, sistémica, o tópica.

29. La composición farmacéutica de la reivindicación 27 o de la reivindicación 30 que se formula para administración oral, nasal, bucal pulmonar, transdérmica, subcutánea, intraduodenal, enteral, parenteral, intravenosa, o intramuscular.

30. La composición farmacéutica de cualquiera de las reivindicaciones 27-29 que se formula para liberación controlada.

31. La composición farmacéutica de cualquiera de las reivindicaciones 27-29 que se formula para administración de dosis sencilla.

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Effect of recombinant factor VIIa variant (NN1731) on platelet function, clot structure and force onset time in whole blood from healthy volunteers and haemophilia patients

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Summary. NN1731 is a novel variant of recombinant factor VIIa (rFVIIa) that binds to activated platelets, but has greater enzymatic activity than rFVIIa in generating FXa and thrombin. The effect of NN1731 on clot structure and platelet function was characterized *ex vivo* in whole blood from healthy volunteers and haemophilic patients. Blood samples from six healthy volunteers, nine haemophilia A patients with and without inhibitors and one acquired haemophilia A patient, were spiked with increasing concentrations (0.32, 0.64 and 1.28 μg mL^{-1}) of rFVIIa and NN1731. Platelet contractile force (PCF) or platelet function, clot elastic modulus (CEM) or clot structure, and force onset time (FOT) or the thrombin generation time (TGT) were determined using the Hemodyne Hemostasis Analysis System (HAS^{TM}). Baseline PCF, CEM and FOT values in patients were abnormal compared to healthy volunteers' baseline values. Overall, haemophilia blood samples with or without inhibitors spiked with NN1731 had significantly greater PCF, CEM and shorter FOT values relative to samples spiked with corresponding doses of rFVIIa. The variability in response to treatment between patients was greater with rFVIIa compared to NN1731. At 1.28 μg mL^{-1} (90 μg kg^{-1}), NN1731 normalized PCF, CEM and FOT in nine of 10 patients, while rFVIIa normalized these parameters in four of 10 patients. Increasing *in vitro* concentrations of NN1731 normalized platelet function, clot structure and thrombin generation consistently in haemophilia blood with or without inhibitors. NN1731 may be a promising haemostatic agent for patients with bleeding disorders. These results should be confirmed in an *in vivo* study.

Keywords: clot elastic modulus, haemophilia, NN1731, platelet contractile force, recombinant factor VIIa, thrombin generation

Introduction

The development of inhibitory antibodies in response to coagulation factor replacement is a very serious complication for patients with haemophilia, occurring in 10–15% of all patients [1,2]. Inhibitors do not necessarily increase the frequency of bleeding episodes, but their presence severely challenges the management of bleeding. Inhibitors develop very early after the start of treatment, most after a median exposure of 10 days [2]. A few inhibitors, predominantly weak ones, may arise later in life, notably after periods of intensive factor replacement treatment. Several types of treatment modalities are used to control bleeding episodes in patients with inhibitors.

Recombinant activated factor FVII (rFVIIa; NovoSeven®, Novo Nordisk A/S, Copenhagen, Denmark) is currently approved for the treatment of bleeding episodes and the prevention of bleeding in surgical interventions or invasive procedures in patients with haemophilia A or B with inhibitors, acquired haemophilia or congenital FVII deficiency [3]. The mechanism of action of rFVIIa is now well established [4]. At standard pharmacological doses (90–120 μg kg^{-1}

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or a plasma level of 1.8 μg mL^{-1}), rFVIIa binds to the surface of locally activated platelets independently of tissue factor (TF) following vascular injury, directly activating FX on the surface of activated platelets, therefore bypassing the need for FVIIIa and FIXa and resulting in enhanced localized thrombin generation and formation of a stable fibrin clot [5–7]. Platelet activation requires binding of FVII to TF, and TF is only exposed during tissue injury [4,5]. The requirement for TF in platelet activation therefore suggests that rFVIIa activity is restricted to the site of injury.

Treatment options for the management of acute bleeding in haemophilic patients with inhibitors depends on both the level of the inhibitor titre, either low or high, and the clinical presentation [8]. Variation in clinical presentation is known to occur even within groups of patients with similar inhibitor titres. Furthermore, some haemophilic patients with inhibitors require higher doses of rFVIIa than others to achieve haemostasis, and cases of rFVIIa resistance have been reported [9]. Given the documented variability in response, a novel variant of rFVIIa with optimized efficacy and a more predictable response profile might be an attractive alternative in treating acute bleeding episodes in special situations where the rapid formation of a strong fibrin plug may be necessary.

NN1731 (Novo Nordisk A/S) is a variant of rFVIIa with three amino acid substitutions. The work leading to the identification of NN1731 has been described previously [10,11]. Similar to rFVIIa, NN1731 binds to TF, generating similar amounts of TF-dependent FXa. NN1731 also binds to activated platelets similar to rFVIIa, but the enzymatic activity of NN1731 is greater [12]. The effects of NN1731 have been characterized previously in animal and *in vitro* human models [13–15]. To further characterize the haemostatic potential of NN1731 we conducted an additional *ex vivo* study using whole blood from healthy and haemophilic patients using the Hemodyne Hemostasis Analysis System (HAS^{TM}; Hemodyne, Inc., Richmond, VA, USA) which provides a global assessment of the integrity of the coagulation system by reporting the parameters such as platelet contractile force (PCF), clot elastic modulus (CEM) and force onset time (FOT; also referred to as TGT) [16]. PCF is the force produced by platelets during clot retraction and occurs at the same moment as the appearance of prothrombin fragment 1.2 in the sample [16,17]. CEM reflects the structural integrity of the blood clot, while FOT is the speed at which thrombin is produced in whole blood [17]. FOT is determined from the PCF kinetics curve; as PCF is absolutely thrombin-dependent, the initial upswing of PCF occurs at the moment of the onset of thrombin production. Therefore, this assay employs the onset of PCF as a surrogate marker of thrombin generation [16,17]. The assays performed by the Hemodyne instrument rapidly yield information about both prothrombin conversion on the platelet surface and clot quality. The normal ranges for PCF, CEM and FOT are 6.6–9.1 kdyn, 13.2–24.4 kdyn cm^{-2}, and 3–8 min, respectively [17].

Platelet contractile force is sensitive to thrombin, platelet number, platelet metabolic status, the presence of thrombin inhibitors and the degree of GPIIb/IIIa exposure. CEM is a complex parameter that is sensitive to changes in clot structure, fibrinogen concentration, the rate of thrombin generation and red cell flexibility. The FOT is sensitive to clotting factor deficiencies, inhibitors or antithrombins (AT) [16]. Unlike TEG, the Hemodyne HAS^{TM} measures PCF and provides a better separation of clot structure and platelet effects [16]. Very low PCF, low CEM and prolonged FOT are associated with increased bleeding risk and very high PCF, elevated CEM and short FOT are associated with disease states such as atherosclerosis [16]. Furthermore, we have used the Hemodyne HAS^{TM} previously to correlate improvements in these parameters with clinical outcomes in haemophiliacs [18].

Materials and methods

The Virginia Commonwealth University (VCU) Institutional Review Board approved this study prior to subject enrolment, and this study was conducted in compliance with the Declaration of Helsinki. All subjects provided written informed consent prior to study commencement. Sixteen subjects were enrolled in this study: six healthy volunteers and 10 patients with haemophilia. The inclusion criteria were aged >18 years for all subjects, and the presence of haemophilia for the haemophilia arm of the study. Exclusion criteria for haemophilic patients included use of factor replacement therapy within the previous 48 h before study. Exclusion criteria for healthy volunteers included having a known clotting factor deficiency, concomitant anticoagulant therapy or thrombocytopenia (platelet count <100 000 mL^{-1}).

Age, gender, factor deficiency information and medications were recorded for each subject. Subject identification numbers were used to maintain patient confidentiality. Platelet counts, factor assays and inhibitor levels were performed by the VCU Health System Department of Pathology. Factor deficiency was determined by the FVIII coagulant activity assay

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(modified activated partial thromboplastin time). Factor inhibitor titres were determined by the Factor VIII Inhibitor Bethesda Assay [19]. The Coagulation Special Studies Laboratory located in the VCU School of Pharmacy performed all Hemodyne HAS^{TM} assays.

Blood samples (23 mL) were obtained from all subjects via aseptic venipuncture and stored in 3.2% citrated containers until use. The Hemodyne HAS^{TM} determined the FOT, PCF and CEM for each subject using methods previously described [17]. Clotting was initiated at time zero by adding $CaCl_2$ and increasing amounts of study material (rFVIIa, NovoSeven® or NN1731 obtained from Novo Nordisk A/S) to 700 μL citrated whole blood samples. Final clotting conditions included: $CaCl_2$ 10 mM, pH 7.4, ionic strength 0.15 M in a final volume of 750 μL. Final rFVIIa and NN1731 concentrations used were 0, 0.32, 0.64 and 1.28 μg mL^{-1} that corresponded to the 0, 23, 45 and 90 μg kg^{-1} doses of rFVIIa, respectively. PCF development was measured for 20 min and recorded in kilodynes. CEM was measured concurrently and reported in kilodynes cm^{-2}, and the time between calcium addition and initial PCF development was designated as the FOT. All samples were run in duplicate. The lower limit of quantification of FOT was 1 min; the coefficient of variation was ±5% for FOT values from 2 to 20 min.

All statistical procedures were performed using JMP version 6.0 statistical software (SAS Institute, Cary, NC, USA). The PCF, CEM and FOT data are continuous variables; therefore the central tendency and dispersion of these data were reported as mean ± SD. Given the lack of statistical independence of the paired blood samples and the relatively small sample size, inter-group comparisons of the individual PCF, CEM and FOT at each dosing level were performed using the non-parametric Wilcoxon signed rank test. Statistical significance was determined at $P < 0.05$.

Results

Sixteen subjects were enrolled in this study. Age ranged from 20 to 76 years (Table 1). Six subjects were healthy volunteers with no bleeding disorders, five subjects had severe FVIII deficiency without inhibitors, one subject had mild FVIII deficiency without inhibitors, three subjects had severe FVIII deficiency with inhibitors and one subject had spontaneously developed FVIII inhibitors (acquired haemophilia).

The mean effect of rFVIIa vs. NN1731 on Hemodyne parameters in blood from healthy volunteers is summarized in Table 2. Baseline values in healthy volunteers were all within the normal range. Both PCF and CEM increased from baseline with increasing rFVIIa and NN1731 concentrations; no significant differences were observed in these parameters between treatments. Increasing doses of rFVIIa and NN1731 shortened the FOT from baseline. FOT was significantly reduced to a greater extent with

Table 1. Demographics and subject characteristics.

Patient ID	Age (years)	Race	Gender	Disease severity	Primary factor replacement treatment	FVIII level (%)	FVIII inhibitor level (BU)
Normal or healthy volunteers							
NV 01	38	Caucasian	F	–	–	–	–
NV 02	39	Caucasian	M	–	–	–	–
NV 03	44	Caucasian	M	–	–	–	–
NV 04	31	Black	M	–	–	–	–
NV 05	51	Caucasian	M	–	–	–	–
NV 06	24	Caucasian	M	–	–	–	–
Haemophilic patients							
P01	54	Caucasian	M	Severe	Recombinate	<1	None
P02	23	Caucasian	M	Severe	Recombinate	<1	None
P03	20	Black	M	Severe with inhibitor	NovoSeven®	<1	1150
P04	36	Caucasian	M	Severe with inhibitor	Recombinate and NovoSeven®	<1	Too weak to titre
P05	65	Caucasian	M	Mild	NovoSeven®	8	None
P06	76	Black	M	Spontaneous inhibitor	NovoSeven®	40	13
P07	36	Caucasian	M	Severe with inhibitor	NovoSeven®	<1	3
P08	55	Caucasian	M	Severe	Hemofil-M	<1	None
P09	21	Black	M	Severe	Recombinate	<1	None
P10	44	Caucasian	M	Severe	Recombinate	<1	None

NV, normal volunteer; P, patient with FVIII deficiency; F, female; M, male; BU, Bethesda Unit.

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Table 2. Mean dose–response to treatment in normal volunteers.

Normal volunteers (N = 6)					
Treatment	Conc. (μg mL^{-1})	Dose (μg kg^{-1})	PCF_{20} (±SD)	CEM (±SD)	FOT (±SD)
rFVIIa	0	0	5.6 (0.7)	20.0 (4.6)	6.7 (0.5)
	0.32	23	6.1 (1.3)	24.0 (4.9)	5.6 (1.1)
	0.64	45	6.2 (1.7)	27.9 (7.8)	5.0 (1.1)
	1.28	90	6.6 (1.3)	31.4 (8.8)	3.8 (0.6)
NN1731	0	0	5.6 (0.7)	20.0 (4.6)	6.7 (0.5)
	0.32	23	6.1 (1.2)	25.7 (5.9)	4.3 (0.8)*
	0.64	45	6.7 (1.3)	30.7 (6.2)	2.8 (0.5)*
	1.28	90	6.8 (1.4)	31.2 (8.4)	2.2 (0.8)*

PCF_{20}, platelet contractile force at 20 min; CEM, clot elastic modulus; FOT, force onset time; rF, recombinant factor.
*Significantly different compared to corresponding rFVIIa values ($P < 0.0001$).

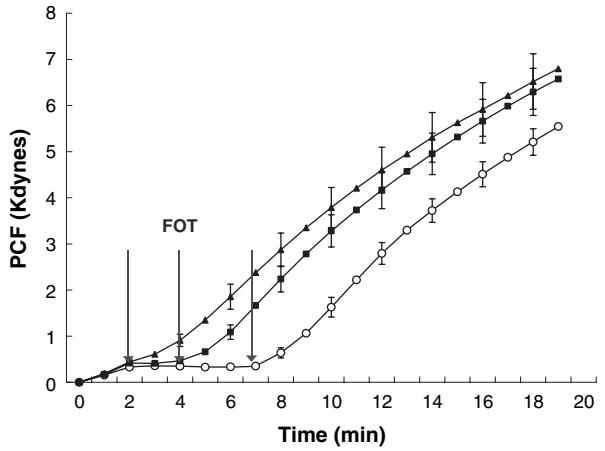


Fig. 1. Platelet contractile force over time in whole blood from healthy volunteers measured at baseline (○) without any haemostatic agent, with 1.28 μg mL^{-1} recombinant factor VIIa (■) or with 1.28 μg mL^{-1} NN1731 (▲). Each point represents mean ± SE, N = 6.

increasing doses of NN1731 compared to the same doses of rFVIIa (Table 2). Figure 1 illustrates the effect of NN1731 vs. rFVIIa on PCF and FOT over time at the recommended rFVIIa dose of 90 μg kg^{-1} (1.28 μg mL^{-1}) in whole blood from healthy volunteers. The figure illustrates that although the FOT is significantly shortened with NN1731 treatment from baseline compared to rFVIIa, no difference in PCF is observed after a 20-min clotting time between treatments in whole blood from healthy volunteers.

The mean effect of rFVIIa vs. NN1731 on Hemodyne parameters in blood from haemophilic patients is summarized in Table 3. Baseline values in haemophilic patients were abnormal compared to baseline values for healthy volunteers (noted in Table 2). With increasing rFVIIa and NN1731 concentrations, both PCF and CEM increased, and the FOT correspondingly decreased, from baseline. Samples spiked with NN1731 had significantly greater PCF, CEM and lower FOT values relative to samples spiked with corresponding doses of rFVIIa. The highest concentrations of rFVIIa and NN1731 had the most dramatic effects on FOT, PCF and CEM in haemophilic patients. In fact, the 1.28 μg mL^{-1} NN1731 concentration yielded PCF, CEM and FOT values that were similar to those reported for the baseline of healthy volunteers.

Table 3. Mean dose–response to treatment in haemophilic patients.

Haemophilic patients (N = 10)								
Treatment	Conc. (μg mL^{-1})	Dose (μg kg^{-1})	PCF_{20} (±SD)	% of NV baseline	CEM (±SD)	% of NV baseline	FOT (±SD)	% of NV baseline
rFVIIa	0	0	0.4 (0.4)	7	0.9 (3.0)	4.5	19.4 (1.9)	290
	0.32	23	1.3 (1.0)	23	4.4 (5.3)	22	16.9 (4.3)	69
	0.64	45	2.5 (2.4)	43	9.4 (9.6)	47	14.1 (4.6)	106
	1.28	90	3.5 (2.2)	63	16.9 (9.4)	85	9.8 (5.1)	139
NN1731	0	0	0.4 (0.4)	7	0.9 (3.0)	4.5	19.4 (1.9)	290
	0.32	23	3.0 (1.6)*	54	13.8 (7.7)*	69	9.3 (4.5)*	139
	0.64	45	4.7 (1.4)*	84	21.2 (8.5)*	106	5.9 (3.0)*	88
	1.28	90	6.3 (2.0)*	113	27.8 (6.9)*	139	3.4 (1.1)*	51

PCF_{20}, platelet contractile force at 20 min; CEM, clot elastic modulus; FOT, force onset time; NV, normal volunteer (refer to Table 2 for NV mean baseline values); rF, recombinant factor.
*Significantly different compared to corresponding rFVIIa dose-response ($P < 0.05$).

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Given the inherent differences in the severity of haemophilia, there was significant inter-subject variability (Table 4). The variability in response to treatment between patients was greater with rFVIIa compared to NN1731. For example, at 1.28 μg mL^{-1} (90 μg kg^{-1}), NN1731 normalized PCF, CEM and FOT in nine of 10 patients, while rFVIIa normalized these parameters in four of 10 patients. An example of patient variability is illustrated in Fig. 2 in the kinetics of PCF development over time in two patients with severe haemophilia with FVIII inhibitors. At baseline both patients had profoundly prolonged FOT (>20 min) and no force development. Patient 03 showed minimal response to increasing rFVIIa doses, but a good normalization response to NN1731. Patient 07 had a similar normalization response to both rFVIIa and NN1731. Although baseline parameters were similar for both patients, patient 03 had a very elevated inhibitor titre vs. patient 07 (1150 vs. 3 Bethesda Inhibitor Units, respectively). Patient 03 normally experiences an average of five bleeding episodes per month and treat bleeds with three doses of rFVIIa (120, 60 and 60 μg kg^{-1}, respectively); patient 07 normally experiences 1–2 bleeds per year and treats bleeds with three doses of rFVIIa 90 μg kg^{-1}.

A second example of patient variability is illustrated in Fig. 3 in the kinetics of PCF development over time in two patients with severe haemophilia without inhibitors. At baseline both patients had profoundly prolonged FOT (>20 min) and no force development. Patient P01 showed minimal response to increasing rFVIIa doses and a good normalization response to NN1731. Patient P02 showed a similar normalization response to both rFVIIa and NN1731. Even though both patients had the same diagnosis with similar FVIII deficiency levels, patient P01 normally experiences an average of four bleeding episodes per month and uses approximately 6000 U of rFVIII replacement therapy monthly; patient P02 experiences an average of two bleeding episodes per month and uses approximately 5000 U of rFVIII replacement therapy monthly.

A comparison of the effect of rFVIIa and NN1731 in two patients with the mean PCF of healthy volunteers is illustrated in Fig. 4. P01 with severe haemophilia and no inhibitors, and P07 with severe haemophilia with inhibitors are presented. As shown in these sample patients, the haemostatic effect of both rFVIIa and NN1731 was greatest in haemophilic patients compared to normal volunteers. Notably, the haemostatic effect of NN1731 in haemophilic patients with or without inhibitors did

Table 4. Percentage normalization of Hemodyne parameters for each hemophilia patient.

			Treatment concentration (μg mL^{-1})											
			PCF (% normalization)				CEM (% normalization)				FOT (% normalization)			
Treatment	ID	Severity/inhibitor	0	0.32	0.64	1.28	0	0.32	0.64	1.28	0	0.32	0.64	1.28
rFVIIa	P05	Mild/none	35	77	135	99	63	100	188	142	21	78	108	140
	P01	Severe/none	0	7	15	28	0	0	12	41	0	0	37	54
	P02	Severe/none	0	35	84	138	0	59	95	194	0	50	70	175
	P08	Severe/none	0	54	57	110	0	61	72	162	0	64	67	233
	P09	Severe/none	0	14	30	57	0	0	43	92	0	0	48	70
	P10	Severe/none	0	8	14	16	0	0	0	0	0	0	0	0
	P03	Severe/high	0	15	8	46	0	5	2	85	0	0	0	50
	P04	Severe/low	0	19	30	59	0	18	41	84	0	0	50	70
	P07	Severe/high	0	17	27	92	0	2	24	182	0	0	47	70
	P06	Spontaneous	0	51	172	174	0	52	156	158	0	47	64	78
NN1731	P05	Mild/none	35	100	124	123	63	92	186	184	21	88	127	156
	P01	Severe/none	0	73	97	106	0	64	102	109	0	100	140	156
	P02	Severe/none	0	36	46	103	0	35	51	131	0	54	78	140
	P08	Severe/none	0	20	74	106	0	28	95	159	0	58	156	200
	P09	Severe/none	0	28	53	112	0	34	63	119	0	58	58	127
	P10	Severe/none	0	11	8	39	0	0	0	23	0	0	0	54
	P03	Severe/high	0	59	57	129	0	38	160	226	0	64	64	74
	P04	Severe/low	0	37	58	88	0	51	83	129	0	78	108	175
	P07	Severe/high	0	27	64	117	0	52	129	199	0	50	67	175
	P06	Spontaneous	0	65	131	161	0	78	120	163	0	50	100	140

P, patient; ID, identification; PCF_{20}, platelet contractile force at 20 min; CEM, clot elastic modulus; FOT, force onset time; rF, recombinant factor.

% normalization = (Value measured in patient/Lowest value in NV)*100.

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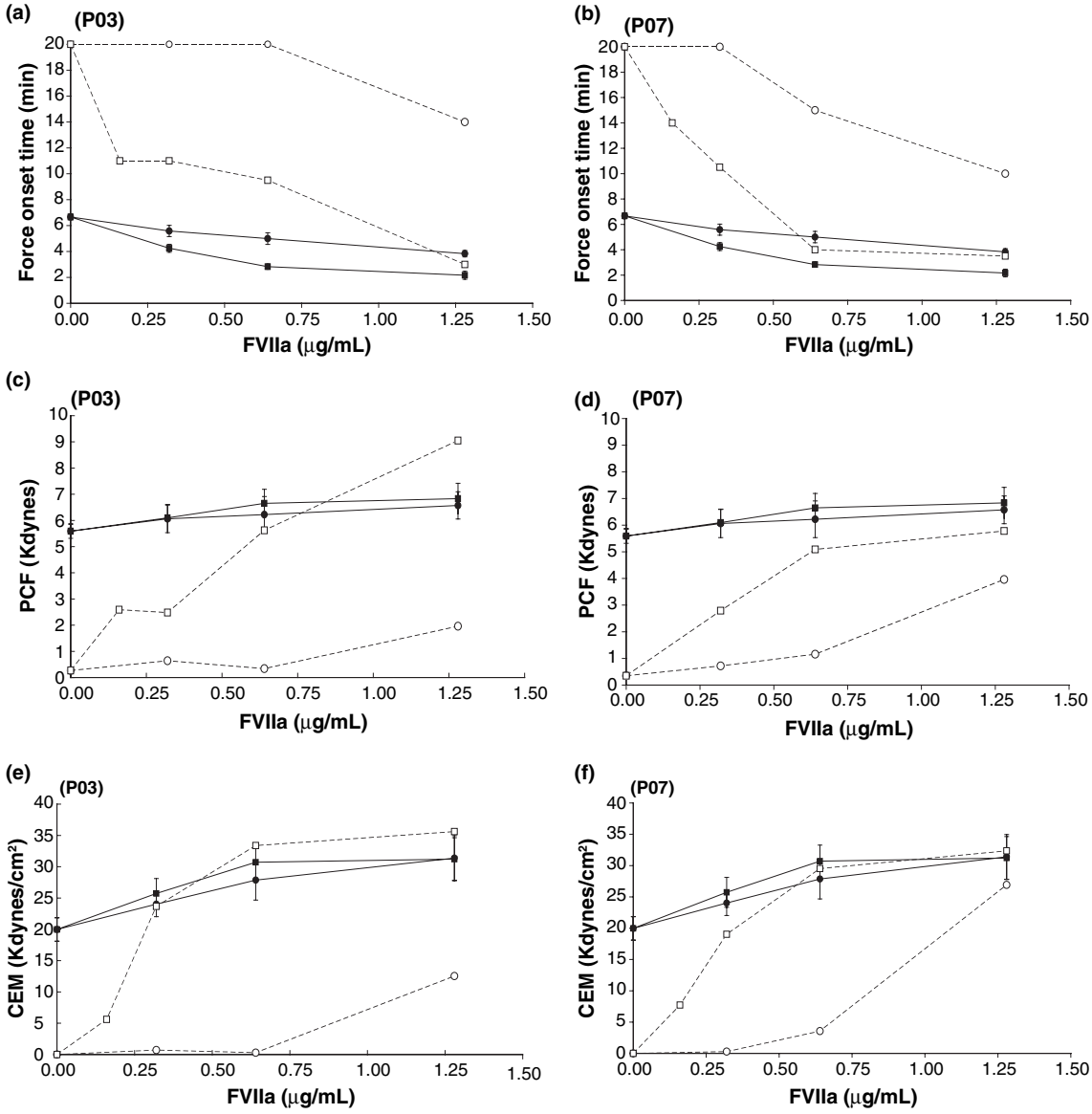


Fig. 2. Haemostatic parameters measured for two individual haemophilic patients with inhibitors (P03 and P07) with increasing concentrations of recombinant factor (rF) VIIa (○) or NN1731 (□) compared to the average in healthy volunteers with increasing concentrations of rFVIIa (•) or NN1731 (■). Force onset time after a 20-min clotting time is presented for patient P03 (a) and P07 (b) compared to healthy volunteers, followed by platelet contractile force after a 20-min clotting time for patient P03 (c) and P07 (d) compared to healthy volunteers, followed by clot elastic modulus after a 20-min clotting time for patient P03 (e) and P07 (f) compared to healthy volunteers. Each point for healthy volunteers represents mean ± SE, $N = 6$.

not exceed the haemostatic response of healthy volunteers.

Discussion

To further characterize the haemostatic potential of NN1731 in comparison with rFVIIa, we conducted an *ex vivo* study using the Hemodyne HAS^{TM} which provides a global assessment of the integrity of the coagulation system by reporting the parameters PCF, CEM and FOT. Our results illustrated that NN1731 provides a greater haemostatic response in blood from FVIII-deficient blood with or without inhibitors compared with rFVIIa. As expected, the magnitude of the haemostatic response to NN1731 in healthy blood was less when compared with the response measured in blood from FVIII-deficient patients. Although variable, most FVIII-deficient patient blood

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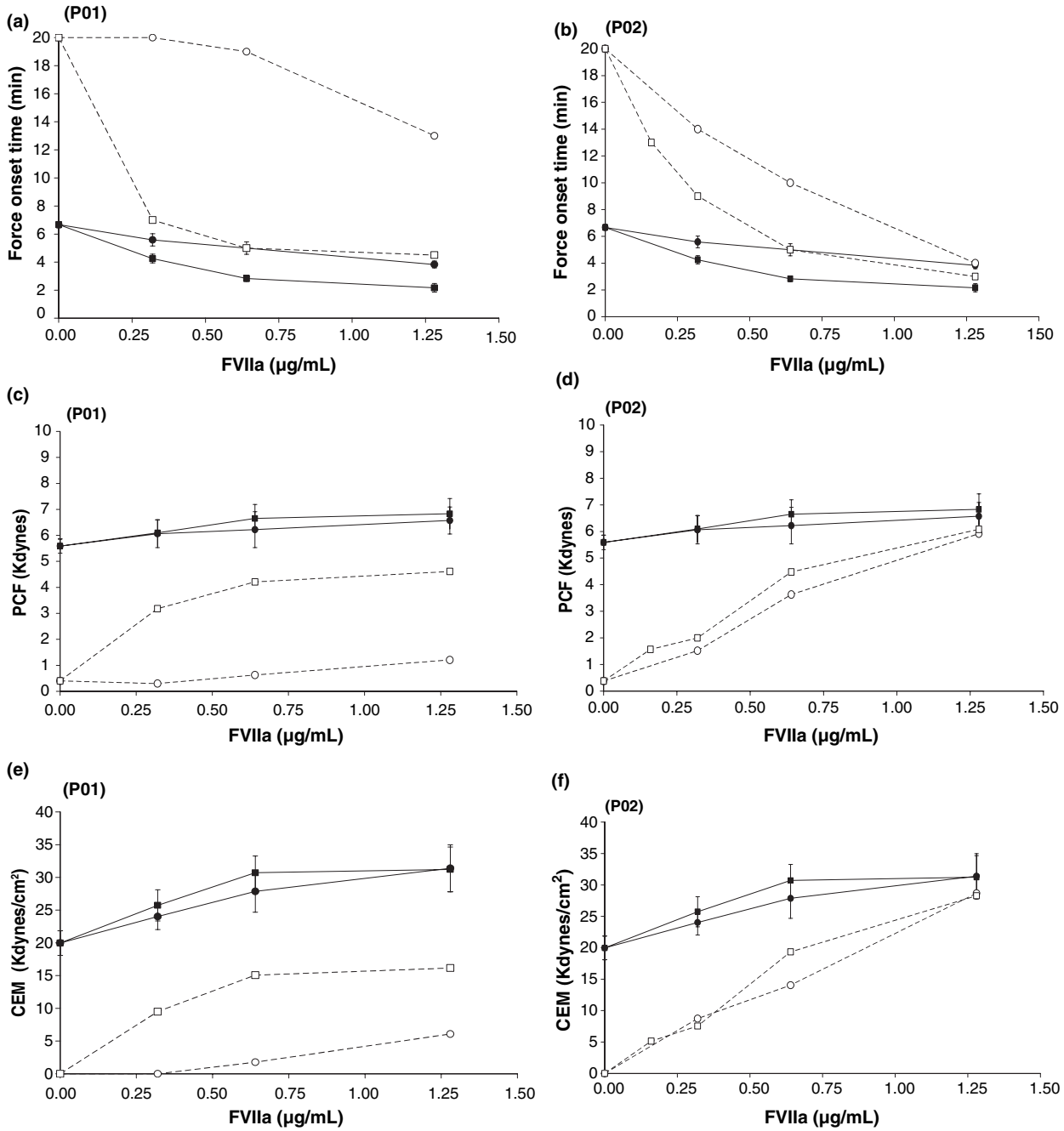


Fig. 3. Haemostatic parameters measured for two individual haemophilic patients without inhibitors (P01 and P02) with increasing concentrations of recombinant factor (rF) VIIa (○) or NN1731 (□) compared to the average in healthy volunteers with increasing concentrations of rFVIIa (•) or NN1731 (■). Force onset time after a 20-min clotting time is presented for patient P01 (a) and P02 (b) compared to healthy volunteers, followed by platelet contractile force after a 20-min clotting time for patient P01 (c) and P02 (d) compared to healthy volunteers, followed by clot elastic modulus after a 20-min clotting time for patient P01 (e) and P02 (f) compared to healthy volunteers. Each point for healthy volunteers represents mean ± SE, $N = 6$.

responded to NN1731 compared to rFVIIa. The results are in agreement with prior *ex vivo* whole blood clotting studies that illustrated that PCF and CEM measurements are sensitive to the dose-dependent effects of rFVIIa and that minimal effects on platelet function and clot structure are observed in normal blood treated with rFVIIa compared to the improved effects seen in FVIII- or FIX-deficient

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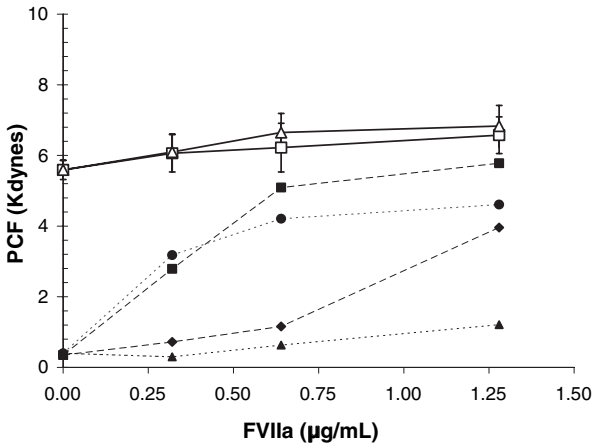


Fig. 4. Platelet contractile force (PCF) in whole blood from healthy volunteers with increasing concentrations of recombinant factor (rF) VIIa (□) or NN173 (△) compared with PCF in blood from a haemophilic patient with inhibitors (P07) with increasing concentration of rFVIIa (♦) or NN1731 (■) and with PCF in blood from a haemophilic patient without inhibitors (P01) with increasing concentration of rFVIIa (▲) or NN1731 (•). Each point represents mean ± SE, $N = 6$.

blood [20]. Variability in response to rFVIIa was also previously reported between individuals as seen in this study.

Most importantly, our study demonstrated that a high dose of NN1731 'normalized' platelet function and FOT in blood from FVIII-deficient patients with or without inhibitors and that the haemostatic response to NN1731 did not exceed the response observed in blood from healthy volunteers. Our results are similar to a study that used thromboelastography (TEG) to characterize the *ex vivo* clot formation in response to NN1731 and rFVIIa [14]. At a concentration of 2 µg mL^{-1}, NN1731 normalized whole blood clot formation and significantly improved whole blood clot stability as measured by TEG in blood from a series of severe haemophilia A patients in a manner superior to that seen with an equivalent amount of rFVIIa. Sorensen *et al.* [14] also found that NN1731 provided greater resistance to tissue plasminogen activator-induced fibrinolysis compared to a similar concentration of rFVIIa. Moreover, similar to our results using the Hemodyne HAS^{TM}, the effects of NN1731 in the TEG study were less variable amongst blood from different individuals compared to rFVIIa suggesting a predictive pharmacodynamic profile.

Our results, in combination with Sorensen *et al.* [14], support that NN1731 provides a consistent haemostatic response with implications for safety. A recent study characterized the *in vitro* regulation and activity of FVIIa variants, including NN1731 [21]. The interaction of FVIIa variants with plasma inhibitors, tissue factor pathway inhibitor (TFPI), AT and heparin was investigated. The authors demonstrated that both TFPI and AT/heparin inhibited the FVIIa variants activity more rapidly compared with wild-type rFVIIa in the absence of TF. The FVIIa variants may provide an increased thrombin burst and clot formation compared with wild-type rFVIIa without an excessive burst as internal levels of TFPI and AT in plasma regulate the response of FVIIa variants [21]. The reason why we observed in our study a haemostatic response to NN1731 in blood from FVIII patients that did not exceed the response observed in healthy blood may be explained by the internal plasma regulation of the thrombin burst by TFPI and AT.

Although our study utilized whole blood samples which provide a global assessment of the haemostatic response, the limitation may lie in the lack of endothelium surfaces that could better predict a clinical response. Ghosh *et al.* [21] demonstrated that although FVIIa variants can generate FXa on native endothelium, the resultant FXa does not enhance thrombin generation. Secondly, our study was limited by the small sample size, but the haemostatic response of NN1731 compared with rFVIIa in our study is consistent with observations on the mechanism of action of FVIIa variants and are in line with the response observed by TEG measurements.

Conclusion

In conclusion, increasing *in vitro* concentrations of NN1731 normalized platelet function, clot structure and thrombin generation consistently in haemophilia blood with or without inhibitors. NN1731 may be a promising haemostatic agent for patients with bleeding disorders. These results should be confirmed in an *in vivo* study.

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This is the first study of the association between ischemic stroke and the AnxA5–1C/T gene polymorphism. We conclude that the polymorphism is not a strong risk factor for ischemic stroke. Although the T-allele has been associated with myocardial infarction, this study does not provide evidence for an important role of the AnxA5–1C/T gene polymorphism in ischemic stroke.

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Homozygous nonsense mutation (Cys72→stop) in the human F7 gene: a not life-threatening mutation despite the absence of circulating factor VII

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Inherited factor (F)VII deficiency is a rare bleeding disorder (estimated prevalence 1 : 500 000), transmitted as an autosomal recessive trait expressed clinically only in homozygotes. In the Algerian population where consanguineous marriages are still frequent, the prevalence of such a recessive coagulation disorder is higher with a clinical and social impact.

We herein report a novel mutation Cys72→stop associated with a moderate bleeding tendency, identified in two unrelated Algerian families: in a young child (family I), in the homozygous state (FVII:C and FVII:Ag < 1%) with severe quantitative FVII deficiency; and in an adult (family II), compound heterozygous, associated with a Cys310→Phe mutation, already reported (FVII:C 3% and FVII:Ag 34%). In family I, the proband, a girl, presented at birth with umbilical hemorrhage and when 7 months old with bilateral epistaxis. The FVII deficiency was diagnosed when she was 18 months old, suffering from recurrent epistaxis, mouth bleeding, spontaneous hematoma after minor trauma. Her parents, first cousins, had no family history of bleeding diathesis. In family II, the proband, a 41-year-old man, had suffered from spontaneous hematoma and extensive bleeding since childhood but the FVII deficiency was diagnosed in adulthood during a preoperative screening for a polypectomy. The patient's daugthers and son had FVII:C and FVII:Ag in the normal range or slightly reduced and were asymptomatic.

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In both families, liver disease and vitamin K deficiency were ruled out as well as thrombophilia (factor V Leiden and antithrombin, protein C or protein S deficiency). Informed consent was obtained in family I from the parents and in family II from the patient and his children. DNA extraction, design of the polymerase chain reaction (PCR) primers derived from the F7 gene sequence and PCR protocols were performed as previously described [1]. Mutations were detected by sequencing the coding regions, the exon–intron boundaries and the 5′ flanking region.

In family I, a C→A mutation at nucleotide 6036 resulted in the substitution of a cysteine codon in position 72, for a stop codon, was found in the homozygous state for the proband, both her parents being heterozygous for the same mutation. In family II, the same mutation, cys72→stop, was found as well as a Cys310→Phe mutation, both in the heterozygous state in the proband. The Cys310→Phe mutation was also present in a heterozygous condition in one daughter and one son whereas the Cys72→stop mutation was present in a heterozygous condition in three daughters.

More than 140 F7 gene lesions have been reported in the literature. Among them only eight nonsense mutations have been characterized [2,3], which means that they are rare compared with the 90 missense mutations found in the F7 gene. Among them, mutation Ser52→stop is the only nonsense mutation found so far in the homozygous state [4].

The absence of cysteine at position 72 prevents the formation of a disulfide bond with the cysteine 61 in the EGF1 domain. Morover, the C→A substitution at nt 6036 in exon 4 generates a premature termination codon in exon 4, leading to a truncated protein devoid of procoagulant activity. Severe bleeding is expected, for the novel Cys72→stop mutation as FVII plays a crucial role in blood coagulation. Our homozygous patient suffered from a moderate but not life-threatening bleeding tendency. In contrast, the other homozygous nonsense mutation (Ser52→stop) producing a short inactive polypeptide [4] belongs to the homozygous life-threatening FVII deficiencies revealed by intracranial and gastrointestinal hemorrhages in childhood. These severely affected patients have severe gene defects such as stop codons [4,5], frameshifts [6] and splice site mutations [5,7–9] leading to the absence of circulating FVII. These data are consistent with F7 gene knockout experiments in mice: F7–/– mice develop normally to term but die at or shortly after birth from abdominal and intracranial hemorrhage [10]. However, Peyvandi *et al.* [11] reported the case of a 5-year-old boy with an homozygous 2 pb deletion in the prepropeptide of FVII: he suffered from gastrointestinal bleeding at the age of 8 months but the diagnosis of FVII deficiency was made later when he was 3 years old, indicating a bleeding tendency less severe than expected.

Even if the mutant Cys72→stop protein is translated, the absence of both EGF domains and the catalytic domain cannot produce an active protein. In these conditions, it is puzzling to have no more than mild bleeding symptoms in the affected 18-month-old girl, despite the absence of circulating FVII. Conversly, the other homozygous nonsense mutation cited previously (Ser52→stop), which interrupts the translation 20 amino acids upstream, was revealed by a lethal intracranial hemorrhage.

The other mutation found in family II, Cys→310Phe, already reported in the literature, is associated in the homozygous state with a severe qualitative FVII deficiency and a mild bleeding tendency [12]. Cys310 is strictly conserved among serine proteases. Modelling of this mutant showed that the loop formed by the disulfide bridge between Cys310 and Cys329 was considerably loosened, inducing a destabilization of the ternary structure of the protein [13] and possibly a less efficient interaction of the mutant protein with tissue factor. For the compound heterozygous patient in family II, it can be hypothesized that the Cys310→Phe mutation allows production of a partially effective FVII which is sufficient to trigger the tissue factor blood coagulation pathway, but no explanation can be given for the homozygous Cys72→stop mutation with no circulating FVII. This case illustrates the difficulty of predicting the bleeding risk in FVII-deficient patients, based upon the phenotype–genotype relationship. The search for an alternative mechanism able to compensate partially for the absence of circulating FVII is still active.

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No effect of polymorphisms in prothrombotic genes on the risk of myocardial infarction in young adults without cardiovascular risk factors

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Recently, the association between nine variants of prothrombotic genes, previously associated with myocardial infarction, were investigated in a large case–control study among young individuals [1]. The focus on the young stems from the assumption that atherosclerosis may play a minor role and that any risk related to a genetic predisposition for a prothrombotic state may be more easily detected. The study followed several reports on the effects of factor (F)V Leiden and prothrombin 20210A in young women [2,3]. The study included 1210 patients with myocardial infarction before age 50 and 1210 controls. Overall, there was no evidence supporting an association between these gene variants and the occurrence of myocardial infarction. However, even in these young individuals the presence of cardiovascular risk factors may have influenced the results. In young individuals who smoke or have hyperlipidemia, atheromatous plaque formation predisposes to myocardial infarction. The aim of the present study was to take the original reasoning even further by the most extreme inclusion criteria, and investigate whether in young individuals amongst whom atherosclerosis is least likely, i.e. in non-smoking, non-obese, non-hypertensive, non-hypercholesterolemic and non-diabetic persons, there would still be no association between prothrombotic gene variants and the development of myocardial infarction. Additionally, we evaluated the influence of a positive family history on developing myocardial infarction.

In the present study data from the same Atherosclerosis, Thrombosis and Vascular Biology Italian Study Group were used [1]. We selected individuals without major cardiovascular risk factors, including smoking, obesity [defined as a body mass index (BMI) > 30.0 kg m^{-2}], hypertension, hypercholesterolemia and diabetes, resulting in 54 cases and 55 controls. Information on cardiovascular risk factors and family history was collected by standardized questionnaires. To decrease statistical variation, we also contrasted the 54 cases with all 1210 controls, under the assumption that in non-diseased individuals the frequency of prothrombotic gene variants will not differ between those with and without cardiovascular risk factors. All participants provided a blood sample for DNA isolation. Subsequently, the following polymorphisms of genes encoding proteins involved in blood coagulation, platelet function, fibrinolysis and homocysteine metabolism were analyzed: the G455A polymorphism in the fibrinogen gene, the G1691A polymorphism in the FV gene (FV Leiden), the G20210A polymorphism in the

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Molecular characterization of a factor VII deficient patient supports the importance of the second epidermal growth factor-like domain

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A B S T R A C T

Background and Objectives. Although a large number of gene mutations have been characterized in patients with factor VII (FVII) deficiency, few naturally occurring mutations have been described in epidermal growth factor (EGF)-like domains. We investigated a 6-year old Italian girl who had low functional and antigenic FVII plasma levels.

Design and Methods. Plasma levels of FVII activity and antigen were evaluated in the propositus and her relatives. Mutation screening was performed by sequencing the FVII gene. The effect of the identified FVII mutations was investigated by protein expression in transfected cells.

Results The propositus was shown to be a compound heterozygote for a known (Arg110Cys) and a novel (Asp123Tyr) missense mutation both occurring in the second EGF-like domain. In transfected cells, expression of the Arg110Cys mutation reduced the amount of intracellular and secreted FVII protein (48% and 18%, respectively). Likewise, cells transfected with the Asp123Tyr mutation gave rise to low intracellular (40%) and extracellular (4%) FVII antigen levels. In conditioned media, FVII procoagulant activity was reduced accordingly (10% and <1%, respectively).

Interpretation and Conclusions Transient expression of the identified FVII mutations caused severely reduced but detectable FVII antigen and activity levels. The present findings suggest that the two naturally occurring missense mutations identified within the second EGF-like domain severely affect FVII protein handling, impairing the correct folding of FVII.

Key words: factor VII, gene, mutations, EGF-like domain, bleeding.

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Human coagulation factor VII (FVII) deficiency is a rare, autosomal recessive trait first described in the early 1950s.[1] It produces severe deficiency in a homozygous state and a moderate deficiency, usually without clinical manifestations, in heterozygotes.[2] Subjects with FVII deficiency show a wide range of symptoms (epistaxis, menorrhagia, spontaneous and post-traumatic bleeding, etc.) that in some instances correlates poorly with the extent of the laboratory abnormalities.[3] Since the first case of FVII deficiency described by Alexander *et al.*,[1] several patients have been described and a high number of different naturally occurring mutations have been reported[4] (for further details see the factor VII database at: *http://europium.csc. mrc. ac.uk*). Most of these mutations are point mutations that severely affect FVII biosynthesis and give a concordant reduction of circulating levels of both functional and immunological activity, but others allow for the presence in the bloodstream of an abnormal protein with impaired activity.[2,5]

FVII is a vitamin K-dependent glycoprotein that, together with the tissue factor in the extrinsic pathway, triggers blood coagulation by activating factor IX and factor X.[6,7] After binding to tissue factor, FVII activation occurs and the resultant two-chain active protein is composed of a light chain and a heavy one linked by a disulphide bond. FVII belongs to a family of homologous proteins that share a common domain organization, involving a N-terminal carboxyglutamic acid-rich (Gla) domain, 2 epidermal growth factor (EGF)-like domains (light chain) and a C-terminal serine protease domain (heavy chain).[8] The serine protease domain contains the active site and is responsible for the activation of FX and FIX.[9] On the other hand, the

Gla domain within the light chain confers the ability to bind to membrane containing negatively charged phospholipids. The first EGF-like domain shares, with the protease domain, the main sites of contact with tissue factor.[10,11] The second EGF-like domain does not appear to play a major role in the ability of FVII to bind tissue factor or to catalyze the activation of other coagulation proteases. Although few naturally occurring mutations have been described in this domain,[12-16] a similar prevalence of mutations in other domains has been reported.

Actually, 9 mutations out of 42 residues in the second EGF-like domain, as well as 10 at 7 out of 37 amino acids within the first EGF-like domain, have been described. This compares with 64 different mutations identified in the 254 residues of the catalytic domain (*http://europium.csc.mrc.ac.uk*).

We now report the molecular characterization of two naturally occurring mutations within the second EGF-like domain suggesting that these residues play an important role in the correct assembly of the FVII molecule.

Design and Methods

Informed consent was obtained from the proband and her relatives, after approval from the local Human Ethics Committee. The studies were carried out according to the Principles of the Declaration of Helsinki.

Materials

Reagents were of analytical grade or the best available commercial grade.

Blood collection and FVII measurements

Blood samples were collected into vacuum plastic tubes containing 3.8% trisodium citrate and centrifuged at 2,000 g for 15 min to obtain platelet-poor plasma. Platelet-poor plasma was kept frozen (< -80°C) until analysis. FVII activity was measured in a one-stage clotting assay using Thromborel S (Behringwerke AG, Marburg, Germany) and a FVII-deficient plasma (International Laboratories, Milan, Italy). FVII antigen was measured by an enzyme immunoassay (EIA) (Asserachrom FVII:Ag, Diagnostica Stago, Asnières, France) and a normal plasma pool as standard. FVII activity and antigen were expressed as percentages of values measured in pooled normal plasma.

DNA analysis

Isolation of DNA and PCR analysis were performed according to standard procedures.[17] All coding regions and intron/exon boundaries of the FVII gene were amplified using sense and antisense oligonucleotides designed on the basis of the known sequence of the FVII gene locus (GenBank accession number J02933). Amplified DNA fragments were purified and subjected to direct cycle sequence analysis using the Taq dye-deoxy terminator method and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

Site-directed mutagenesis

To study the effect of mutations identified, mutagenesis of the pED4 mammalian expression plasmid, containing the full-length FVII cDNA, was made by overlapping PCR. The mutagenic antisense primers (Life Technologies, Paisley, UK) 5'-GCACCGACAGGAGCACTTGGTGCCCGT-3' (underlined letter indicates the mismatch) corresponding to nucleotide positions 8398-8372 (numbered according to GenBank accession number J02933) and 5'- GCACCGACAGGAGCTCTTGGTGCCCGT -3' were used to replace Arg110 with a Cys or a Ser codon, respectively.[18] A 543bp construct was made using a sense primer (*comFw*), which extended from nucleotide 501 to 518 (5'-CAGCACTGCAGAGATTTC-3'). The remaining 3' end of FVII cDNA was obtained employing the sense primer 5'-ACGGGCACCAAGTGTCCTGTCGGTGC-3' (Cys110) or the sense primer 5'-ACGGGCACCAAGAGTCCTGTCGGTGC-3' (Ser110) (nucleotide 8372-8398) and the antisense primer *comRev* 5'-TTGGCTTTCTTCACAGG-3' (nucleotide 11674-11692) to obtain a 939bp product. For the Asp123 to Tyr substitution, a 580bp construct was made using the sense primer *comFw* and a mutagenic antisense primer 5'-GGACACCCCGTATGCCAGCAGAGAGTA-3' (nucleotide 8434-8408). Then, the mutagenic sense primer 5'-TACTCTCTGCTGGCATACGGGGTGTCC-3' (nucleotide 8408-8434) and the antisense *comRev* were used to obtain a 903bp product. A second round of amplification, using the outermost primers *comFw* and *comRev,* was employed to obtain a 1,455bp product containing the entire FVII coding region. The resulting mutant plasmids were checked by sequencing. Plasmids used in transfection experiments were extracted by the EndoFree Plasmid Midi Kit (Qiagen, Hilden, Germany).

Cell cultures and transfections

COS-7 cells (SV40-transformed green monkey kidney cells) were cultured according to standard procedures.[13] Transfection experiments were performed using the Lipofectamine 2000 reagent (Invitrogen) essentially as previously described.[13]

FVII measurements in conditioned media and cell lysates

FVII activity and antigen levels were measured 72 hours after transfection in conditioned media and cell lysates by a one-stage clotting assay and EIA. Standard curves were constructed with reference plasma diluted

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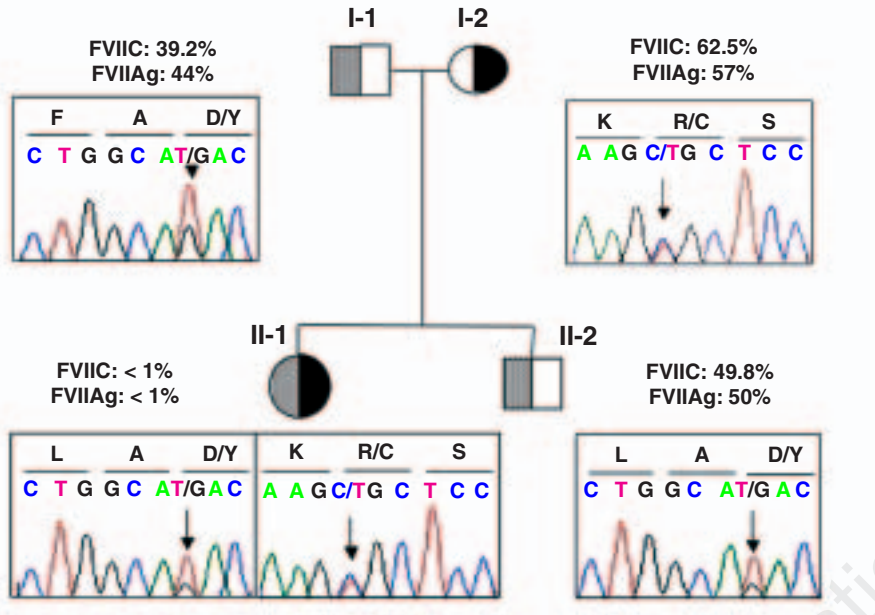


Figure 1. Pedigree of the FVII-deficient family, and electropherograms showing the identified missense mutation. Sequence electropherograms of the region surrounding each mutation (whose position is indicated by an arrow) are reported for individual I-1 and II-2 (both heterozygous for the 8423T>G transversion [half shaded]), I-2 (heterozygous for the 8384 C→T transition [half filled]), and II-1 (compound heterozygous). The predicted translations of the nucleotide sequences are also reported (one letter code).

1:100 to 1:6400 in Tris-buffered saline (50 mM Tris, 150 mM NaCl), pH 7.5. Conditioned media were collected in pre-chilled tubes containing a protease inhibitor (Complete; Roche, Basel, Switzerland), and stored at -80°C, after removal of cell debris by centrifugation. To obtain cell lysates, cells were washed three times with pre-chilled phosphate-buffered saline (PBS) and lysed for 1 hour on ice with lysis buffer containing 1× PBS, 1% Triton X-100, and 1× Complete. Cell lysates were centrifuged to remove cell debris.

Results

Case presentation

The patient was a 6-year old white girl whose parents were not consanguineous (Figure 1). Her family history was negative for a bleeding tendency. She had a moderate bleeding diathesis manifested by easy bruising, hematomas, and bleeding from the mouth. A diagnosis of FVII deficiency was made based on a normal activated partial thromboplastin time (aPTT), a very prolonged prothrombin time (PT), and undetectable FVII function (>1%; normal range: 70-130%) as well as quantitative (>1%; normal range: 50-150%) plasma levels. Both parents of the propositus, as well as her brother, had low functional and quantitative levels of FVII (Figure 1).

Genetic characterization

Fragments covering the entire coding region of the FVII gene were amplified from the proband's genomic DNA. All the amplified segments proved to be identical to those obtained in controls except for that spanning exon 5. Direct DNA sequencing of the PCR product from the patient showed a heterozygous C→T transition at cDNA position 8384 [numbered according to GenBank accession number J02933],[18] leading to an Arg to Cys (CGC→TGC) substitution at amino acid position 110 (numbering omits the signal peptide) (Figure 1). The same mutation was found in the heterozygous state in her mother (I-2) who displayed reduced FVII levels. In addition, a single nucleotide heterozygous transversion (G→T) was identified at nucleotide 8423, leading to an Asp to Tyr (GAC→TAC) substitution at amino acid position 123. The same mutation was found in the heterozygous state in her father (I-1) and in her brother (II-2), who had reduced FVII levels (Figure 1). Screening for both the C→T and the G→T mutations failed to identify these molecular changes in 51 healthy subjects (102 chromosomes).

Transient expression of wild-type and mutant recombinant FVII alleles

To investigate the effect of the Arg110Cys and Asp123Tyr mutations on the secretion of the FVII antigen, recombinant mutated FVII alleles (Cys110 and Tyr123) were transiently and separately expressed in COS-7 cells, which do not express endogenous FVII. The 8384G→A nucleotide substitution was introduced into the pED4/FVII expression plasmid by site-directed mutagenesis to obtain the mutated constructs. Cells were transfected with either the wild-type or one of the mutant constructs. Serum-free media as well as cell extracts were analyzed for the presence of FVII antigen by EIA.

The concentration of FVII antigen in conditioned media of cells expressing the wild-type construct ranged

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Table 1. Transient expression of wild-type and mutant FVII constructs.

	Wild-type	*Cys110*	*Ser110*	*Tyr123*
FVII antigen				
Conditioned media ng/mL (%)	410+125	74.0+5.7(18)	193.0+9.3(64)	17.5+0.7 (4)
Cell lysates ng/mL (%)	31.5+2.1	15.0+0.9 (49)	20.0+1.2 (47)	12.5+0.6 (39)
FVII activity Conditioned media %	100	9.7+0.3	39.2+2.6	<1

pED4/FVII (wild-type), pED4/FVII-Cys110, pED4/FVII-Asp123, or pED4/FVII-Ser110 were transiently transfected in COS-7 cells. Antigen levels of recombinant FVII were measured in both conditioned media and in the corresponding cell lysates by an EIA assay, 72 hours after transfection. Results represent means + standard deviation of three independent experiments, each performed in duplicate. The mean value of wild-type FVII is set as 100%.

from 321 ng/mL to 499 ng/mL. However, in conditioned media of cells transfected with pED4/FVII-Cys110 plasmid, an about 5-fold reduction in FVII antigen concentrations was recorded (74.0±5.7 ng/mL) (Table 1). Transfection with the pED4/FVII-Tyr123 plasmid gave rise to extracellular FVII antigen levels about 4% of the wild-type (17.5±0.7 ng/mL) (Table 1). In lysates of cells expressing the mutant alleles, FVII antigen levels were reduced to approximately 50 to 40% (15.0±0.9 ng/mL and 12.5±0.6 ng/mL, in cells transfected with the pED4/FVII-Cys110 or the pED4/FVII-Tyr123 plasmid, respectively) of those measured in cells expressing the wild-type allele (31.5±2.1 ng/mL) (Table 1). The importance of the Arg110 residue was further investigated by expressing a mutated plasmid containing a C-to-A transition (Arg110Ser) at cDNA position 8384. In conditioned media of cells transfected with the pED4/FVII-Ser110, FVII:Ag levels were reduced to 47% of the wild-type, whereas the levels in cell lysates were decreased to 64% (193.0±9.3 and 20.0±1.2 ng/mL, respectively). No FVII procoagulant activity was detectable in conditioned media of cells transfected with the pED4/FVII-Tyr123 plasmid (<1%), whereas the FVII activity of cells transfected with the pED4/FVII-Cys110 (9.7±0.3%) or the pED4/FVII-Ser110 (39.2±2.6%) plasmid was lower than antigen levels found.

Discussion

FVII is crucial for the correct maintenance of hemostasis, since patients presenting with a severe reduction of circulating levels suffer from a moderate to severe bleeding disorder.[19] We have investigated the mechanisms responsible for FVII deficiency in an Italian patient presenting with a moderate bleeding diathesis and undetectable FVII levels (<1%). Sequencing of the entire coding sequence and intron/exon boundaries of the FVII gene revealed that the patient was a compound heterozygote for two missense mutations, Arg110Cys and Asp123Tyr. Both of them occurred within the second EGF-like domain and are highly conserved among different species (Figure 2). This domain is not involved in the direct contact between tissue factor and FVII but is necessary for optimal binding, merely imparting structure to the rest of the molecule.[10,20] The EGF-like domains have been found to mediate protein-protein interactions. Experimental studies have shown that both the EGF-like and the serine protease domains are essential for the interaction between the tissue factor and FVII.[21] The second EGF-like and the serine protease domains are known to interact with each other and a synthetic peptide corresponding to the N-terminal part of the second EGF-like domain exerts an antithrom-

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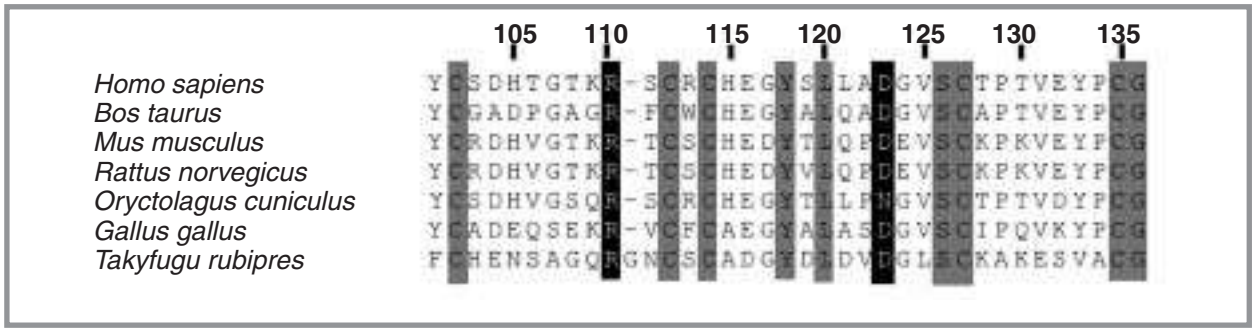


Figure 2. Conservation of Arg110 and Asp123 residues in the second EGF-like domain. Multiple alignment of FVII proteins from different species in the region containing the both Arg110Cys and Asp123Tyr mutations: human, bovine, mouse, rat, rabbit, chicken, and pufferfish FVII (accession numbers NP_062562, I46932, NP_034302, NP_690059, KFB07, AA033363 and AA033368, respectively). Identical residues are highlighted in gray, amino acid Arg110 and Asp123 in the human FVII molecule and the corresponding residues of the other FVII proteins are boxed in black. Numbering does not include the signal peptide and refers to the human sequence.

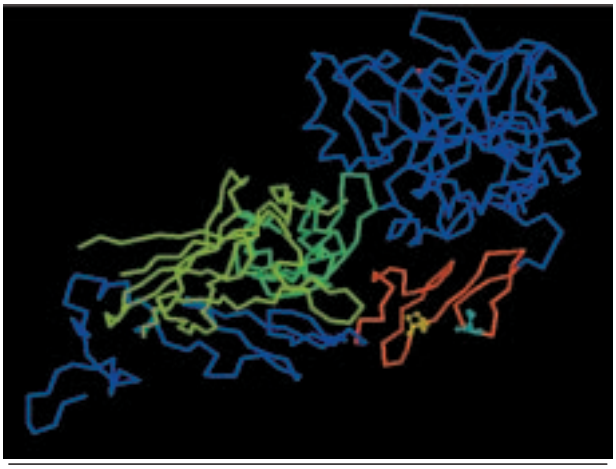


Figure 3. Structural analysis of the FVIIa (1DAN.PBD)[10] using RasMol. The second EGF-like domain is indicated in red. The positions of Arg110 (yellow) and Asp123 (cyan) are indicated as a ball and stick.

botic effect disrupting the interaction between the two FVII domains.[22] In keeping with this, FVII-deficient patients carrying a Glu100Arg missense mutation displayed very low to undetectable FVII antigen levels and activity.[5,13,23] Actually, almost all natural mutations occurring within the second EGF-like domain have been identified between Cys91 and Cys102. To further unravel the role of mutations within this portion of the domain, we performed transient expression studies in COS-7 cells with cDNA encoding FVIIwt, FVIICys110, and FVIITyr123 and showed that the secretion of the two mutant proteins was severely impaired. In conditioned media, cells transfected with the FVIICys110 or FVIITyr123 mutant constructs showed much lower FVII antigen levels, about 5 and 25-fold, respectively, than concentrations measured in conditioned media from cells transfected with the wild-type allele. However, concentrations of FVII antigen in cell lysates of COS-7 cells transfected with the mutant constructs were reduced, by about 40 to 50% of the values found in control cells. Thus, these data are consistent with the possibility that the Arg110Cys and Asp123Tyr substitutions severely impair the processing/stability and the secretion of the FVII molecule causing intracellular degradation of the mutant protein by lysosomal proteolysis or by pre-Golgi or endoplasmic reticulum mechanism.

The Arg110Cys substitution has been recently found in a family with mild to moderate FVII deficiency.[16] The proband was homozygous for the missense mutation and showed FVII activity level of 25% and FVII antigen level of 28% of the normal value, without severe bleeding episodes. These circulating values differ from those (<1%) found in the present patient. On the other hand, the latter were in agreement with FVII concentrations found in conditioned media. It is likely that differences in other regions of the FVII gene locus, i.e. the promoter, may explain differences in plasma levels. The Arg110Cys mutation implies the substitution of an amino acid with a positively charged polar side chain, arginine, by a different amino acid containing an uncharged polar side chain, cysteine. The Arg 110 residue is located within one of the two hairpins that characterize the second EGF-like domain (Figure 3). Therefore, we speculated that the substitution might lead to an abnormal three-dimensional conformation of the FVII molecule, which would have a significant impact on the protein structure. To confirm this, we analyzed a FVII mutant containing a different amino acid with an uncharged polar side chain, Ser110. The Arg110Ser mutation leads to reduced levels of FVII in cell lysates (64%) and in conditioned media (FVII antigen levels: 47%; FVII activity: 39%). In addition to the different charge of the side chain, cysteine has a thiol group, which may form a disulphide bond with other cysteines through oxidation. The second EGF-like domain contains six cysteines that are arranged to form three intramolecular disulphide bonds, Cys91-Cys102, Cys98-Cys112, and Cy114-Cys127. We postulate that the formation of a new cysteine residue may interfere with the correct formation of disulphide bridges and further severely impair the structural integrity of the FVII molecule.

At variance with this, the Asp123Tyr missense mutation is a novel variant. The Asp123 residue is located in a turn of the second EGF-like domain (Figure 3) and the substitution with a tyrosine implies the presence of an amino acid containing an uncharged polar side chain instead of one with a negative charged polar side chain. The residue is conserved in other vitamin K-dependent coagulation proteins, being an aspartic acid in protein C (Asp129) and in protein Z (Asp121) and an asparagine in factor IX (Asn120), factor X (Asn121), and protein S (Asn155). In all these proteins, the homologous residue is found in a β turn and the substitution of Asn120 in the factor IX resulted in loss of circulating factor IX antigen,[24] further supporting a pivotal role of this residue in the correct folding of vitamin K-dependent coagulation proteins. As suggested for the Glu100Arg missense mutation,[23] mutation of FVII residues not directly involved in the interaction with the tissue factor, such as the Asp123Tyr substitution, may result in molecular variants of FVII unable to take part in the initiation of coagulation.

Transient expression of FVII mutations caused reduced but detectable FVII antigen and activity levels, whereas virtually undetectable FVII levels were measured *in vivo*. Since the patient was a compound heterozygote, the two abnormal FVII molecules are expect-

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ed to have a joint effect *in vivo*, leading to intermediate FVII levels. The presence of unrecognized different common or rare variants within the FVII gene locus and/or related gene loci, which may modulate the FVII gene expression or protein handling, has to be taken into account. The assay employed to evaluate the effect of mutant FVII molecules may be not informative enough to fully appreciate the effect of missense mutations on protein secretion and folding. In keeping with this, folding and/or disulphide linkage disturbances in the second EGF-like domain are likely to affect protein stability severely and account for the absence of measurable FVII antigen and coagulant activity in the patient investigated.

In conclusion, we have identified two naturally occurring missense mutations within the second EGF-like domain. These mutations severely affect FVII intracellular protein handling, probably profoundly impairing the correct folding of the native protein leading to FVII deficiency and further suggest the importance of the second EGF-like domain in the correct orientation of different FVII domains.

GD'A, AB: gave important contributions to the conception, design, analysis, and interpretation of data; for drafting the article and revising it critically for important intellectual content; MRL: gave a substantial contributions to the conception, and interpretation of data; for drafting the article and revising it critically for important intellectual content; and gave her final approval of the version to be published; FP, GM, EG, MM: gave an important contribution to the conception, design, and interpretation of data; drafting the article and revising it critically for important intellectual content; and gave her final approval of the version to be published. The authors reported no potential conflicts of interest.

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Description

TECHNICAL FIELD

The present invention is related to modified factor VII/VIIa's, DNA sequences coding for such modified factors and a process for their production.

BACKGROUND ART

Factor VIIa is a serine protease that participates in blood coagulation by activating factor X and/or factor IX. Factor VIIa is produced from its precursor, factor VII, which is synthesized in the liver and secreted into the blood where it circulates as a single-chain glycoprotein (Mw = 50,000). Factor VII can in vitro be converted into the two-chain form factor VIIa by factor Xa, factor XIIa, factor IXa or thrombin. In the presence of tissue factor and calcium ions, factor VIIa, in vivo is believed to convert factor X to factor Xa by limited proteolysis. The latter enzyme in turn converts prothrombin to thrombin in the presence of factor Va, calcium ions, and phospholipid. Factor VIIa will also convert factor IX to factor IXa in the presence of tissue factor and calcium.

Factor VII can be purified from plasma and activated into factor VIIa by the methods described by Broze and Majerus, J. Biol.Chem. 255 (4): 1242 - 1247, 1980 and Hedner and Kisiel, J.Clin.Invest. 71: 1836 - 1841, 1983.

Factor VIIa may also be produced by recombinant DNA-technology by culturing in an appropriate medium mammalian cells transfected with a DNA-sequence encoding factor VII, isolating the protein produced and activating said protein to factor VIIa (vide European patent application No. 86302855.1).

The cDNA coding for human factor VII has been characterized (Hagen et al., Proc.Natl.Acad.Sci. USA, 83: 2412 - 2416, 1986). The amino acid sequence deduced from the cDNAs indicates that factor VII is synthesized with a prepro-leader sequence of 60 or 38 amino acids. The mature factor VII that circulates in plasma is composed of 406 amino acid residues. The amino acid sequence analysis of the activated protein and the amino acid sequence deduced from the cDNAs indicate that factor VII is converted to factor VIIa by the cleavage of a single peptide bond between arginine (152) and isoleucine (153). This results in the formation of a two-chained molecule consisting of a light chain (152 amino acid residues) and a heavy chain (254 amino acid residues) that are held together by one disulphide bond. The light chain contains a γ-carboxyglutamic acid (Gla) domain and two potential epidermal growth factor domains, while the heavy chain contains the serine protease portion of the molecule.

Factor VIIa may be used in treating patients who have developed inhibitors to factor VIII (Hedner, U. and Kisiel, W, J.Clin.Invest., 71: 1836 - 1841, 1983) and for the treatment of patients suffering from bleeding disorders such as platelet disorders including thrombocytopenia, von Willebrand's disease and others typically present in association with severe tissue damages (European patent application No. 86309197.1).

According to observations of the inventors hereof factor VIIa has been found to be a protein susceptible to proteolytic cleavage giving rise to a number of degradation products without clotting activity. The proteolytic cleavage may occur at different steps of the recovery procedure and also during storage. Degradation products have been observed both for factor VIIa derived from plasma as well as for factor VIIa produced by recombinant DNA-technology. The degradation may occur before factor VII has been activated into factor VIIa, i.e. during production and isolation of factor VII, during the activating step itself or during isolation, purification and/or storage of the activated product.

As the degradation products are inactive molecules their occurrence in the factor VIIa preparation will lead to a lower specific activity of the final preparation. Furthermore, the amount and nature of the degradation products may vary from one production batch to another giving rise to preparations with a variable content of biologically active factor VIIa.

Factor VIIa preparations containing inactive degradation products will as mentioned have a less specific activity as compared to preparations in which all or a major part of the protein material is active. Accordingly, higher and more frequent doses are necessary to obtain and sustain a therapeutic or prophylactic effect as compared to a preparation with higher specific activity.

Variable amounts of inactive degradation products and as a consequence variable content of biologically active factor VIIa will furthermore make calculation of appropriate doses troublesome and difficult, if not in some circumstances impossible.

Finally, a content of non-physiological degradation products in the final preparation may trigger the immune system of the patient. Readministration may then result in allergic reactions, which in severe cases may have a lethal course. Patients may also develop high titers of antibodies against factor VIIa rendering subsequent treatment difficult or ineffective. Accordingly, a factor VIIa preparation with less tendency to proteolytic degra-

dation in vitro will be more satisfactory and potentially more useful in factor VIIa therapy.

Factor VIIa is probably, like other circulating proteins, removed from the bloodstream by means of enzymatic degradation. In the initial step of this regulatory process the biologically active enzyme is cleaved at one or a few sensitive peptide bonds to produce an inactive degraded molecule. It is very likely that the peptide bonds which are the most sensitive to enzymatic hydrolysis in vivo are identical to the labile peptide bonds which are most frequently observed to be hydrolyzed during production, purification and/or storage of factor VIIa (George J. Broze, Jr., Scot Hichman and Joseph P. Miletich, J.Clin.Invest. 76 (1985) 937-946).

Factor VII contains 17 lysine (positions 18, 32, 38, 62, 85, 109, 137, 143, 148, 157, 161, 197, 199, 316, 337, 341, 389) and 24 arginine (positions 9, 15, 28, 36, 79, 110, 113, 144, 152, 202, 223, 224, 247, 266, 271, 277, 290, 304, 315, 353, 379, 392, 396, 402) residues that in principle all are susceptible to proteolytic degradation, but usually a number of these residues are not active as cleaving sites.

Although the exact halflife of circulating factor VIIa is unknown, preliminary results suggest that factor VIIa procoagulant activity is rapidly cleared from the bloodstream upon intravenous administration (Ulla Hedner and Walter Kisiel, J.Clin.Invest. 71 (1983) 1836-1841).

The treatment and the lives of the patients will be negatively influenced by the observed short in vivo half life of native factor VIIa. Relatively high doses and frequent administration will be necessary to reach and sustain the desired therapeutic or prophylactic effect. As a consequence adequate dose regulation will be difficult to obtain and the need for frequent intravenous administrations will impose restrictions on the patients' way of living.

Consequently, there exists a need in the art for factor VIIa preparations which are stable during production, purification and storage even at high concentrations, and which furthermore have a longer half life and slower clearance from the blood than the native or recombinant factor VIIa. The present invention fulfills this need by providing certain modified factor VII/VIIa.

DESCRIPTION OF THE INVENTION

In its broadest aspect the present invention provides a modified factor VII/VIIa being stabilized against proteolytic cleavage at certain positions in the molecule. More specifically the present invention provides modified factor VII/VIIa in which one or more proteolytically sensible peptide bond(s) in native factor VII/VIIa has/have been replaced by a proteolytically more stable peptide bond.

According to the present invention this is achieved by modifications at certain positions in the native human factor VII/VIIa molecule. Such modifications may include removal of certain amino acid residues or replacement of one or more amino acid residues with a different amino acid residue. For instance a trypsin like proteolytic cleavage may be hindered by stabilizing the peptide bond on the C-terminal end of certain Arg and/or Lys residues and/or by replacement of certain Arg and/or Lys residues with other amino acid residues and/or by removal of certain Arg and/or Lys residues.

Examples of trypsin-like cleavage sites within the human factor VII molecule at which cleavages have been observed include

(i) lysine(38)-leucine(39),
(ii) lysine(32)-aspartate(33),
(iii) lysine(143)-arginine(144),
(iv) arginine(290)-glycine(291),
(v) arginine(315)-lysine(316),
(vi) lysine(316)-valine(317),
(vii) lysine(341)-glycine(342),
(viii) arginine(392)-serine(393),
(ix) arginine(396)-proline(397) and
(x) arginine(402)-alanine(403).

Minor chymotrypsin-like cleavages have also been observed after

(xi) isoleucine(42) and
(xii) tyrosine(44).

Of these the cleavage sites (i), (ii), (iv) and (v) have been found to be the ones most susceptible to proteolytic degradation, while the remaining are of less quantitative importance.

When considering the stabilization of factor VII/VIIa it is an important aspect that the resulting modified factor VII should retain its activity. This is according to the invention obtained by comparing the sequence of native factor VII/VIIa in the area to be modified with corresponding sequences in related proteins such as factor IX, factor X, factor II and protein C. Homologue sequences around the major cleavage sites are shown below:

```
                                  32     38
                                   |     |
Factor II          EEAFEALESSTATDVFWAKY
Factor VII         EEAREIFKDAERTKLFWISY
Factor X           EEAREVFEDSDKTNEFWNKY
Factor IX          EEAREVFENTERTTEFWKQY
Protein C          EEAKEIFQNVDDTLAFWSKH

                                        290
                                         |
Factor II          RVTGWGNLKETWTANVGKGQPSV-L
Factor VII         LVSGWGQL--------LDRGATALEL
Factor X           IVSGFGRT--------HEKGRQSTRL
Factor IX          YVSGWGRV--------FHKGRSALVL
Protein C          LVTGWGYH--------SSREKEAKRN

                              315                                341
                               |                                  |
Factor II          C-KDSTRI----RITDNMFCAGYKPDEGKRGDACEGDSGGPF
Factor VII         CLQQSRKVGDSPNITEYMFCAGYS--DGSK-DSCKGDSGGPH
Factor X           C-----KLSSSFIITQNMFCAGYD--TKQE-DACQGDSGGPH
Factor IX          CLR-STKFT----IYNNMFCAGFH--EGGR-DSCQGDSGGPH
Protein C          CSEVMSNM-----VSENMLCAGIL--DGRQ-DACEGDSGGPM
```

Consequently, it is an object of the present invention to provide for modified factor VII/VIIa wherein one, more or all of the lysine, arginine, isoleucine and tyrosine residues:

(i) lysine(38)
(ii) lysine(32)
(iii) lysine(143)
(iv) arginine(290)
(v) arginine(315)
(vi) lysine(316)
(vii) lysine(341)
(viii) arginine(392)
(ix) arginine(396)
(x) arginine(402)
(xi) isoleucine(42) and
(xii) tyrosine(44)

have been stabilized by substitution or deletion.

In a preferred embodiment of the invention one, more or all of the amino acid residues in positions (32), (38), (290) and (315) have been stabilized by substitution or deletion.

According to the present invention Lys in position 32 (ii) and/or 38 (i) may be replaced by another amino acid residue. Lys(38) may preferably be replaced by Thr, Asp, Leu, Gly, Ala, Ser, Asn or His and Lys(32) may preferably be replaced by Gln, Glu, His, Gly, Thr, Ala, or Ser.

Also Arg in position 290 (iv) may be replaced by another amino acid residue, for instance Gly, Ala, Ser, Thr or Lys, preferably Ser, Ala or Gly.

Arg(315) (v) may preferably be substituted by Gly, Thr, Ala, Ser or Gln.

Furthermore Lys(341) (vii) may be substituted by Glu, Gln, Gly, Thr, Ala or Ser, preferably Glu or Gln.

Besides substitution of the above mentioned Arg respective Lys residues with another amino acid residue removal of the Arg or Lys amino acid residues may also be considered in order to avoid proteolytic cleavage. Furthermore, one or more of the amino acid residues on either the N- or C-terminal side of such Arg or Lys residues may be substituted by another amino acid residue exerting a stabilizing effect on the proteolytically sensible peptide bond. An example of such modifications is substitution of the amino acid residue linked to the C-terminal end of a Lys or Arg residue with Pro.

To avoid proteolytic cleavage at position 42 (xi) and 44 (xii), Ile(42) and/or Tyr(44) may be substituted by

Asn, Ser, Ala or Gln.

The present invention is contemplated to cover any combination of the above mentioned substitutions and deletions.

Other aspects of the invention will become evident upon reference to the following detailed description and attached drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the amino acid sequence given by one letter abbreviations and the tentative structure for factor VII,
Figure 2 illustrates the construction of plasmid pFW10-3/6,
Figure 3 illustrates the construction of plasmid pFW60-3/6, and
Figure 4 illustrates the construction of plasmid pFWx-3/6.

DEFINITIONS

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms to be used hereinafter.

Complementary DNA or cDNA:

A DNA molecule or sequence which has been enzymatically synthesized from the sequences present in a mRNA template or a clone of such molecule.

DNA Construct:

A DNA molecule, or a clone or such a molecule, either single- or double-stranded, which may be isolated in partial form from a naturally occurring gene or which has been modified to contain segments of DNA which are combined and juxtaposed in a manner which would not otherwise exist in nature.

Plasmid or Vector:

A DNA construct containing genetic information which may provide for its replication when inserted into a host cell. A plasmid generally contains at least one gene sequence to be expressed in the host cell; as well as sequences encoding functions which facilitate such gene expression, including promoters and transcription initiation sites. It may be a linear or closed circular molecule. As used herein, the term expression vector shall mean a plasmid or vector containing a transcription promoter and terminator operably linked to a DNA sequence encoding a protein or polypeptide of interest. Expression vectors may further contain other elements, including selectable markers, enhancers, polyadenylation signals, etc., which will be determined in part by the particular host cell chosen.

Biological Activity:

A function or set of functions performed by a molecule in a biological context (i.e. in an organism or an in vitro facsimile). Biological activities of proteins may be divided into catalytic and effector activities. Catalytic activities of clotting factors generally involve the activation of other factors through the specifid cleavage of precursors. Effector activities include specific binding of the biologically active molecule to calcium or other small molecules, to macromolecules such as proteins or to cells. Effector activity frequently augments, or is essential to, catalytic activity under physiological conditions. Catalytic and effector activities may, in some cases, reside within the same domain of a protein.

For factor VIIa biological activity is characterized by the mediation of blood coagulation through the extrinsic pathway. Factor VIIa activates factor X to factor Xa, which in turn converts prothrombin to thrombin thereby initiating the formation of a fibrin clot.

The modified factor VIIa according to the present invention has a biological activity that is substantially the same as that of native factor VIIa.

"Factor VII/VIIa" as used in this application means a product consisting of either the unactivated form (factor VII) or the activated form (factor VIIa) or mixtures thereof. "Modified factor- VII/VIIa" shall mean a biologically active molecule derived from factor VII/VIIa by the substitution or deletion of one or more amino acid residues.

As the modifications according to the present invention is made on gene expression level modifications introduced in the factor VII molecule will also be found in the activated product (factor VIIa).

"Factor VII/VIIa" within the above definition includes proteins that have the amino acid sequence of native human factor VII/VIIa. It also includes proteins with a slightly modified amino acid sequence for instance a modified N-terminal end including N-terminal amino acid deletions or additions so long as those proteins substantially retain the activity of factor VIIa.

"Factor VII" within the above definition also includes natural allelic variations that may exist and occur from one individual to another. Also degree and location of glycosylation or other post-translation modifications may vary depending on the chosen host cells and the nature of the host cellular environment.

The number system of the amino acid sequence of factor VII/VIIa used herein appears from Figure 1 in which the N-terminal alanine is numbered 1 and the C-terminal proline is numbered 406.

The three letter and one letter abbreviations used for the amino acids are those as normally used in the art, i.e.:

Amino acid	Three letter abbreviation	One letter abbreviation
Alanine	Ala	A
Cysteine	Cys	C
Asparatate	Asp	D
Glutamate	Glu	E
Phenylalanine	Phe	F
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Lysine	Lys	K
Leucine	Leu	L
Methionine	Met	M
Asparagine	Asn	N
Proline	Pro	P
Glutamine	Gln	Q
Arginine	Arg	R
Serine	Ser	S
Threonine	Thr	T
Valine	Val	V
Tryptophan	Trp	W
Tyrosine	Tyr	Y
γ-carboxyglutamic acid	Gla	γ

Best Mode for Carrying out the Invention

The amino acid changes are preferably introduced by oligonucleotide-directed site specific mutagenesis in factor VII cDNA. After screening E. Coli cells the mutated factor VII gene is isolated and recloned into a suitable expression vector. The expression vector is then transfected into an appropriate host cell which when cultured in a suitable culture medium expresses and secretes the modified factor VII which after recovery from

the culture medium is converted into the corresponding modified factor VIIa by known means.

Various host cells may be used including mammalian cells, yeast and other fungi, and bacteria. However, mammalian cells are preferred. A particularly preferred mammalian cell line is the BHK cell line tk⁻tsl3 (Waechter and Basserga, Proc.Natl.Acad.Sci. USA 79: 1106-1110, 1982). Methods for expressing cloned genes in each of these hosts are known in the art, vide for instance EP published patent application No. 200,421 (expression of factor VII and IX in mammalian cells), EP published patent application No. 191,606 (expression of protein C in bacterial cells and EP published patent application No. 167,420 (expression of factor IX in yeast).

For expression of modified factor VII according to the invention in cultured mammalian cells, expression vectors containing cloned modified factor VII sequences are introduced into the cells by appropriate transfection techniques, such as calcium phosphate-mediated transfection (Graham and Van der Eb, Virology 52: 456-467, 1973; as modified by Wigler et al., Proc.Natl.Acad.Sci. USA 77: 3567-3570, 1980). Electroporation transfection technique may also be used (Neuman et al., EMBO.J. I: 841-845, 1982). A DNA-calcium phosphate precipitate is formed, and this precipitate is applied to the cells. A portion of the cells take up the DNA and maintain it inside the cell for several days. A small fraction of the cells integrate the DNA into the genome of the host cell. These integrants are identified by cotransfection with a gene that confers a selectable phenotype (a selectable marker). A preferred selectable marker is the mouse dihydrofolate reductase (DHFR) gene, which imparts cellular resistance to the drug methotrexate (MTX). After the host cells have taken up the DNA, drug selection is applied to select for a population of cells that are expressing the selectable marker in a stable fashion.

Modified factor VII produced by the transfected cells may be removed from the cell culture media by adsorption to barium citrate. Spent medium is mixed with sodium citrate and barium chloride and the precipitate collected. The precipitated material may then be assayed for the presence of the appropriate clotting factor. Further purification may be achieved through immunoadsorption. It is preferred that the immunoadsorption column comprise a high-specificity monoclonal antibody. Alternatively, purification of the barium citrate precipitated material may be accomplished by more conventional biochemical methods or by high-performance liquid chromatography (HPLC).

Conversion of single-chain modified factor VII to active two-chain modified factor VIIa may be achieved using factor XIIa as described by Hedner and Kisiel (J.Clin.Invest. 71: 1836-1841, 1983), or with other proteases having trypsin-like specificity (Kisiel and Fujikawa, Behring Inst. Mitt. 73: 29-42, 1983). Alternatively modified factor VII may be activated by passing it through an ion-exchange chromatography column, such as mono Q® (Pharmacia Fire Chemicals) or the like (Bjoern et al., Research Disclosures, 269, September 1986, pp. 564 - 565).

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

MATERIALS AND METHODS

Restriction enzymes were obtained from Bethesda Research Laboratories (BRL), New England Biolabs, and Stratagene and were used as indicated by the producer, unless otherwise stated, Oligonucleotides were synthesized on an automatic DNA synthesizer using phosphoramidite chemistry on a controlled pore glass support (S.L. Beaucage and M.H. Caruthers (1981) Tetrahydron Letters 22, 1859 - 1869). E. coli cells were transformed as described by Maniatis et al. (Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory, 1982).

A representative modified factor VII was prepared by changing amino acid No. 32 from Lys - → Gln and amino acid No. 38 from Lys - → Thr by oligonucleotide directed mutagenesis.

The oligonucleotides with these changes are of the sequences:

```
I)                                  BglII       Lys(32)

cDNA:                    ... ... GAG ... ... AAG ... ... ...
                                    ↓           ↓
oligonucleotide: 5' GCC CGG GAA ATC TTC CAG GAC GCG GAG 3'
                                    ↑           ↑
     nucleotide position            228         235
                                    Glu         Gln
```

which is a 27-mer with changes at nucleotide position 228 which destroy a BglII site without changing the amino acid and at position 235 changing amino acid Lys(32) to Gln.

```
II)                                 Lys(38)

cDNA                     ... ... AAG ... ... ... ...
                                    ↓
oligonucleotide: 5' AGG ACG ACG CTG TTC TGG ATT 3'
                                    ↑
     nucleotide position            254
                                    Thr
```

which is a 21-mer with changes at nucleotide position 254 changing amino acid Lys(38) to Thr.

A further representative modified factor VII was prepared by changing amino acid No. 290 from Arg -- Ser and amino acid No. 315 from Arg -- Ser by oligonucleotide directed mutagenesis.

The oligonucleotides with these changes are of the sequences:

```
(III)                                   Arg

cDNA:                    ...  ... ... CGT ... ... ... ... ..
                                      ↓
oligonucleotide: 5' GCTG CTG GAC AGT GGC GCC ACG GCC CT
                                      ↑
     nucleotide position              1009
                                      Ser
```

which is a 27-mer with changes at nucleotide position 1009 changing the amino acid Arg (290) to Ser.

```
(IV)                                    Arg

cDNA                    .... ... ... CGG ... ... ... ... .
                                     ↓ ↓
oligonucleotide: 5' GCAG CAG TCA AGT AAG GTG GGA GAC T
                                     ↑ ↑
     nucleotide position:      1084 1086
                                     Ser
```

which is a 26-mer with changes at nucleotide position 1084 and 1086 changing amino acid Arg(315) to Ser.

Example 1

Production of a modified factor VIIa in which Lys(32) has been replaced with Gln (factor VIIa(Gln(32)).

Recloning Factor VII cDNA

Factor VII cDNA with a 38 amino acid long leader (Berkner, K.L. et al., Cold Spring Harbor Symposium on Quantitative Biology, Vol. LI, 531-541, 1986) was cloned in the EcoRI site of pGEM3 vector (Promega Biotec) and propagated in E. coli MC 1061 (dam^+) or MC 1000 (dam^-) bacteria strain.

Briefly, plasmid FVII(565 + 2463)/pDK was cut with EcoRI and the factor VII cDNA was ligated to EcoRI cut pGEM3. The construction of plasmid FVII(565 + 2463)/pDX is described in EP patent application No. 86302855.1. The plasmid has also been deposited at American Type Culture Collection (ATTC No. 40205).

Small and large scale DNA preparations were prepared as described in for example Maniatis et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbour 1982. One of these plasmid preparations is termed pFW 10-3/6. The construction of pFW 10-3/6 is illustrated in fig. 2.

Addition of 5′ phosphate groups to oligonucleotides

Addition of either labelled or unlabelled phosphate groups to oligonucleotides was carried out as described (Maniatis et al. as above).

Oligonucleotide directed site specific mutagenesis using double-stranded plasmid DNA

The site directed mutagenesis reaction was carried out by modifying the method by Morinaga et al. 1984 (BIO/TECHNOLOGY vol. 2 p. 636). Plasmid pFW10-3/6 containing FVII cDNA was digested with BglI, a unique site in the plasmid. This cleavage generated fragment a) shown in fig. 3 and fig. 4 destroying the ampicillin resistance. Fragment a) was purified by electroelution from agarose gel and treated with calf intestinal alkaline phosphatase (CIAP) as described in Maniatis et al. as above.

Another sample of pFW 10-3/6 was digested with BssHII and SacII generating fragment b) in fig. 3 with a window of 575 bp in the FVII cDNA. Fragment b) was purified by electroelution from agarose gel after electrophoresis.

Fragments a) and b) were further purified by several phenol extractions, phenol/chloroform (1:1, v/v) and chloroform/isoamyl alcohol (24:1, v/v) extractions, precipitated with 0.3 M Na-acetate and 70% (v/v) ethanol and dissolved in TE (10 mM Tris, 1 mM EDTA pH 7.6).

Then 0.1 - 0.15 pmol of both fragment a) and b) were mixed with 25 pmol of the phosphorylated synthetic oligonucleotide I) in an Eppendorf tube.

Then 10 µl of 5Xpolymerase-ligase buffer (0.5 M NaCl, 33 mM Tris HCl pH 7.5, 40 mM $MgCl_2$, 5 mM 2-ME) were added.

From the final mixture 15 µl samples were removed and stored on ice until later use as marker in gel electrophoresis. The remaining mixture was incubated in a boiling water bath for 4 min. to denature the DNA fragments. After incubation the mixture was gradually cooled. Upon reannealing heteroduplexes were formed and using agarose gel electrophoresis the formation of a new circular DNA with the correct mutation was dem-

onstrated by comparison with the non-heated sample from above.

Then 10 µl of the four deoxyribonucleoside triphosphate (2.5 mM each), 3 µl of 20 mM ATP, 1 µl of Klenow fragment of DNA polymerase I (5 U/µl) and 1 µl of T4 DNA ligase (10 U/µl) were added to the mixture (20 µl) of heteroduplexes (final volume 40 µl). The final mixture was incubated at 12°C overnight.

Transformation of E. coli MC 1061 and MC 1000 with the incubation mixture resulted in ampicillin resistant transformants. Transformants carrying the mutant FVII gene were selected by colony hybridization (Maniatis et al.) with the 5′-^{32}P-labelled 27-mer and 21-mer synthetic oligonucleotides.

After retransformation plasmid DNA was purified from selected colonies, analysed, and sequenced (by the Maxam-Gilbert method and the dideoxy method) to verify the mutation caused by the synthetic oligonucleotide.

The construction of plasmid pFW 60-3/6 harbouring a mutated factor VII gene in which Lys(32) has been replaced with Gln is illustrated in fig. 3.

pFW 60-3/6 was digested with EcoRI and the EcoRI-EcoRI factor VII fragment was ligated into EcoRI cut pDx plasmid to obtain plasmid pFW 78-3/6 harbouring the factor VII(Gln32) gene in the same orientation as in plasmid FVII(565 + 2463)/pDX. Plasmid pFW 78-3/6 was then transfected into BHKtk$^-$tsl3 cells following the general procedure described above.

The modified factor VII produced by the cells is then precipitated with barium citrate; purified by immunoadsorption; and activated to modified factor VIIa by passing it through an ion-exchange chromatography column as described by Bjoern et al., supra.

Test for Activity

As activated native factor VIIa, the activated modified factor VIIa shortened the coagulation period in a one-stage clotting assay. The activated modified factor VIIa was incubated at a concentration of approximately 0,9 mg/ml in a 10 mM Tris-HCl buffer at pH 8.5 comprising 390 mM NaCl and 5 mM EDTA. The degradation was monitored by SDS-PAGE of reduced samples and when significant degradation had occured an aliqot was withdrawn and applied to an HPLC column. The preparative chromatography served mainly to exclude Tris from the sample for amino acid sequencing as intact and degraded modified factor VIIa coeluted from the column. N-terminal amino acid sequencing revealed that no hydrolysis had occurred of the peptide bond between glutamine residue No. 32 and aspartic acid residue No. 33. In contrast profound degradation at lysine residue No. 32 was observed when activated native factor VIIa was subjected to the same treatment and analysis as performed in a parallel investigation.

Example 2

Production of a modified factor VIIa in which Lys(38) has been replaced with Thr (factor VIIa(Thr 38)).

By following the procedure of example 1 with the only exception that the synthetic oligonucleotide II) was used instead of I) an expression plasmid was obtained harbouring the mutated factor VII gene.

This plasmid is then transfected into BHKtk$^-$tsl3 cells and factor VII(Thr38) is recovered from the cell supernatant and activated to factor VIIa(Thr38) as described.

Example 3

Production of a modified factor VIIa in which Lys(32) and Lys(38) have been replaced with Gln and Thr, respectively (factor VIIa(Gln32, Thr38)).

By following the procedure of example 1 with the only exception that 12.5 pmol of both oligonucleotide I) and II) are used in the site directed mutagenesis reaction, an expression plasmid harbouring the mutated factor VII gene was obtained.

This plasmid is then transfected into BHKtk$^-$tsl3 cells and factor VII(Gln32, Thr38) is recovered from the cell supernatant and activated to factor VIIa(Gln32, Thr38) as described.

Example 4

Production of a modified factor VIIa in which Arg(290) has been replaced with Ser (factor VIIa(Ser(290)).

The construction of plasmid pFW A-3/6 harbouring a mutated factor VII gene in which Arg(290) has been replaced with Ser is illustrated in fig. 4.

Plasmid pFW 10-3/6 was used to produce the fragment a) of Example 1; and another sample of pFW 10-3/6 was digested with SacII and DraIII generating fragment b) in fig. 4 with a window of 1366 bp in the FVII cDNA.

Fragments b) and a) were subsequently treated as described in Example 1 except for the use of oligonuc-

leotide III) instead of I).

pFW A-3/6 was digested with EcoRI and the EcoRI-EcoRI factor VII fragment was ligated into EcoRI cut pDx plasmid to obtain plasmid pFW X-3/6 harbouring the factor VII(Ser290) gene in the same orientation as in plasmid FVII(565 + 2463)/pDX. Plasmid pFW X-3/6 was then transfected into BHKtk$^-$tsl3 cells following the general procedure described above.

The modified factor VII produced by the cells is then precipitated with barium citrate; purified by immunoadsorption; and activated to modified factor VIIa by passing it through an ion-exchange chromatography column as described by Bjoern et al., supra.

Example 5

Production of a modified factor VIIa in which Arg(315) has been replaced with Srr (factor VIIa(Ser315)).

By following the procedure of example 4 with the only exception that the synthetic oligonucleotide IV) was used instead of III) an expression plasmid was obtained harbouring the mutated factor VII gene.

This plasmid is then transfected into BHKtk$^-$tsl3 cells and factor VII(Ser315) is recovered from the cell supernatant and activated to factor VIIa(Ser315) as described

Example 6

Determination of Three Active Proteolytic Cleavage Sites

In order to identify some active cleavage sites a heavily degraded preparation of recombinant factor VII was submitted to N-terminal sequence analysis by automated Edman degradation using an Applied Biosystems model 470 A gas-phase sequencer. The results are shown in Table 1 below

Table 1

Cycle No.	PTH-a.a.	Yield (pmol)	PTH-a.a.	Yield (pmol)	PTH-a.a.	Yield (pmol)	PTH-a.a.	Yield (pmol)
1	Leu	593	Ile	756	Gly	706	Lys	425
2	Phe	497	Val*	1183	Ala	232	Val*	1183
3	Trp	341	Gly*	1128	Thr	38	Gly*	1128
4	Ile	458	Gly	780	Ala	166	Asp	413
5	Ser*	213	Lys	936	Leu	262	Ser*	213
6	Tyr	397	Val	885	Glu	154	Pro	386
7	Ser	94	(1/2Cys)	n.d.	Leu	240	Asn**	n.d.
8	Asp	312	Pro	690	Met	136	Ile	416
9	Gly	400	Lys	815	Val	289	Thr	104
10	Asp	278	Gly	647	Leu	225	Glu	354
11	Gln	274	Glu	670	Asn	122	Tyr	389
12	(1/2Cys)	n.d.	(1/2Cys)	n.d.	Val	267	Met	363
13	Ala	379	Pro*	604	Pro*	604	Phe	370
14	Ser	31	Trp	8	Arg	270	(1/2Cys)	n.d.
15	Ser	66	Gln	394			Ala	427

(*) The total amount of amino acid residues occuring from two sequences is given

(**) Glycosylated Asn

n.d. Not determined

In this sample four N-terminals were deduced Leu-39 (column 1), Gly-291 (column 3) and Lys-316 (column 4) corresponding to proteolytic cleavage and Ile-153 (column 2) corresponding to activation of FVII to FVIIa.

Claims

1. Modified factor VII/VIIa, characterized in that one, more, or all the lysine, arginine, isoleucine, and tyrosine residues

(i) Lys(38)
(ii) Lys(32)
(iii) Lys(143)
(iv) Arg(290)
(v) Arg(315)
(vi) Lys(316)
(vii) Lys(341)
(viii) Arg(398)
(ix) Arg(396)
(x) Arg(402)
(xi) Ile(42)
(xii) Tyr(44)

have been substituted and/or deleted.

2. Modified factor VII/VIIa according to claim 1, characterized in that one, more or all of the amino acid residues (i), (ii), (iv), and (v) have been substituted or deleted.

3. Modified factor VII/VIIa according to claim 1 or 2, characterized in that Lys(38) has been replaced with Thr, Asp, Leu, Gly, Ala, Ser, Asn, or His.

4. Modified factor VII/VIIa according to claim 3, characterized in that Lys(38) has been replaced with Thr.

5. Modified factor VII/VIIa according to claim 1 or 2, characterized in that Lys(32) has been replaced with Gln, Glu, His, Gly, Thr, Ala, or Ser.

6. Modified factor VII/VIIa according to claim 5, characterized in that Lys(32) has been replaced with Gln.

7. Modified factor VII/VIIa according to claim 1 or 2, characterized in that Arg(290) has been replaced with Gly, Ala, Ser, Thr, or Lys.

8. Modified factor VII/VIIa according to claim 1 or 2, characterized in that Lys(38) has been replaced with Ser, Ala, or Gly.

9. Modified factor VII/VIIa according to claim 1 or 2, characterized in that Arg(315) has been replaced with Gly, Thr, Ser, Ala, or Gln.

10. Modified factor VII/VIIa according to claim 1 or 2, characterized in that Lys(341) has been replaced with Glu, Gln, Thr, Ser, Ala, or Gly, preferably Glu or Gln.

11. Modified factor VII/VIIa according to claim 1, characterized in that Ile(42) has been replaced with Asn, Ser, Ala, or Gln.

12. Modified factor VII/VIIa according to claim 1, characterized in that Tyr(44) has been replaced with Asn, Ser, Ala, or Gln.

13. Modified factor VII/VIIa according to claim 1 or 2, characterized in that it comprises any combination of the substitutions according to any of the claims 3 to 12.

14. Modified factor VII/VIIa according to claim 1 or 2, characterized in that Lys(38) has been replaced with Thr, and Lys(32) has been replaced with Gln.

15. DNA-sequences, characterized in that they encode for a modified factor VII/VIIa according to any of the claims 1 to 14.

16. Expression vectors, characterized in that they contain a DNA-sequence according to claim 15.

17. Cells, characterized in that they are transformed to produce a modified factor VII/VIIa according to any of the claims 1 to 14

18. A method for the production of modified factor VII/VIIa, characterized in that a gene encoding for the expression of factor VII is mutated in one, more or all of the codons responsible for the expression of the lysine, arginine, isoleucine, and tyrosine residues

(i) Lys(38)
(ii) Lys(32)
(iii) Lys(143)
(iv) Arg(290)
(v) Arg(315)

(vi) Lys(316)
(vii) Lys(341)
(viii) Arg(398)
(ix) Arg(396)
(x) Arg(402)
(xi) Ile(42)
(xii) Tyr(44)

in native factor VII, generating a mutated gene capable of expressing a modified factor VII, wherein one, more or all of said amino acid residues are substituted or deleted, said mutated gene is inserted into a suitable host capable of expressing said mutated gene, said host is cultured in an appropriate growth medium thereby expressing the modified factor VII/VIIa, which subsequently is isolated and optionally activated to generate a modified factor VIIa product.

19. The method of claim 18, characterized in that in the produced modified factor VII/VIIa one, more or all of the amino acid residues (i), (ii), (iv), and (v) have been substituted or deleted.

20. The method of claim 18 or 19, characterized in that in the produced modified factor VII/VIIa Lys(38) has been replaced with Thr, Asp, Leu, Gly, Ala, Ser, Asn, or His.

21. The method of claim 20, characterized in that in the produced modified factor VII/VIIa Lys(38) has been replaced with Thr.

22. The method of claim 18 or 19, characterized in that in the produced modified factor VII/VIIa Lys(32) has been replaced with Gln, Glu, His, Gly, Thr, Ala, or Ser.

23. The method of claim 22, characterized in that in the produced modified factor VII/VIIa Lys(32) has been replaced with Gln.

24. The method of claim 18 or 19, characterized in that in the produced modified factor VII/VIIa Arg(290) has been replaced with Gly, Ala, Ser, Thr, or Lys.

25. The method of claim 18 or 19, characterized in that in the produced modified factor VII/VIIa Lys(38) has been replaced with Ser, Ala, or Gly.

26. The method of claim 18 or 19, characterized in that in the produced modified factor VII/VIIa Arg(315) has been replaced with Gly, Thr, Ser, Ala, or Gln.

27. The method of claim 18, characterized in that in the produced modified factor VII/VIIa Lys(341) has been replaced with Glu, Gln, Thr, Ser, Ala, or Gly, preferably Glu or Gln.

28. The method of claim 18, characterized in that in the produced modified factor VII/VIIa Ile(42) has been replaced with Asn, Ser, Ala, or Gln.

29. The method of claim 18, characterized in that in the produced modified factor VII/VIIa Tyr(44) has been replaced with Asn, Ser, Ala, or Gln.

30. The method of claim 18, characterized in that in the produced modified factor VII/VIIa any combination of the substitutions according to any of the claims 20 to 29 is present.

31. The method of claim 18 or 19, characterized in that in the produced modified factor VII/VIIa Lys(38) has been replaced with Thr, and Lys(32) has been replaced with Gln.

Revendications

1. Facteur VII/VIIa modifié, caractérisé en ce que un, plusieurs, ou tous les résidus lysine, arginine, isoleucine et tyrosine

(i) Lys(38)
(ii) Lys(32)
(iii) Lys(143)
(iv) Arg(290)
(v) Arg(315)
(vi) Lys(316)
(vii) Lys(341)
(viii) Arg(398)
(ix) Arg(396)
(x) Arg(402)
(xi) Ile(42)
(xii) Tyr(44)

ont été substitués et/ou supprimés par délétion.

2. Facteur VII/VIIa modifié selon la revendication 1, caractérisé en ce que un, plusieurs, ou tous les sidus

acides aminés (i), (ii), (iv), et (v) ont été substitués ou supprimés par délétion.

3. Facteur VII/VIIa modifié selon la revendication 1 ou 2, caractérisé en ce que Lys(38) a été remplacé par Thr, Asp, Leu, Gly, Ala, Ser, Asn, ou His.

4. Facteur VII/VIIa modifié selon la revendication 3, caractérisé en ce que Lys(38) a été remplacé par Thr.

5. Facteur VII/VIIa modifié selon la revendication 1 ou 2, caractérisé en ce que Lys(32) a été remplacé par Gln, Glu, His, Gly, Thr, Ala, ou Ser.

6. Facteur VII/VIIa modifié selon la revendication 5, caractérisé en ce que Lys(32) a été remplacé par Gln.

7. Facteur VII/VIIa modifié selon la revendication 1 ou 2, caractérisé en ce que Arg(290) a été remplacé par Gly, Ala, Ser, Thr, ou Lys.

8. Facteur VII/VIIa modifié selon la revendication 1 ou 2, caractérisé en ce que Lys(38) a été remplacé par Ser, Ala, ou Gly.

9. Facteur VII/VIIa modifié selon la revendication 1 ou 2, caractérisé en ce que Arg(315) a été remplacé par Gly, Thr, Ser, Ala, ou Gln.

10. Facteur VII/VIIa modifié selon la revendication 1 ou 2, caractérisé en ce que Lys(341) a été remplacé par Glu, Gln, Thr, Ser, Ala, ou Gly, de préférence par Glu ou Gln.

11. Facteur VII/VIIa modifié selon la revendication 1, caractérisé en ce que Ile(42) a été remplacé par Asn, Ser, Ala, ou Gln.

12. Facteur VII/VIIa modifié selon la revendication 1, caractérisé en ce que Tyr(44) a été remplacé par Asn, Ser, Ala, ou Gln.

13. Facteur VII/VIIa modifié selon la revendication 1 ou 2, caractérisé en ce qu'il comprend une combinaison quelconque des substitutions selon l'une quelconque des revendications 3 à 12.

14. Facteur VII/VIIa modifié selon la revendication 1 ou 2, caractérisé en ce que Lys(38) a été remplacé par Thr, et Lys(32) a été remplacé par Gln.

15. Séquences de DNA, caractérisées en ce qu'elles codent pour un facteur VII/VIIa modifié selon l'une quelconque des revendications 1 à 14.

16. Vecteur d'expression, caractérisés en ce qu'ils contiennent une séquence de DNA selon la revendication 15.

17. Cellules, caractérisées en ce qu'elles sont transformées pour produire un facteur VII/VIIa modifié selon l'une quelconque des revendications 1 à 14.

18. Procédé pour la production d'un facteur VII/VIIa modifié, caractérisé en ce qu'un gène codant pour l'expression du facteur VII est muté en un, plusieurs ou tous les codons responsables de l'expression des résidus lysine, arginine, isoleucine, et tyrosine.

(i) Lys(38)
(ii) Lys(32)
(iii) Lys(143)
(iv) Arg(290)
(v) Arg(315)
(vi) Lys(316)
(vii) Lys(341)
(viii) Arg(398)
(ix) Arg(396)
(x) Arg(402)
(xi) Ile(42)
(xii) Tyr(44)

dans le facteur VII natif, générant un gène muté capable d'exprimer un facteur VII modifié, dans lequel un, plusieurs ou tous les résidus acides aminés sont substitués ou supprimés par délétion, ledit gène muté est inséré dans un hôte convenable capable d'exprimer ce gène muté, ledit hôte est cultivé dans un milieu de croissance approprié exprimant de ce fait le facteur VII/VIIa modifié, qui par la suite est isolé et facultativement activé pour générer un produit du facteur VIIa modifié.

19. Procédé selon la revendication 18, caractérisé en ce que dans le facteur VII/VIIa modifié produit, un, plusieurs ou tous les résidus acides aminés (i), (ii), (iv) et (v) ont été substitués ou supprimés par délétion.

20. Procédé selon la revendication 18 ou 19, caractérisé en ce que dans le facteur VII/VIIa modifié produit, Lys(38) a été remplacé par Thr, Asp, Leu, Gly, Ala, Ser, Asn, ou His.

21. Procédé selon la revendication 20, caractérisé en ce que dans le facteur VII/VIIa modifié produit, Lys(38) a été remplacé par Thr.

22. Procédé selon la revendication 18 ou 19, caractérisé en ce que dans le facteur VII/VIIa modifié produit, Lys(32) a été remplacé par Gln, Glu, His, Gly, Thr, Ala, ou Ser.

23. Procédé selon la revendication 22, caractérisé en ce que dans le facteur VII/VIIa modifié produit,

Lys(32) a été remplacé par Gln.

24. Procédé selon la revendication 18 ou 19, caractérisé en ce que dans le facteur VII/VIIa modifié produit, Arg(290) a été remplacé par Gly, Ala, Ser, Thr, ou Lys.

25. Procédé selon la revendication 189 ou 19, caractérisé en ce que dans le facteur VII/VIIa modifié produit, Lys(38) a été remplacé par Ser, Ala, ou Gly.

26. Procédé selon la revendication 18 ou 19, caractérisé en ce que dans le facteur VII/VIIa modifié produit, Arg(315) a été remplacé par Gly, Thr, Ser, Ala, ou Gln.

27. Procédé selon la revendication 18, caractérisé en ce que dans le facteur VII/VIIa modifié produit, Lys(341) a été remplacé par Glu, Gln, Thr, Ser, Ala, ou Gly, de préférence par Glu, ou Gln.

28. Procédé selon la revendication 18, caractérisé en ce que dans le facteur VII/VIIa modifié produit, Ile(42) a été remplacé par Asn, Ser, Ala, ou Gln.

29. Procédé selon la revendication 18, caractérisé en ce que dans le facteur VII/VIIa modifié produit, Tyr(44) a été remplacé par Asn, Ser, Ala, ou Gln.

30. Procédé selon la revendication 18, caractérisé en ce que dans le facteur VII/VIIa modifié produit, une quelconque combinaison des substitutions selon l'une quelconque des revendications 20 à 29 est présente.

31. Procédé selon la revendication 18 ou 19, caractérisé en ce que dans le facteur VII/VIIa modifié produit, Lys(38) a été remplacé par Thr, et Lys(32) a été remplacé par Gln.

Patentansprüche

1. Modifizierter Faktor VII/VIIa, dadurch gekennzeichnet, daß einer, mehrere oder alle der Lysin-, Arginin-, Isoleucin- und Tyrosin-Reste

(i) Lys(38)
(ii) Lys(32)
(iii) Lys(143)
(iv) Arg(290)
(v) Arg(315)
(vi) Lys(316)
(vii) Lys(341)
(viii) Arg(398)
(ix) Arg(396)
(x) Arg(402)
(xi) Ile(42)
(xii) Tyr(44)

substituiert und/oder entfernt worden sind.

2. Modifizierter Faktor VII/VIIa nach Anspruch 1, dadurch gekennzeichnet, daß einer, mehrere oder alle der Aminosäure-Reste (i), (ii), (iv) und (v) substituiert oder entfernt worden sind.

3. Modifizierter Faktor VII/VIIa nach Anspruch 1 oder 2, dadurch gekennzeichnet, daß Lys(38) durch Thr, Asp, Leu, Gly, Ala, Ser, Asn oder His ersetzt worden ist.

4. Modifizierter Faktor VII/VIIa nach Anspruch 3, dadurch gekennzeichnet, daß Lys(38) durch Thr ersetzt worden ist.

5. Modifizierter Faktor VII/VIIa nach Anspruch 1 oder 2, dadurch gekennzeichnet, daß Lys(32) durch Gln, Glu, His, Gly, Thr, Ala oder Ser ersetzt worden ist.

6. Modifizierter Faktor VII/VIIa nach Anspruch 5, dadurch gekennzeichnet, daß Lys(32) durch Gln ersetzt worden ist

7. Modifizierter Faktor VII/VIIa nach Anspruch 1 oder 2, dadurch gekennzeichnet, daß Arg(290) durch Gly, Ala, Ser, Thr oder Lys ersetzt worden ist.

8. Modifizierter Faktor VII/VIIa nach Anspruch 1 oder 2, dadurch gekennzeichnet, daß Lys(38) durch Ser, Ala oder Gly ersetzt worden ist

9. Modifizierter Faktor VII/VIIa nach Anspruch 1 oder 2, dadurch gekennzeichnet, daß Arg(315) durch Gly, Thr, Ser, Ala oder Gln ersetzt worden ist.

10. Modifizierter Faktor VII/VIIa nach Anspruch 1 oder 2, dadurch gekennzeichnet, daß Lys(341) durch Glu, Gln, Thr, Ser, Ala oder Gly, bevorzugt durch Glu oder Gln, ersetzt worden ist.

11. Modifizierter Faktor VII/VIIa nach Anspruch 1, dadurch gekennzeichnet, daß Ile(42) durch Asn, Ser, Ala oder Gln ersetzt worden ist.

12. Modifizierter Faktor VII/VIIa nach Anspruch 1, dadurch gekennzeichnet, daß Tyr(44) durch Asn, Ser, Ala oder Gln ersetzt worden ist.

13. Modifizierter Faktor VII/VIIa nach Anspruch 1 oder 2, dadurch gekennzeichnet, daß er irgendeine Kombination der Substitutionen nach einem der Ansprüche 3 bis 12 aufweist.

14. Modifizierter Faktor VII/VIIa nach Anspruch 1 oder 2, dadurch gekennzeichnet, daß Lys(38) durch Thr ersetzt worden ist und Lys(32) durch Gln ersetzt worden ist.

15. DNA-Sequenzen, dadurch gekennzeichnet, daß sie für einen modifizierten Faktor VII/VIIa nach einem der Ansprüche 1 bis 14 codieren.

16. Expressionsvektoren, dadurch gekennzeichnet, dadurch gekennzeichnet, daß sie eine DNA-Sequenz nach Anspruch 15 enthalten.

17. Zellen, dadurch gekennzeichnet, daß sie zum Erzeugen eines modifizierten Faktors VII/VIIa nach einem der Ansprüche 1 bis 14 transformiert werden.

18. Verfahren Für die Herstellung von modifiziertem Faktor VII/VIIa, dadurch gekennzeichnet, daß ein für die Expression des Faktors VII codierendes Gen in eines, mehrere oder alle der Codone mutiert wird, die für die Expression der Lysin-, Arginin-, Isoleucin- und Tyrosin-Reste

(i) Lys(38)
(ii) Lys(32)
(iii) Lys(143)
(iv) Arg(290)
(v) Arg(315)
(vi) Lys(316)
(vii) Lys(341)
(viii) Arg(398)
(ix) Arg(396)
(x) Arg(402)
(xi) Ile(42)
(xii) Tyr(44)

im nativen Faktor VII verantwortlich sind, der ein mutiertes Gen erzeugt, welches zum Exprimieren eines modifizierten Faktors VII fähig ist, in dem einer, mehrere oder alle der Aminosäure-Reste substituiert oder entfernt worden sind, wobei das mutierte Gen in einen zweckmäßigen Wirt eingefügt wird, der fähig ist, das mutierte Gen zu exprimieren, wobei der Wirt in einem geeigneten Wachstumsmedium kultiviert wird, so daß der modifizierte Faktor VII/VIIa exprimiert wird, welcher anschließend isoliert und wahlweise aktiviert wird, um ein Modifizierter-Faktor-VIIa-Produkt zu erzeugen.

19. Verfahren nach Anspruch 18, dadurch gekennzeichnet, daß in dem erzeugten modifizierten Faktor VII-/VIIa einer, mehrere oder alle der Aminosäure-Reste (i), (ii), (iv) und (v) substituiert oder entfernt worden sind.

20. Verfahren nach Anspruch 18 oder 19, dadurch gekennzeichnet, daß in dem erzeugten modifizierten Faktor VII/VIIa Lys(38) durch Thr, Asp, Leu, Gly, Ala, Ser, Asn oder His ersetzt worden ist.

21. Verfahren nach Anspruch 20, dadurch gekennzeichnet, daß in dem erzeugten modifizierten Faktor VII-/VIIa Lys(38) durch Thr ersetzt worden ist.

22. Verfahren nach Anspruch 18 oder 19, dadurch gekennzeichnet, daß in dem erzeugten modifizierten Faktor VII/VIIa Lys(32) durch Gln, Glu, His, Gly, Thr, Ala oder Ser ersetzt worden ist.

23. Verfahren nach Anspruch 22, dadurch gekennzeichnet, daß in dem erzeugten modifizierten Faktor VII-/VIIa Lys(32) durch Gln ersetzt worden ist.

24. Verfahren nach Anspruch 18 oder 19, dadurch gekennzeichnet, daß in dem erzeugten modifizierten Faktor VII/VIIa Arg(290) durch Gly, Ala, Ser, Thr oder Lys ersetzt worden ist.

25. Verfahren nach Anspruch 18 oder 19, dadurch gekennzeichnet, daß in dem erzeugten modifizierten Faktor VII/VIIa Lys(38) durch Ser, Ala oder Gly ersetzt worden ist.

26. Verfahren nach Anspruch 18 oder 19, dadurch gekennzeichnet, daß in dem erzeugten modifizierten Faktor VII/VIIa Arg(315) durch Gly, Thr, Ser, Ala oder Gln ersetzt worden ist.

27. Verfahren nach Anspruch 18, dadurch gekennzeichnet, daß in dem erzeugten modifizierten Faktor VII-/VIIa Lys(341) durch Glu, Gln, Thr, Ser, Ala oder Gly, bevorzugt durch Glu oder Gln, ersetzt worden ist.

28. Verfahren nach Anspruch 18, dadurch gekennzeichnet, daß in dem erzeugten modifizierten Faktor VII-/VIIa Ile(42) durch Asn, Ser, Ala oder Gln ersetzt worden ist.

29. Verfahren nach Anspruch 18, dadurch gekennzeichnet, daß in dem erzeugten modifizierten Faktor VII-/VIIa Tyr(44) durch Asn, Ser, Ala oder Gln ersetzt worden ist.

30. Verfahren nach Anspruch 18, dadurch gekennzeichnet, daß in dem erzeugten modifizierten Faktor VII-/VIIa irgendeine Kombination der Substitutionen nach einem der Ansprüche 20 bis 29 vorliegt.

31. Verfahren nach Anspruch 18 oder 19, dadurch gekennzeichnet, daß in dem erzeugten modifizierten Faktor VII/VIIa Lys(38) durch Thr ersetzt worden ist und Lys(32) durch Gln ersetzt worden ist.

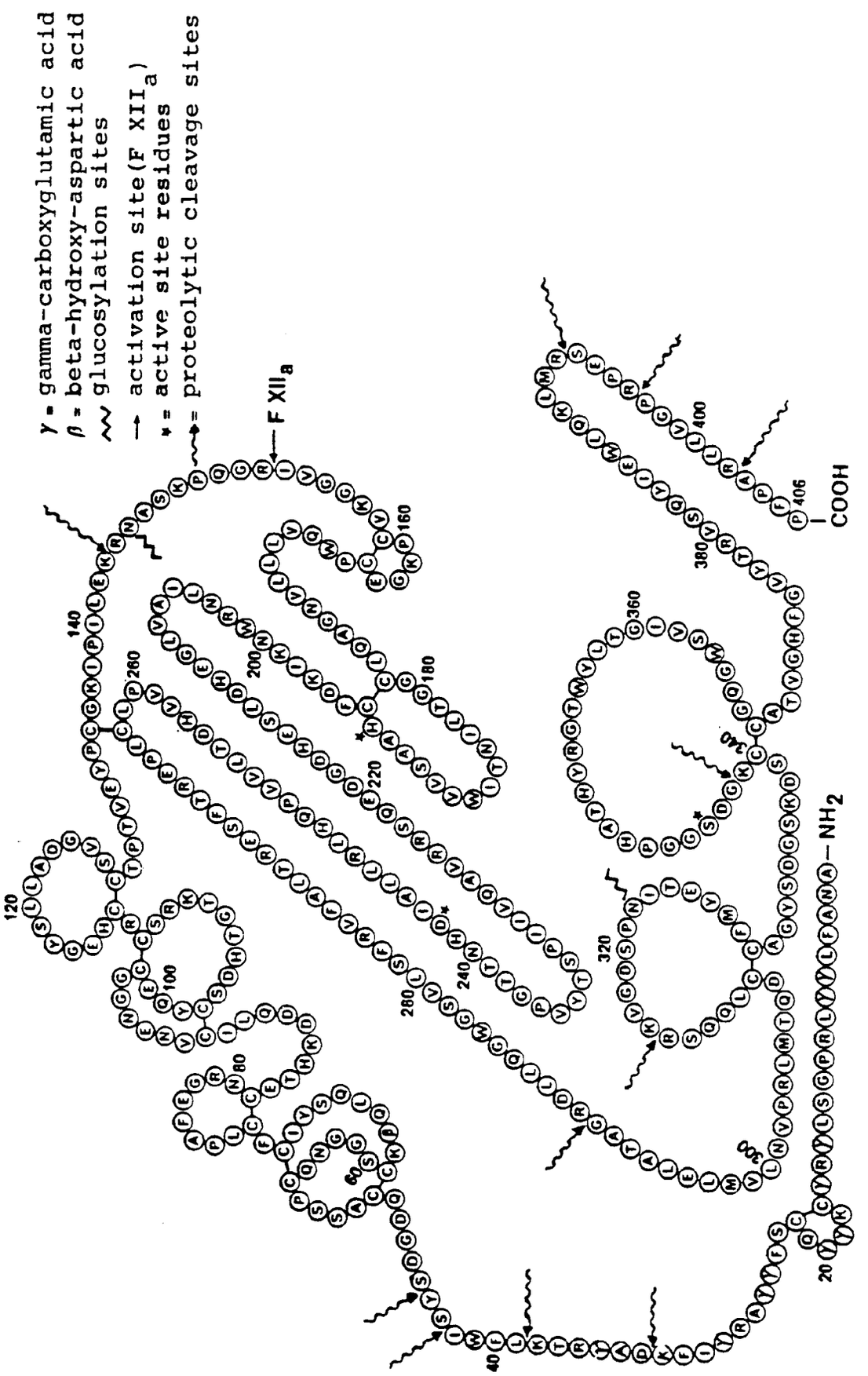

γ = gamma-carboxyglutamic acid
β = beta-hydroxy-aspartic acid
glucosylation sites
activation site(F XII$_a$)
* = active site residues
proteolytic cleavage sites
F XII$_a$
COOH
NH$_2$

FIG. 1

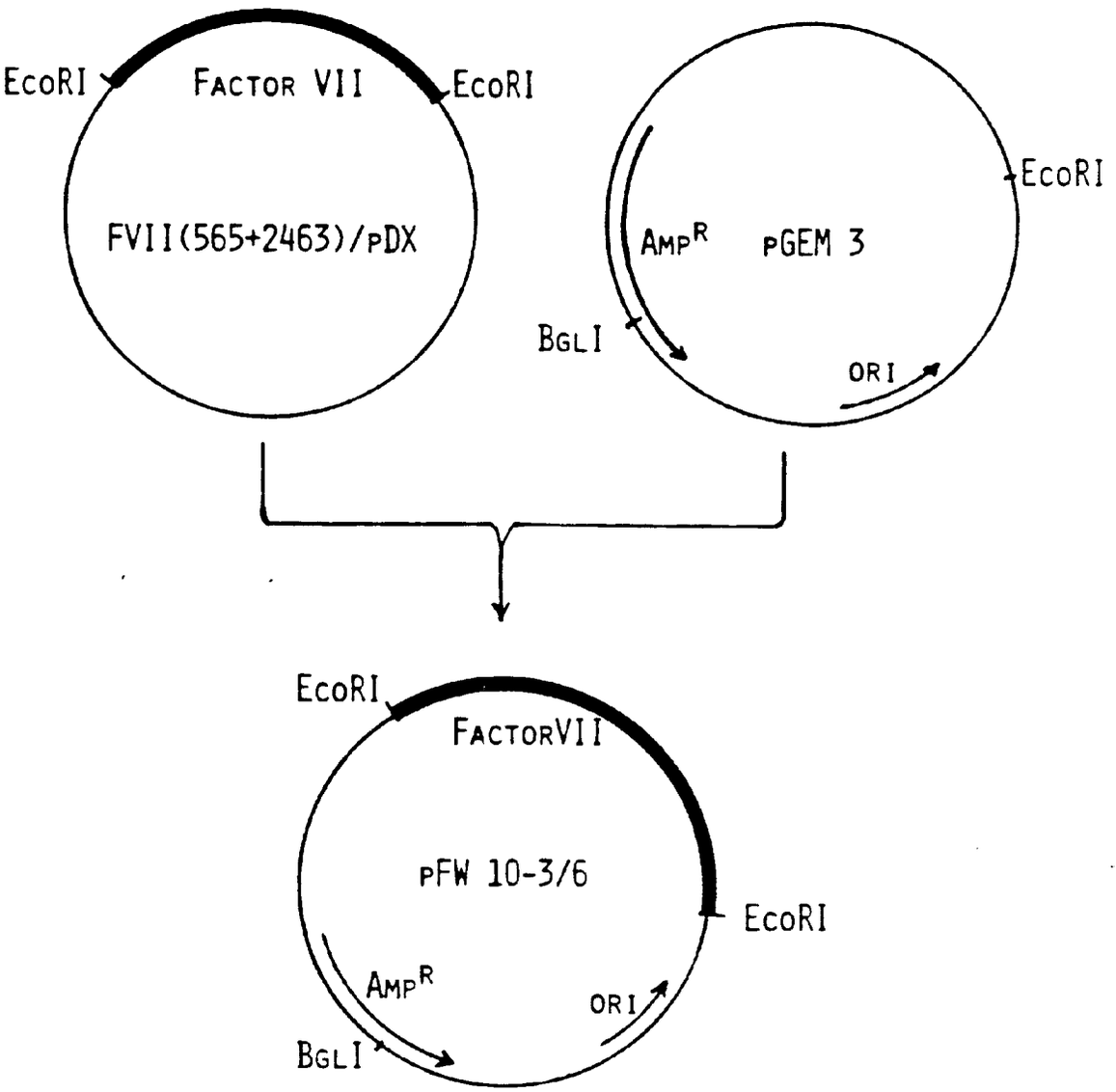

EcoRI
FACTOR VII
EcoRI
FVII(565+2463)/pDX
AMPR
pGEM 3
EcoRI
BGLI
ORI
EcoRI
FACTORVII
pFW 10-3/6
EcoRI
AMPR
ORI
BGLI

FIG. 2

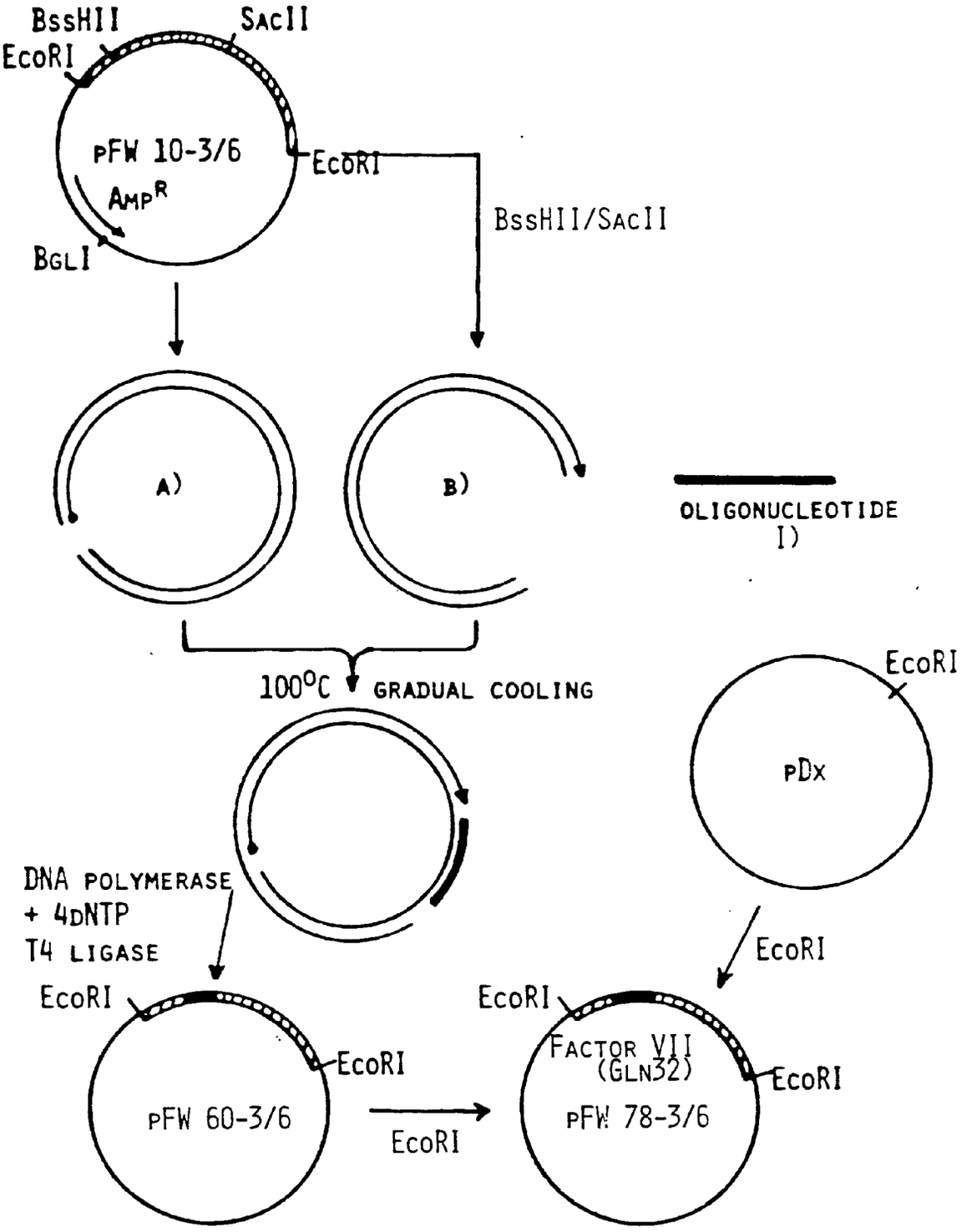

BssHII
SacII
EcoRI
pFW 10-3/6
AmpR
EcoRI
BglI
BssHII/SacII
A)
B)
OLIGONUCLEOTIDE
I)
100°C GRADUAL COOLING
EcoRI
pDx
DNA POLYMERASE
+ 4dNTP
T4 LIGASE
EcoRI
EcoRI
EcoRI
pFW 60-3/6
EcoRI
EcoRI
Factor VII
(Gln32)
EcoRI
pFW 78-3/6

Fig. 3

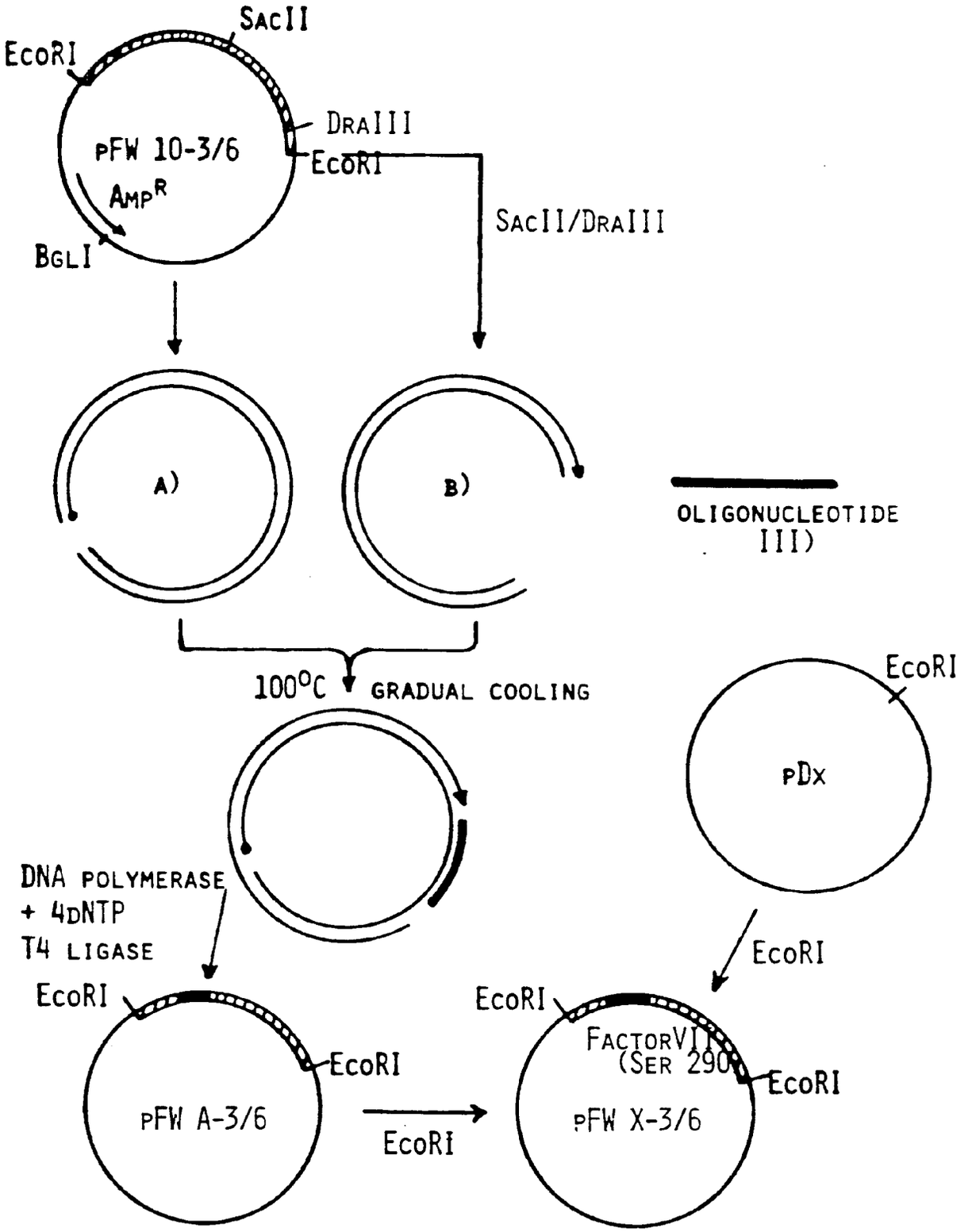

SacII
EcoRI
DraIII
pFW 10-3/6
EcoRI
AmpR
BglI
SacII/DraIII
A)
B)
OLIGONUCLEOTIDE
III)
100°C GRADUAL COOLING
EcoRI
pDx
DNA POLYMERASE
+ 4dNTP
T4 LIGASE
EcoRI
EcoRI
EcoRI
EcoRI
pFW A-3/6
EcoRI
EcoRI
FactorVII
(Ser 290)
EcoRI
pFW X-3/6

Fig. 4

The Gly331Ser mutation in factor VII in Europe and the Middle East

The Gly331Ser mutation in factor VII (FVII) was found in the homozygous condition in several unrelated FVII deficient subjects from Southern Italy and from Germany, one of Turkish origin. Genotyping for several polymorphisms revealed that the mutation, occurring at a CpG site, is associated with two haplotypes, suggesting multiple occurrence.

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(*http://www.haematologica.org/2003_12/1434.htm*)

Following vascular injury, factor VII (FVII) binds to tissue factor and triggers blood coagulation. FVII deficiency,[1] a rare inherited clotting factor deficiency, produces a wide range of hemorrhagic phenotypes and is caused by a variety of molecular lesions.[2] The association between phenotype and the underlying clotting defect, and the threshold FVII levels able to guarantee coagulation are still debated.

We present here the study of the distribution and the haplotyping of the Gly331Ser mutation in several FVII deficient patients. Ten subjects from Southern Italy (group A) with FVII coagulant activity (FVIIc) ≤4% and normal FVII antigen (FVIIAg) were initially studied. Subjects A1-5 have been previously described[3] as PFVII33-37. Subjects A6 and A7 were asymptomatic. Patient A8 experienced gum-bleeding and was treated with recombinant activated FVII (rFVIIa) (NovoNordisk, Bagsvaed, Denmark), A9 had a central nervous system bleed at the age of six years and A10 was treated with rFVIIa after post-trauma hemorrhage.

Mutations were searched by MspI restriction analysis for the Gly331Ser change[3] and sequencing. Five subjects (A1-5) were homozygous for the Gly331Ser mutation and one (A6) was doubly heterozygous for the Gly331Ser and Cys310Phe changes (Table 1). Three patients (A7-9) were homozygous for the Cys310Phe, Ala294Val and Arg304Gln mutations in the FVII catalytic domain. All these mutations are responsible for dysfunctional FVII molecules.[4-6] No mutation in coding regions was detected in subject A10. The frequency of Gly331Ser among unrelated subjects from Southern Italy prompted us to investigate 22 additional subjects with mild to asymptomatic FVII deficiency (group B, mean FVIIc 31%, range 10-63%) from this geographical area. The Gly331Ser mutation was present in four heterozygotes (Table 1).

Moreover, five unrelated carriers of this change were identified among 189 German subjects (group C) included in the Greifswald Research Study on FVII Deficiency[7] (Table 1).

The Gly 331Ser-FVII variant is associated with a cross-reacting material positive (CRM+) FVII deficiency, as indicated by

Table 1. Geographical, coagulation and clinical features of the Italian (I) and German (G) carriers of the FVII Gly331Ser mutation.

Patient (G-PIN)	*Center*	*FVIIc (FVIIAg) %*	*Genotype (second mutation)*	*Clinical phenotype*	*Haplotype*
A1	Bari (I)	2	Homozygous	Asymptomatic	A1-G1-H1-b-M1
A2	Naples (I)	0.7 (85)	Homozygous	Asymptomatic	A1-G1-H1-b-M1
A3	Salerno (I)	0.7 (100)	Homozygous	Asymptomatic	A1-G1-H1-b-M1
A4	Napoli (I)	0.6 (100)	Homozygous	Mild	A1-G1-H1-b-M1
A5	Palermo (I)	1.1	Homozygous	Mild	A1-G1-H1-b-M1
A6	Naples (I)	4 (95)	D. het. (C310F)	Asymptomatic	A1-G1-H1-b-M1
B1	Naples (I)	25 (85)	Heterozygous	Asymptomatic	A1-G1-H1-b-M1
B2	Naples (I)	37 (85)	Heterozygous	Asymptomatic	A1-G1-H1-b-M1
B3	Naples (I)	41 (80)	Heterozygous	Asymptomatic	A1-G1-H1-b-M1
B4	Napoli (I)	47 (100)	Heterozygous	Asymptomatic	A1-G1-H1-b-M1
C1 (9490)	Hamburg (G)	6 (100)	D. het. (A294V)	Mild	A1-G1-H1-b-M1
C2 (9254)	Frankfurt (G)*	5.6	D. het. (A294V)	Asymptomatic	A2-G2-H2-a-M2
C3 (9291)	Kaiserslautern (G)	21	Heterozygous	Asymptomatic	#G1/2-H1/2-M1/2
C4 (9496)	Halle (G)	45	Heterozygous	Asymptomatic	#G1/2-H1/2-M1/2
C5 (9933)	Greifswald (G)	35	Heterozygous	Asymptomatic	A1-G1-H1-b-M1

*G-PIN: Identification Number of German Patients, enrolled in the Greifswald Research Study on FVII Deficiency. D. het.: doubly heterozygous #Family members were not available for this study. *Turkish origin.*

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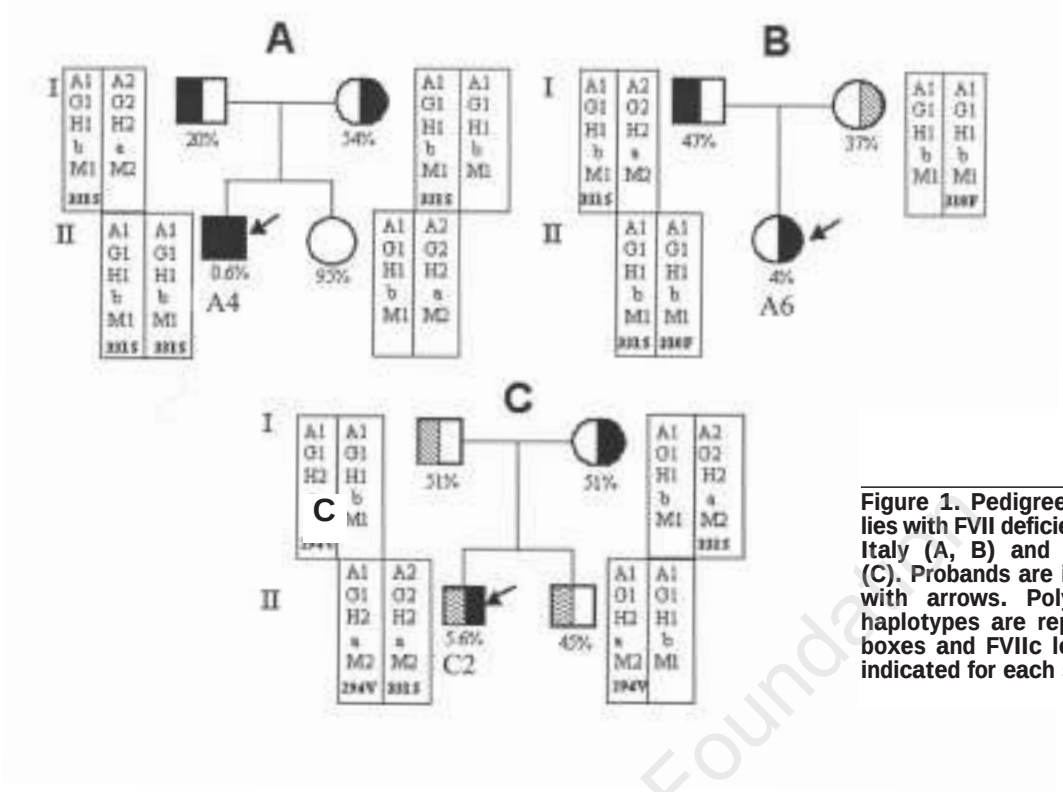


Figure 1. Pedigree of families with FVII deficiency from Italy (A, B) and Germany (C). Probands are indicated with arrows. Polymorphic haplotypes are reported in boxes and FVIIc levels are indicated for each subject.

the decreased FVIIc and the normal FVIIAg levels observed in patients' plasma (Table 1) and confirmed by previous and present expression studies performed in our laboratory. Expression of the FVII variant and functional assays were conducted as reported elsewhere.[3] FXa generation activity in patients' plasma was 0.7±0.2% of that observed in pooled normal plasma and, in reconstituted systems, the Gly 331Ser-FVII activity was 2.7±1.1% that of wild type FVII. These findings predict that this variant is able to trigger coagulation thus explaining the mild to asymptomatic phenotype observed in homozygotes, and the spread of the Gly331Ser mutation in Europe. One British heterozygous carrier of the Gly331Ser mutation and one French compound heterozygote for the Gly331Ser and the Gln100Arg mutations have been described (*http://europium.csc.mrc.ac.uk*).

The slightly different FVIIc levels observed among the Gly331Ser homozygotes (Table 1) could be explained by the different thromboplastins used in routine clotting assays. Carriers of the Gly331Ser mutation were further characterized for five FVII gene polymorphisms:[8-9] a 10bp insertion at -323 (A1/A2 alleles), 73G to A transition (G1/G2), 7880C to T transition at His115 (H1/H2), intron 7 repeat polymorphism (a/b/c/d) and Arg353Gln substitution (M1/M2).

The availability of three families allowed segregation studies to be performed. In the A4 pedigree (Figure 1A) the M2 allele, present in the father who was heterozygous for the Gly331Ser mutation, contributed to further reduction of FVII level.[10] Pedigree analysis of A6 (Figure 1B) revealed segregation of the Ser331 and Phe310 alleles. Segregation of the Ser331 and Val294 alleles was observed in family C2 (Figure 1C) of Turkish origin.

Genotyping for polymorphisms showed that the Gly331Ser mutation is associated with the same genetic background, A1-G1-H1-b-M1, in the Italian and German subjects, but it was associated with the A2-G2-H2-a-M2 haplotype in the Turkish family (Table 1). These findings support the independent origin of the mutation in Europe and in Turkey.

The recurrence of this transition may be considered in the light of its position (nt 10908), in a CpG dinucleotide, one of the several *hot spots* in the FVII gene prone to recurrent mutations.

The high incidence of the Gly331Ser mutation among unrelated subjects from Southern Italy, and its association with the same haplotype, led to a scenario of a founder effect in the past, likely followed by consanguineous spread. We suggest screening for this mutation in FVII deficient patients from this geographical area, through a simple Msp I restriction analysis. This strategy also reveals the frequent M1/M2 polymorphism able to predict reduced FVII levels. Analysis for the presence of the Gly 331Ser-FVII variant, whose residual activity prevents life-threatening bleeding symptoms in FVII deficient patients with very reduced FVIIc levels might also have implications for prevention and treatment of this disease.

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Key words: mutation, CRM+ FVII deficiency, founder effect.

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Genetic determinants of iron metabolism plasma phenotypes and their relationship with risk of thrombosis

This study evaluates the relative contributions of genetic and environmental factors to 6 iron-related phenotypes and the relationship between these phenotypes and the risk of thrombosis. All of these phenotypes were influenced significantly by genetics, with heritability ranging between 18% for transferrin saturation to 55% for soluble transferrin receptor (sTfR). Only sTfR exhibited a significant genetic correlation with thrombosis.

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The physiologic and biochemical pathways involved in iron metabolism are very complex. Given the spectrum of iron metabolism-related phenotypes, it is likely that there are a number of interacting genetic and environmental factors that jointly determine the variable expression. The primary objective of the present investigation was to examine the relative roles of genetic and environmental factors in determining iron-related phenotypes. To this end, we studied a series of extended Spanish kindreds, half of which were enrolled through individuals with thrombophilia. They were recruited in a project called GAIT (Genetic Analysis of Idiopathic Thrombophilia) designed to look for new genetic risk factors for thrombosis through the analysis of intermediate phenotypes.[1-3] Recent data on a possible association between the hemochromatosis gene mutation HFE Cys282Tyr and cardiovascular diseases[4-6] motivated us to include iron metabolism-related phenotypes in the GAIT Project. In 1981, Sullivan proposed a possible relationship between iron and ischemic heart disease. He hypothesized that iron depletion protects against myocardial infarction and could explain the sex differences in the rates of heart disease.[7] The ascertainment criteria for our families also allowed us to study the potential relationship of iron metabolism phenotypes with thromboembolic disease.

The details on the enrollment of families and statistical methods used in the GAIT Project can be found elsewhere.[1-3] Plasma iron, unsaturated iron binding capacity (UIBC) and soluble transferrin receptor (sTfR) were measured in a Hitachi 911 biochemical analyzer by colorimetric and immunoassay methods, respectively (Fe, UIBC and sTfR Tinaquant). Total iron binding capacity (TIBC) was obtained as the sum of plasma iron and UIBC. Circulating ferritin was evaluated using an electrochemiluminiscent immunoassay (Ferritina) in a biochemical analyzer (ELECSYS). All assays and the analyzer were from Roche Diagnostics (Mannheim, Germany). Transferrin saturation (SAT) was obtained by dividing iron values by TIBC values and was expressed as a percentage. Intra- and inter-assay coefficients of variation were less than 10% for all of the phenotypes. We logarithmically (ln) transformed the values of sTfR and ferritin to obtain normal distributions.

Table 1 presents the estimated components of the residual variance for the iron metabolism-related phenotypes. All of the traits had significant heritability (h^2), ranging between 18% and 55% of the residual phenotypic variability. The proportion of the residual phenotypic variability accounted for by shared *household effects* (χ^2) tended to be considerably smaller than that accounted for by genetic effects. Household effects were significant for only 2 traits: ferritin and SAT. The remaining variance (not accounted for in Table 1) was attributed to individual-specific random environmental influences and random error.

Table 2 shows the results of bivariate genetic analyses of thrombosis with each of the quantitative iron metabolism-

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ORIGINAL ARTICLE

Characterization of mutations causing factor VII deficiency in 61 unrelated Israeli patients

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Summary. Inherited factor (F)VII deficiency is rare in most populations but relatively common in Israel. The aim of this study was to characterize the molecular and functional defect in unrelated Israeli patients with FVII deficiency. Mutations were identified by direct sequencing of PCR-amplified genomic DNA fragments. Selected mutations were expressed in baby hamster kidney (BHK) cells and tested for binding to tissue factor (TF), activation by FXa and activation of FX. In 61 patients with FVII deficiency, the causative mutation in the FVII gene was discerned. The predominant mutation found in this and a previously reported cohort of 27 unrelated patients in Israel was Ala244Val substitution; of 121 independent mutant alleles defined in all 88 patients ascertained in Israel, 102 (84%) bore this alteration. Eleven additional mutations were identified of which one, Cys22Arg, is novel. Expression of the mutations in BHK cells revealed that four (Ala244Val, 11128delC, Leu300-Pro and Cys22Arg) were cross-reacting material (CRM)-negative, and three (Ala294Val, Cys310Phe and Phe24del) were CRM-positive. As predicted by modeling, we observed no binding to TF of FVII Phe24del, diminished binding of FVII Cys310Phe and normal binding of FVII Ala294Val. The main defect of FVII Ala294Val was its inability to activate FX in the presence of TF. Coexpression of Ala294Val and Arg353Gln, a polymorphism known to affect FVII secretion, did not reveal an additive effect on FVII secretion, while coexpression of Ala244Val and Arg353Gln did yield an additive effect.

Keywords: *F7* gene mutation, factor VII deficiency, factor VII, tissue factor.

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Introduction

Inherited factor (F)VII deficiency is a rare autosomal recessive disorder manifested by a bleeding tendency in homozygotes or compound heterozygotes. The bleeding tendency in patients with severely reduced FVII activity is variable, ranging from hemarthroses and intracerebral bleeding to a mild or moderate bleeding tendency, and does not always correlate with the extent of FVII deficiency [1]. Another common feature of FVII deficiency is discordance between plasma FVII activity and FVII antigenicity displayed by normal or reduced FVII antigen levels and very low FVII activity [2]. About 130 mutations causing FVII deficiency have been identified and are listed in a human genome mutation database (http://europium.csc.mrc.ac.uk). We previously determined the molecular basis in 27 unrelated Israeli patients with FVII deficiency [3,4]. The aim of this study was to identify the molecular defects in additional 61 unrelated patients and to characterize functional defects caused by mutations pertinent for analysis.

Materials and methods

Patients

Sixty-one unrelated Israeli patients were referred for evaluation because of a bleeding tendency, prolonged prothrombin time and normal partial thromboplastin time, or a known FVII deficiency. The ethnic origin of the patients was defined by the country of birth of their grandparents. All patients consented to participate in the study. The Institutional Review Board of Sheba Medical Center approved the study.

Materials

Chemicals were of high analytical grade and obtained from Sigma (St. Louis, MO, USA) unless otherwise stated. Restriction enzymes were obtained from New England Biolabs (Beverly, MA, USA).

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Mutation identification

DNA extracted from patients' leukocytes by a desalting procedure was used as template for PCR amplifications of coding regions and intron/exon boundaries of the *F7* gene. Specific primers and conditions are available upon request. PCR fragments were subjected to direct sequencing by automatic sequencer.

Restriction enzyme analysis

The Arg353Gln polymorphism, Arg304Gln and Ala244Val mutations were detected as described [3,5]. Cys310Phe, Thr359Met and Cys22Arg mutation were detected by *Cac*8I, *Bsa*BI and *Nci*I digestion, respectively.

Construction of expression vectors

Human FVII cDNA in puC19 vector was provided by Dr Earl Davie (University of Washington, Seattle). It was subcloned into *Eco*RI site of pGEM7 (Promega, Madison, WA, USA) for mutagenesis. For production of Ala244Val, Ala294Val, Leu300Pro, Cys310Phe, Arg353Gln, 11128delC and Ala294-Val/Arg353Gln mutants, the respective sequence alterations were introduced into FVII cDNA by site-directed mutagenesis, using two overlapping oligonucleotide primers incorporating the single base pair substitution and two external primers (specific primers for each mutation are available upon request). A Phe24del mutant and a double mutant Ala244Val/Arg353Gln were introduced into normal FVII cDNA by PCR amplification of the DNA from patients bearing the respective two genotypes and using external primers.

PCR products, each containing one of eight identified mutants (except Phe24del), were cut by *Blp*I and *Bsi*WI for replacing normal cDNA in pGEM7-FVII. For Phe24del, the 5′ and 3′ enzymes were *Bss*HII and *Bgl*II, respectively. Wild-type (WT) and mutated cDNAs were subcloned into *Eco*RI site of pcDNA3 mammalian expression vector carrying the geneticine resistance gene as selection marker (Invitrogen, Carlsbad, CA, USA). The Cys22Arg mutant was created by kit (QuickChange™, Stratagene, Cambridge, UK) using pcDNA3WT-FVII as a template and two mutant primers. The sequence and orientation of created mutant clones were verified by sequencing and restriction analysis.

Cell culture and transfection

Baby hamster kidney (BHK) cells were grown and transfected with either WT or mutants of FVII in pCDNA3 as described [6]. Mock cells were produced by transfecting BHK cells with 1 μg of pcDNA3. Selection of stably transfected cells was carried out by growing the cells in a medium containing 0.7 μg mL^{-1} G418 (Gibco BRL, Paisley, UK) for at least 2 weeks. The medium was collected for FVII assays from confluent plates containing about 10^7 cells grown for 24 h with 7 mL serum-free medium (Optimem 1, Gibco BRL) containing 5 μg mL^{-1} vitamin K (Konakion, Roche, Basel, Switzerland), and was filtered and kept frozen at − 70 °C until assayed. Cells were washed twice with phosphate buffered saline, and lysed for 10 min in 1 mL lysis buffer [20 mmol L^{-1} Tris HCl (pH 7.5), 150 mmol L^{-1} NaCl, 1% Nonident-P-40 supplemented with a 1 : 10 dilution of an inhibitor cocktail (Complete™, Bohringer, Mannheim, Germany) and 2 mmol L^{-1} phenylmethylsulfonyl fluoride]. The lysates were centrifuged at 14 000 × *g* for 10 min at 4 °C and kept frozen at − 70 °C. All mutated clones were transfected three times except for the Cys22Arg which was transfected only twice. The WT clone was transfected six times. Total protein in medium and lysate were calculated by Bradford procedure (Bio-Rad, Hercules, CA, USA).

FVII activity and antigen measurements

FVII activity was measured by a PT-based assay using FVII-depleted plasma. This assay was sensitive to FVII levels up to 0.2 U dL^{-1}. FVII antigen was determined by ELISA, using a kit (Asserachrom Stago Diagnostica, Asnieres, France). FVII activity and antigenicity in plasma were expressed as U dL^{-1} and in media or lysates of transfected cells as percent of WT.

Western blot analysis

Proteins were concentrated two- to 20-fold by Centricon-30 (Amicon, Millipore, Bedford, MA, USA), analyzed by NuPAGE® Bis-Tris 4–12% gels (Invitrogen) and transferred to polyvinylidene difluoride membrane (Immobilon P, Millipore) incubated with 1 : 1000 dilution of polyclonal rabbit anti-FVII (Stago Diagnostica). Immunoreactive complexes were detected using 1 : 10 000 dilution of peroxidase conjugate goat antirabbit antibody (Jackson, West Grove, PA, USA) which was enhanced by chemiluminescence (ECL) kit (Amersham, Buckinghamshire, UK) and displayed by X-ray film exposure.

Binding of mutant FVII to tissue factor (TF)

Binding of recombinant FVII to immobilized TF was analyzed as described by Kavlie *et al.* [7]. Optical density at wavelength 490 nm of each mutant protein was expressed as percent of the optical density of WT at the same concentration, after subtraction of nonspecific binding.

Activation of mutant FVII by FXa

Reaction mixtures in Tris-buffered saline (TBS; 20 mmol L^{-1} Tris HCl pH 7.5, 150 mmol L^{-1} NaCl) containing 1.4 nmol L^{-1} recombinant FVII, lipidated TF (Innovin®, Dade Behring, Marburg, Germany) in a 1 : 1 molar ratio and 5 mM calcium were incubated at 37 °C. FVII activation was initiated by 25 pmol L^{-1} FXa (Enzyme Research, South Bend, IN, USA). At selected intervals, subsamples were removed and subjected to gel electrophoresis under reducing condition and Western blotting. Rate of FVII activation was

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estimated by densitometric analysis assessing the proportion of the 28-kDa (the heavy chain of activated FVII) and the 50-kDa (of nonactivated FVII). Mean results of two experiments were expressed as percent of mean results obtained with WT FVII.

Activation of FX by mutant FVII and TF

Reaction mixture in TBS containing 1 mg mL^{-1} BSA, WT or mutant FVII (2.5–25 pmol L^{-1}), lipidated TF in a 1 : 1 molar ratio in the presence of 5 mmol L^{-1} calcium and 2 nmol L^{-1} purified FX (Enzyme Research) was incubated at 37 °C. After 30 min, activation of FX was stopped by adding 25 mM EDTA. Generated FXa was measured by adding the chromogenic substrate S2765 (0.4 mmol L^{-1}) (Chromogenix, Molndal, Sweden) and recording the increased absorbance at 405 nm. The minute amount of FXa present in purified FX was essential for initial activation of FVII but had no detectable effect on the chromogenic substrate. The results were expressed as percentage of WT V_{max} (mOD min^{-1}).

Structural analysis of FVII mutants

Crystal structures of active site-inhibited TF–FVIIa complex [8], des-Gla TF–FVIIa complex inhibited by E-76 peptide [9] and TF–FVIIa complex-inhibited by BPTI mutant [10], were obtained from Protein Data Bank (PDB entries 1dan, 1dva and 1fak, respectively). Superimpositions were generated by the C-Alpha Match program [11,12].

Results

Mutations causing FVII deficiency in Israel

Sixty-one unrelated patients with FVII deficiency were characterized. Table 1 shows the genotypes, FVII levels, clinical data and ethnic origin of a total of 88 patients examined in our present and previous studies [3,4]. None of 40 patients with one mutated allele and FVII activity more than 17 U dL^{-1} had significant bleeding manifestations except for a newborn who exhibited gastrointestinal bleeding. Of 48 patients with mutations affecting both alleles, 45 had mild to moderate bleeding manifestations. The remaining three (two with an activity of < 1 U dL^{-1} and one with an activity of 8 U dL^{-1}) had hemarthroses and cerebral hemorrhage which was fatal in two patients.

Of 121 independent mutant alleles discerned in 88 patients, 102 (84%) bore the Ala244Val substitution. This predominant mutation was present particularly in Moroccan and Iranian Jewish patients but was also detected in two homozygotes of Arab and Iraqi-Jewish origin, respectively, and in four heterozygotes of Iraqi, Turkish, Bucharan and Libyan Jewish origin, respectively. Interestingly, another mutation at the same location, Ala244Thr, was detected in a Moroccan Jewish heterozygote.

The second most frequent mutation, Cys310Phe, was detected in four unrelated Druze patients, of whom three were homozygotes and one was a heterozygote. This mutation was also detected in a Yemenite Jewish patient who was a compound heterozygote for Leu300Pro and Cys310Phe mutations [4].

Altogether, 12 mutations were identified, of which 11 were earlier reported and one was novel, a T to C transversion at nucleotide 3886 in exon 2 [nucleotides numbering according to O'Hara *et al*. (13)]. This mutation, identified in a heterozygous Arab newborn, predicts Cys22Arg transition in the Gla domain and creates a restriction site of *Nci*I. Amplification of exon 2 from the parents and digestion with *Nci*I disclosed that the mother was heterozygous and the father was normal.

One patient of Russian origin was homozygous for two mutations, an Ala294Val alteration and 11128delC. This double homozygosity was observed in Polish patients [14]. The 11128delC alteration causes a frameshift, giving rise to a termination codon, 84-bp downstream to the natural one.

Plasma FVII activity and antigenicity in homozygotes disclosed concordantly decreased levels in patients bearing Ala244Val and Ala294Val/11128delC alterations, cross reacting material reduced $(CRM)^{reduced}$ FVII in a patient bearing the Phe24del mutation and $CRM^{positive}$ FVII in patients bearing Arg304Gln and Cys310Phe mutations, respectively (Table 1).

Interaction between Ala294Val mutation and Arg353Gln polymorphism

All homozygotes for Ala244Val are also homozygotes for Arg353Gln [3], a polymorphism that causes decreased FVII secretion from cells thereby augmenting the defect caused by Ala244Val mutation [15,16]. Because the identified homozygote for the Ala294Val mutation was also homozygous for Arg353Gln polymorphism, we examined the relative effects of the mutation and the polymorphism on FVII secretion by transfected BHK cells. Table 2 shows that compared to WT cells, cells bearing only Ala294Val or Ala294Val/Arg353Gln secreted similar amounts of dysfunctional FVII (41 and 55%, respectively). These results contrasted with the effect of Arg353Gln on Ala244Val; cells bearing only Ala244Val secreted 35% of WT FVII antigenicity, cells harboring Arg353Gln secreted 55% and cells bearing both Ala244Val and Arg353Gln secreted only 7%.

CRM-negative mutations

FVII activity and antigenicity in the medium of BHK cells transfected with Ala244Val, 11128delC, Leu300Pro and Cys22Arg mutations were concordantly decreased. However, measurement of the cellular content of FVII antigen in cells bearing these mutations yielded variable results: In cells harboring Ala244Val and Leu300Pro, FVII content was similar to WT, in cells harboring 11128delC approximately half the amount of FVII present in WT cells was detected and in cells bearing Cys22Arg the amount of FVII was 50% higher than WT (Table 2). These results suggest that in cells bearing Ala244Val, Leu300Pro and Cys22Arg there is a

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Table 1 Molecular, laboratory and clinical data in unrelated Israeli patients with FVII deficiency

Genotype	*n**	Plasma FVII		Ethnic origin	Clinical severity
		Activity (U dL^{-1})	Antigen (U dL^{-1})		
Homozygotes or compound heterozygotes					
Ala244Val/Ala244Val	37 (13)	5.5 ± 1.8†	6 (5)‡	Moroccan, Iranian and Iraqi Jewish, Arab	Mild or asymptomatic
Cys310Phe/Cys310Phe	3	< 1	83 (3)‡	Druze	Mild or asymptomatic
Ala244Val/Arg304Gln	2 (1)	7.5	ND	Moroccan/Egyptian and Iraqi Jewish	Mild
Arg304Gln/Arg304Gln	1	7	60	Iraqi Jewish	Asymtomatic
Cys310Phe/Leu300Pro	1 (1)	< 1	52	Yemenite Jewish	Mild
Thr359Met/Thr359Met	1	8	ND§	Arab	Severe
Ala294Val; 11128delC/Ala294Val; 11128delC	1	< 1	1	Russian	Moderate
IV52 + 1 G > C/IV52 + 1 G > C	1 (1)	< 1	ND§	Arab	Severe
Phe24del/Phe24del	1 (1)	< 1	30	Arab	Severe
Heterozygotes					
Ala244Val/N	35 (9)	32 ± 10.5†	41 (2)‡	Moroccan, Iranian, Iraqi, Turkish, Bucharan and Libyan Jewish	Asymptomatic
Ala244Thr/N	1	34	ND	Moroccan Jewish	Asymptomatic
Cys310Phe/N	1	36	68	Druze	Asymptomatic
Arg304Gln/N	1	21	71	Arab	Asymptomatic
Cys22Arg¶//N	1	17	36	Arab	Mild
Arg277His/N	1 (1)	25	44	Yemenite Jewish	Asymptomatic

ND, not determined; N, normal allele; *n** denotes total number of patients, numbers in parenthesis represent previously described patients [3,4]. †Mean ± SD. ‡Mean values are given. They were derived from the number of patients depicted in parenthesis. §Died of intracerebral hemorrhage soon after birth. ¶A novel mutation.

Table 2 Characteristics of FVII mutants expressed in BHK cells

Mutation	Secreted FVII, % of WT		Cellular content of FVII antigen, % of WT
	Activity	Antigen	
WT*	100	100	100
CRM negative			
Ala244Val	25 ± 6	35 ± 9	83, 89
Arg353Gln	65 ± 23	55 ± 26	98 ± 26
Ala244Val/Arg353Gln	8 ± 2	7 ± 0.6	100
11128delC	7 ± 3	16 ± 0.7	48 ± 9
Leu300Pro	< 1	10 ± 4	92 ± 12
Cys22Arg	< 1	0.6, 1	138, 166
CRM positive/reduced			
Ala294Val	7 ± 2	41 ± 21	95
Ala294Val/Arg353Gln	7 ± 2	55 ± 27	100
Cys310Phe	< 1	36 ± 10	91 ± 5
Phe24del	< 1	22 ± 9	107 ± 12

Total protein in media and lysates were about 10 μg mL^{-1} and 1 mg mL^{-1}, respectively. *In six transfections absolute data of WT FVII was (mean ± SD): activity 25 ± 10 U dL^{-1}, secreted antigen 171 ± 63 ng mL^{-1} and intracellular antigen 102.5 ± 24 ng mL^{-1}.

secretion defect, whereas in cells bearing 11128delC there is either instability and intracellular degradation of FVII or a defect in its production. A small amount of FVII 11128delC was nonetheless secreted which had a higher molecular weight due to a predictable extension of FVII by 28 amino acids which was demonstrable by Western blot analysis (Fig. 1).

Mutations causing dysfunctional FVII

Expression of Ala294Val, Cys310Phe and Phe24del mutations produced FVII proteins that were categorized as $CRM^{positive}$ or $CRM^{reduced}$ (Table 2). The Arg304Gln, another $CRM^{positive}$ mutation, was characterized by O'Brien *et al.* [17]. The intracellular content of FVII antigen in all three mutants was normal.

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Figure 2 shows that binding of FVII Phe24del to nonlipidated TF was completely abolished, of FVII Cys310Phe substantially decreased, and of FVII Ala294Val or FVII Ala294Val/Arg353Gln not significantly different from WT FVII or commercial rFVIIa. The experiment with FVII Phe24del mutant was repeated with lipidated TF and the same result was obtained (data not shown).

Figure 3 shows the results of experiments in which the FVII mutants were activated by FXa. At 60 min, Ala294Val FVII was activated by FXa similarly to WT FVII (89% of WT). The proportion of Ala294Val/Arg353Gln FVIIa was 70% of WT. In contrast, activation of FVII Cys310Phe mutant by FXa was delayed and reduced to 20% of WT FVII.

FX activation by FVII Ala294Val, Ala294Val/Arg353Gln and Cys310Phe mutants in the presence of TF and Ca^{2+} was then tested. V_{max} for Ala294Val, Ala294Val/Arg353Gln and Cys310Phe mutants at a concentration of 2.5 pmol L^{-1} were 5, 5.1 and 0.4 mOD min^{-1}, respectively, compared to 43.8 mOD min^{-1} for WT FVII. Partial correction for Ala294Val and Ala294Val/Arg353Gln was achieved by using 10-fold higher concentrations (25 pmol L^{-1}) of the recombinant proteins yielding V_{max} of 78.7 and 74.2 mOD min^{-1}, respectively, compared to 92.1 mOD min^{-1} for WT FVII at the same concentration. Increased concentrations of FVII Cys310Phe did not correct the V_{max}.

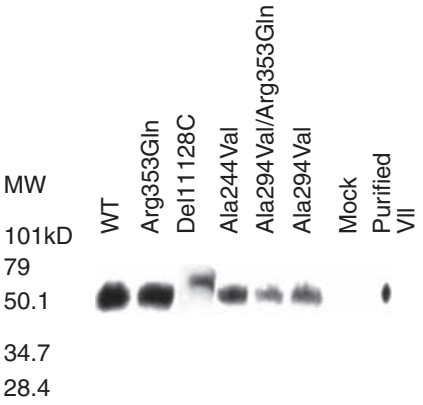


Fig. 1. Western blot analysis of recombinant proteins. Notice the higher band representing 11128delC FVII containing 28 additional amino acids.

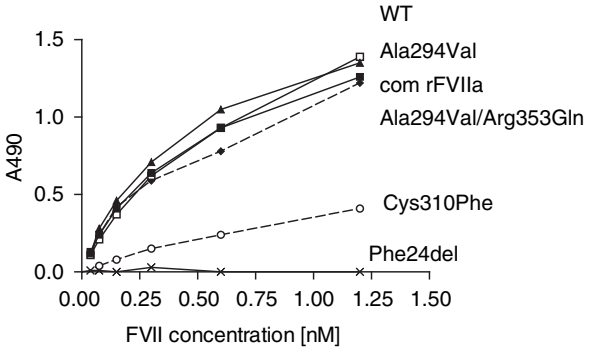


Fig. 2. Binding of recombinant WT FVII, mutant FVII and commercial recombinant FVIIa (rFVIIa) to non-lipidated TF. Corrected absorbance at 490 nm is plotted against different concentrations of FVII.

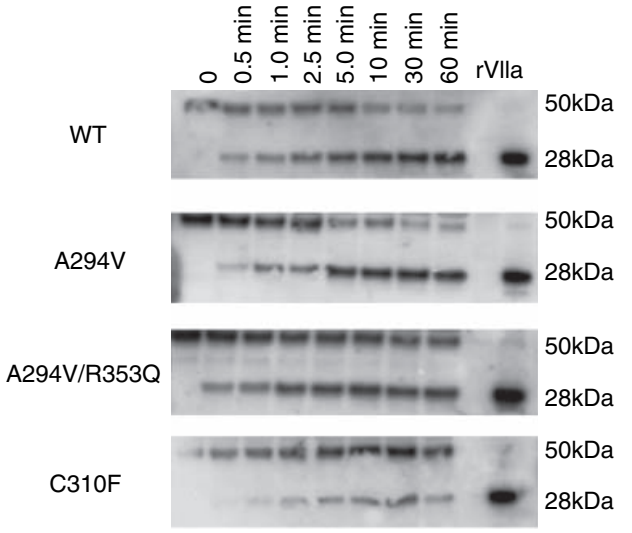


Fig. 3. Time course of activation of WT or mutant FVII by purified FXa in the presence of TF and Ca^{2+} shown by Western blotting. rFVIIa (5 ng) is shown as a positive control. A polyclonal rabbit anti FVII detected the 50-kDa single chain nonactivated FVII and the 28-kDa heavy chain of activated FVII.

Structural analysis of Ala294Val and Phe24Del mutants

Modeling was carried out only for FVII mutants secreted from transfected cells, i.e. Ala294Val and Phe24del. Cys310Phe was not analyzed because the mutation disrupts the 170s disulfide bonded loop that is essential for binding of FVII to TF [18]. A model of Ala294Val (not shown) predicted that replacement of Ala by Val, a β-branched amino acid, imposes clashes between the side chain of Val and neighboring amino acids, predicting a conformational alteration in the 140s loop which is essential for recognition of FIX and FX [9,19]. An analogous effect of a conformational change in the 140s loop is presented by superimposition of the heavy chain of FVIIa–TF complex covalently bound with an inhibitor (1dan) or with a peptide exosite inhibitor (1dva) (Fig. 4A). Notably, the inhibitory peptide in the FVIIa–TF–peptide complex inflicts conformational movement of the 140s loop causing allosteric inhibition of FVIIa thereby interfering with substrate recognition [9].

The 'hydrophobic stack' of the Gla helix (residues 34–46) comprises the main interface of the Gla domain and TF [20]. Phe24 interacts with Trp41 located on this helix and therefore the Phe24del mutant is predicted to disrupt the interface between the Gla domain and TF. This prediction was evaluated by analysis of a known structure of FVII in which the Gla domain is truncated. Figure 4(B) represents superimposition of FVIIa with full-length Gla domain (1dan) and FVIIa with truncated Gla domain missing the 35 N-terminal amino acids (1fak). Notably, a shorter Gla domain causes a shift of the hydrophobic helix imitating the effect of Phe24 deletion.

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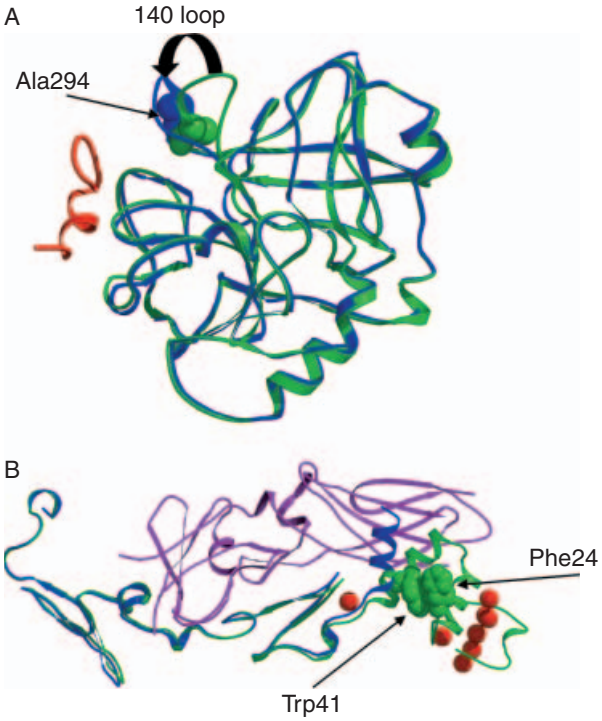


Fig. 4. Superimposition of FVII structures. (A) Superimposition of the heavy chain of 1dan (green) and 1dva (blue) structures. The E-76 inhibitor is demonstrated by a red ribbon. Ala294 in the 140s loop of both structures is represented by space-filled models. The black arrow demonstrates the conformational movement of the 140s loop in the presence of the inhibitor. Such a movement is expected to occur when there is Ala294Val substitution. (B) Superimposition of the light chain of FVIIa (1dan, green) and Gla-truncated light chain of FVIIa (1fak, blue). The TF is represented by a purple ribbon. Phe24 contacts Trp41 located on the hydrophobic helix. Both are represented by space-filled models. Ca^{2+} ion bonds in the Gla domain are shown as red balls. The diagrams were prepared using MolScript [30] and Raster3d [31].

Discussion

The present data and our earlier reports [3,4] established that the predominant mutation causing FVII deficiency in Israel is Ala244Val, accounting for 84% of independent mutant alleles defined in 88 patients. This mutation was confined mainly to patients of Iranian and Moroccan Jewish origin and was shown to have stemmed from a single founder and to be relatively frequent in normal subjects belonging to these ethnic groups [3]. A Lebanese patient [21], an Arab patient and an Iraqi Jewish patient (Table 1), all homozygotes for Ala244Val, had the same founder haplotype. The bleeding tendency in homozygotes for Ala244Val is relatively mild which is probably related to their plasma FVII levels ranging between 3 and 10 U dL^{-1}. Stable transfection of Ala244Val FVII in BHK cells revealed decreased secretion (35% of WT cells) (Table 2), as demonstrated in transient transfection in COS cells [16]. Coexpression of Ala244Val with the common Arg353Gln polymorphism further decreased secretion to 7% of WT cells which is consistent with similarly reduced plasma levels of FVII that are typical for homozygotes for Ala244Val who are always homozygotes for the Arg353Gln polymorphism [3,16]. Interestingly, this effect of Arg353Gln could not be reproduced in cells bearing Ala294Val. Cells transfected with cDNA coding for Ala294Val mutation secreted $CRM^{reduced}$ FVII, but inclusion of Arg353Gln polymorphism in the transfected cDNA did not further decrease FVII secretion (Table 2). The cause for the difference between the effects of Arg353Gln on Ala244Val and on Ala294Val remains to be clarified.

The second most frequent mutation identified in Israel was Cys310Phe substitution (4% of 121 independent alleles). This mutation was described in other populations [5,21,22]. Four of five patients carrying the mutation were Druze (Table 1) whose population in 2002 according to Israel's Bureau of Statistics was 108 000. Although three Druze homozygotes for Cys310Phe were offspring of related parents, it is conceivable that this mutation is prevalent in Druze. Bearers of Cys310Phe, including the Druze patients, carry a common haplotype, but it is difficult to assess whether this finding indicates common ancestry because this haplotype is very frequent in the general population [21]. The Cys310Phe mutation was shown to be $CRM^{positive}$ and structural analysis predicted that it causes a defect in binding FVII to tissue factor by abrogating loop 170s between Cys310 and Cys329 [22]. Although this loop is not in direct contact with TF, chemical modification experiments indicated that the disulfide bond between Cys310 and Cys329 stabilizes a structure essential for interaction with TF [18]. Moreover, this loop probably plays an important role in TF-induced conformational transition of FVIIa affecting its catalytic efficiency [23]. We proved that indeed, recombinant Cys310Phe displays diminished binding to TF (Fig. 2). We also showed that its activation by FXa was substantially reduced (Fig. 3) and that in the presence of TF it did not activate FX (1% from WT). Conceivably, the decreased binding of Cys310Phe to TF and reduced catalytic efficiency were responsible for the additional derangement observed.

The Arg304Gln mutation observed in a homozygote, two compound heterozygotes and a heterozygote (Table 1), was described in other populations [5,17,22]. This is a recurrent mutation located in a CpG dinucleotide hot spot. Arg304Gln FVII is $CRM^{positive}$, exhibiting impaired binding to TF and defective activations of FIX and FX [17]. Notably, Arg304Trp also exhibits defective binding to TF [24] and plasma of homozygotes for Arg304Gln displays 1% of normal FVII activity when rabbit TF is used, but 8–12% when human TF is used [5,17,22]. The low FVII activity (7% of normal) observed in the homozygote of this study when measured with human TF, probably stems from the concomitant presence of homozygosity for Arg353Gln polymorphism. This additive effect of Arg353Gln was further supported by finding an FVII antigen level of 60 U dL^{-1} in the patient, whereas other described homozygotes for Arg304Gln had much higher FVII antigen levels [5,17,22].

The triple homozygosity for Ala294Val, 11128delC and Arg353Gln gene alterations observed in one patient was observed in other populations [14,21]. We dissected the effect of the gene alterations on the secretion of FVII by BHK cells. Expression of Ala294Val produced $CRM^{reduced}$ FVII

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(Table 2), adding Arg353Gln alteration had no additional effect but expression of 11128delC yielded profoundly decreased secretion of FVII. These results imply that among the three gene alterations, the 11128delC mutation which predicts an extension of FVII by 28 amino acids is the predominant cause for the severe FVII deficiency. Similarly to Toso *et al*. [19], we found that FVII Ala294Val displayed greatly diminished activation of FX in the presence of TF (11% from WT). This finding is consistent with our structural analysis which predicts that replacement of Ala294 by Val causes conformational alteration of the 140 s loop which is essential for recognition of FX and FIX [25]. Our experiments did not reveal any defect in binding FVII Ala294Val to TF (Fig. 2), while another study did report an apparent defect [19]. This discrepancy is probably related to the different methodologies used: Our data were based on direct binding of Ala294Val FVII to TF, whereas Toso *et al*. [19] interpreted the need to increase the concentration of TF for achieving measurable FVII amidolytic activity as an indication of defective binding of Ala294Val FVII to TF.

The unique Phe24del mutation was investigated in this study. The Gla domain, and particularly the hydrophobic stack residues 39–44, is part of the interface between TF and FVII [20,26,27]. The Gla domain is crucial for the optimal conformation of FVII and any change in its structure affects the overall interaction with TF [8,10,20,26]. A truncation [10], a mutation at codon Glu35 [28] or deletion of single residue in the Gla domain disrupt the binding of FVII to TF. Also, a structure containing a truncated Gla domain reveals that the hydrophobic stack assumes a different conformation and that the interaction with TF is impaired (Fig. 4B). These observations suggest that the deletion of Phe24 disrupts the interface between the Gla domain and TF, and indeed this $CRM^{reduced}$ FVII did not bind at all to human nonlipidated and lipidated TF (Fig. 2).

The novel Cys22Arg mutation is also located in the Gla domain. Cys17 and Cys22 are conserved residues in vitamin K-dependent coagulation factors forming a disulfide bond important for holding together two Gla residues in a proper position. This position enables capturing Ca^{2+} ions that are critical for proper function of the vitamin K-dependent factors [29]. However, our results suggest that Cys22Arg substitution abolishes the structure of the molecule because BHK cells expressing this mutation failed to secrete FVII and accumulated it intracellulary (Table 2).

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ARTICLE

Analysis of the genotypes and phenotypes of 37 unrelated patients with inherited factor VII deficiency

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Severe inherited factor VII (FVII) deficiency is a rare autosomal recessive disorder with a poor relationship between FVII coagulant activity and bleeding tendency. Both clinical expression and mutational spectrum are highly variable. We have screened for mutations the FVII gene of 37 unrelated patients with a FVII coagulant activity less than 5% of normal pooled plasmas. The nine exons with boundaries and the 5′ flanking region of the FVII gene were explored using a combination of denaturing gradient gel electrophoresis and direct DNA sequencing. This strategy allowed us to characterise 68 out of the 74 predicted FVII mutated alleles. They corresponded to a large panel of 40 different mutations. Among these, 18 were not already reported. Genotypes of the severely affected patients comprised, on both alleles, deleterious mutations which appeared to be related to a total absence of activated FVII. We suggest that this absence of functional FVII can explain the severe clinical expression. Whether a small release of FVII is sufficient to initiate the coagulation cascade and to prevent the expression of a severe phenotype, requires further investigations. *European Journal of Human Genetics* (2001) **9**, 105–112.

Keywords: factor VII deficiency; haemorrhagic disorder; single base-pair mutation

Introduction

Human coagulation factor VII (FVII) is a vitamin-K-dependent glycoprotein that consists of a 406 amino-acid modular protein with a N-terminal-gamma-carboxyglutamic acid rich (Gla) domain, two epidermal growth factor-like (EGF) domains and a C-terminal serine proteinase domain. After cleavage of an Arg-Ile peptide bond at residues 152/153, FVII is converted to the active form, factor VIIa (FVIIa) comprising two chains connected by a disulphide bridge between Cys 135 and Cys 262. FVIIa on its own has virtually no catalytic activity but triggers the coagulation at sites of injury by binding to the cell surface receptor tissue factor (TF) in the presence of calcium ions, ultimately resulting in thrombin generation and the formation of a fibrin clot.[1]

Inherited FVII deficiency is a rare autosomal recessive disorder with an estimated incidence of 1 in 500 000. The bleeding tendency in affected patients is highly variable and correlates poorly with plasma FVII activity levels.[2] The clinical features vary considerably, from easy bruising, epistaxis, modest or severe postoperative bleeding, to life-threatening intracranial haemorrhages.[1–3] On the other hand, several asymptomatic patients with a FVII coagulant activity level less than 1% of normal have been reported.[1] In addition, up to 70 different mutations underlying the FVII deficiency phenotype have already been described in the

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human FVII mutation database (http://europium.csc.mrc.ac.uk/usr). Study of the heterogeneous molecular basis of FVII deficiency offers the opportunity to investigate the mechanisms through which genotype is able to modulate the residual FVII activity and to produce a large spectrum of clinical phenotypes. For this purpose, we have collected clinical features and characterised the FVII genotypes of a large cohort of patients displaying FVII coagulant activity less than 5% of normal pool plasmas. This study is one of the largest ever reported on severe FVII deficiency.[4–6]

Materials and methods

Patients

The panel consisted of 37 unrelated patients (22 females and 15 males) with inherited FVII deficiency attending 16 French centres specialising in the follow-up of haemorrhagic disorders. Patients who entered the study were selected on the basis of a factor VII coagulant activity level less than 5% of normal (Table 1). All patients gave written informed consent in accordance with the French law. A family history of consanguinity was known for six patients.

Clinical and biological data

Clinical features were recorded by the physician in charge of each patient in a standardised questionnaire. Collected data focused on the diagnostic circumstances and on the bleeding tendency. The type, severity and frequency of the haemorrhagic symptoms were noted. The FVII coagulant activity (FVIIc) was assayed in each centre by a one-stage method based on the prothrombin time using thromboplastin reagents and FVII deficient plasma as a substrate. Factor VII antigen (FVIIAg) was determined using the Asserachrom FVII:Ag Kit (Diagnostica Stago, Asniere, France) according to the manufacturer's instructions (Table 1).

Blood collection

Blood samples were obtained from the 16 centres and mailed to the laboratory at room temperature within 24 h, for DNA extraction.

DNA isolation and conditions for polymerase chain reaction

Genomic DNA was isolated according to standard methods. The coding portions, intron/exon boundaries and the 5′ flanking region (5′FR) of the FVII gene were amplified from genomic DNA by polymerase chain reaction (PCR). Primer sets were designed according to the published FVII gene sequence[7] and are listed in Table 2.

Mutation detection by denaturing gradient gel electrophoresis (DGGE)

DGGE was used to screen for sequence alterations, PCR fragments containing the portions of interest of FVII gene. MELT 87 and SQHTX computer programs,[8] which allow simulation of the melting behaviour of any DNA sequence, were used to optimise the DGGE conditions. To ensure that the DNA portion of interest was within the lowest melting temperature domain, a GC rich region was added at one end of the fragment to be screened as described.[8] As the melting map of exons 1a, 2, 3, 4, 6 and 7 did not easily allow the use of DGGE, these PCR fragments were directly sequenced. DGGE was performed for the mutation screening of exons 1b, 5 and 8. Three sets of primers were used to independently amplify the three melting domains of exon 8, generating fragments 8A, 8B and 8C. Migration gels consisted of 6.5% acrylamide in a linear 50–90% denaturing gradient and were run as previously described[9] (Table 2). Samples displaying abnormal DGGE profiles were sequenced.

Mutation detection by direct sequencing

PCR amplified fragments for coding regions 1a, 2, 3, 4, 6, 7, exon-intron boundaries, 5′FR and samples with an abnormal DGGE pattern were directly sequenced using the Big dye Terminator sequencing kit (Perkin Elmer, Norwalk, CT, USA) on an Applied 310 sequencing apparatus (PE, Applied Biosystems,Warrington, UK).

Mutation screening strategy

In this study, we have made an exhaustive genetic analysis of all FVII regions of interest so as not to miss double-mutations or polymorphisms that could be involved in the phenotypic variability. For each patient, the sequences of the nine exons and corresponding boundaries of FVII gene were analysed. Each nucleotide sequence alteration was confirmed on at least two independent PCR products by either restriction enzyme digestion, when the mutation modified a restriction site, or another independent sequencing analysis. If no mutation was found after the first screening-step, exons 1b, 5 and 8 were tested for heteroduplex formation under the same DGGE conditions, and the 5′FR was sequenced. If one or two mutations were still missing, exons 1b, 5 and 8 were then entirely sequenced.

Results

Clinical and biological phenotypes

The 37 patients investigated in this study were classified into three clinical groups (Table 1). Group A comprised asymptomatic patients with no bleeding history (n=15). Group B included patients who experienced menorrhagia, epistaxis or bruising with no need for substitutive therapy (n=13). Group C comprised patients with a history of severe bleeding (haematoma, haemarthrosis, intracranial haemorrhages) (n=9). The severe disease group comprised only females, whereas the mild disease groups included 15 males and 13 females. No thromboembolic episode was related.

Table 1 Identified phenotypes and genotypes of the 37 FVII deficient patients

				Biological phenotypes (%)		*Genotypes and haplotypes*		
Lab number	*Sex*	*Non French origin*	*Clinical group*	*FVII c*	*FVII Ag*	*Allele 1*	*Allele 2*	*FVII haplotypes*
F7-11	F		A	<1	64	**Gly331→Ser**	Gln100→Arg	(A1 H1 h6 I1 M1) X2
F7-16	M	N Afr	A	<5[a]	25	**Ala191→Thr**	**Ala191→Thr**	(A1 H1 h6 I1 M1) X2
F7-22	M		A	2[a]	72	Arg304→Gln	Arg304→Gln	(A1 H1 h6 I1 M1) X2
F7-24	M	N Afr	A	5[a]	44	Arg304→Gln	Ala244→Val	A1A1 H1H1 h6h7 I1I2 M1M2
F7-34	F		A	<5[a]	55	Arg304→Gln	Cys135→Arg	A1A1 H1H1 h6h7 I1I1 M1M1
F7-39	M		A	3[a]	10	Ala244→Val	**Gly179→Arg**	A2A2 H1H2 h7h7 I2I2 M2M2
F7-29	M	N Afr	A	1	9	Met298→Ile	Cys310→Phe	(A1 H1 h6 I1 M1) X2
F7-12	F	N Afr	A	2[a]	na	Arg304→Trp	Arg304→Trp	(A1 H1 h6 I1 M1) X2
F7-33	F		A	<1	na	**10543 del gcgagcacgacctca**	Cys310→Phe	(A1 H1 h6 I1 M1) X2
F7-38	F		A	<5[a]	na	Arg304→Gln	Arg304→Gln	(A1 H1 h7 I1 M1) X2
F7-40	M		A	<1[a]	na	Cys310→Phe	Cys310→Phe	(A1 H1 h6 I1 M1) X2
F7-17	M	N Afr	A		37	Met298→Ile	wt	A1A1 H1H1 h6h7 I1I1 M1M1
F7-31	F		A	2	19	**Cys102→Tyr**	wt	A1A1 H1H1 h6h7 I1I1 M1M1
F7-37	M	A		5[a]	7	Ala244→Val	wt	(A1 H2 h7 I2 M2) x2
F7-15	M	N Afr	A	1	<2	wt	wt	(A1 H1 h7 I1 M1) x2
F7-41	F		B	4[a]	48	11125 delC and Ala294→Val	Ala294→Val	(A2 H2 h7 I2 M2) x2
F7-2	F		B	<1	8	Gln100→Arg	Gly97→Cys	A1A1 H1H2 h6h7 I1I2 M1M2
F7-9	M	N Afr	B	<1	47	**Met327→Ile**	**Met327→Ile**	(A1 H1 h6 I1 M1) X2
F7-25	F	N Afr	B	5	76	Met298→Ile	Met298→Ile	(A1 H1 h6 I1 M1) X2
F7-1	M	N Afr	B	<1	6	**Arg379→Gly**	**Arg379→Gly**	(A2 H2 h7 I2 M2) x2
F7-4	F		B	2	62	64+5G→A	**Gly179→Arg**	A1A1 H1H1 h6h7 I1I1 M1M1
F7-5	M		B	<1	17	3933+1G→A	**3762G→A**	(A1 H1 h6 I1 M1) x2
F7-6	M		B	3	23	Arg304→Gln	**Gly180→Arg**	A1A2 H1H2 h6h7 I1I2 M1M2
F7-8	M		B	2[a]	15	**10743 delG**	Gly96→Ser	(A1 H1 h6 I1 M1) X2
F7-10	F		B	2[a]	na	11125 delC and Ala294→Val	**Leu13→Gln**	(A1 H2 h7 I2 M2) x2
F7-27	M	Swiss	B	2[a]	na	Arg277→Cys	**Arg28→Gly**	A1A2 H1H2 h7h7 I1I2 M1M2
F7-42	F		B	5[a]	na	Met298→Ile	**Trp364→Stop**	(A1 H1 h6 I1 M1) X2
F7-19	F		B	2	na	**Met1→Val**	wt	A1A1 H1H2 h6h7 I1I2 M1M2
F7-13	F		C	2	7	6070+1G→A	Cys135→Arg	(A1 H1 h6 I1 M1) x2
F7-20	F		C	<1	25	**5886+5G→A**	Glu16→Lys	A1A2 H1H1 h6h7 I1I1 M1M1
F7-26	F		C	<1	2	**Asn57→Ile**	**Asn57→Ile**	(A1 H1 h6 I1 M1) x2
F7-30	F		C	<1	65	Phe328→Ser	**Asp343→Asn**	(A1 H1 h6 I1 M1) X2
F7-32	F		C	<1[a]	na	11125 delC and Ala294→Val	Arg152→Gln	A1A1 H1H2 h7h7 I1I2 M1M2
F7-14	F		C	1	<2	Thr359→Met	Thr359→Met	(A1 H1 h6 I1 M1) X2
F7-18	F		C	<1[a]	na	**Gln49→Stop**	Gln100→Arg	(A1 H1 h6 I1 M1) X2
F7-23	F	Asian	C	2	2	His348→Gln	His348→Gln	(A1 H1 h6 I1 M1) X2
F7-35	F		C	2[a]	na	−61T→G	−55C→T	A1A1 H1H1 h6h7 I1I1 M1M1

The FVIIc column corresponds to the FVII coagulant activity levels. The recombinant human TF (Instrumentation laboratory, Lexington, USA) was used for the coagulant assays except for the samples denoted with a superior ([a]), which were tested with Thromboplastins from human or rabbit origins. The corresponding FVII haplotype is associated with each mutation when possible. A1: allele 73G, A2: allele 73A, H1: allele His 115 CAC, H2: allele His 115 CAT, h6: allele including 6 × 37 bp repeats at IVS7, h7: allele including 7 × 37 bp repeats at IVS7, I1: allele 10523G, I2: allele 10523A, M1: allele Arg 353 CGG, M2: allele Gln 353 CAG.[10–13] The mutations which are not reported in the FVII mutation database 2000, are indicated in bold. The numbering is according to O'Hara *et al*.[7] 'wt' indicates a wild type sequence in the screened area. 'na', data not available. 'N Afr' indicates origins from North Africa.

FVII antigen levels could be determined in 26 patients (Table 1). Four patients were classified as 'cross-reacting material negative' (CRM−) as they had very low or absent antigen levels (2% or less). Seventeen patients had reduced antigen levels (CRM^{red}). Five patients displayed normal antigen levels (>60%) (CRM+), maybe due to the presence in the plasma of functionally altered FVII molecules.[2] As can be seen in Table 1, none of these antigen-groups was related to the clinical distribution. For example, the clinically severely affected patient F7-30 had normal FVII antigen levels (65%) whereas an asymptomatic subject, F7-15, can have very low FVII antigen levels (<2%).

FVII mutations screening

DGGE analysis allowed us to characterise 46 out of 74 mutated alleles. Figure 1A,B shows typical electrophoretic patterns of different mutations within exons 5 and 8B respectively. All the PCR products displaying an abnormal DGGE pattern revealed a sequence alteration. The other exons were explored by direct sequencing and showed 22 additional gene defects. After this first screening-step, one or two mutations were still missing in five patients (F7-15, F7-17, F7-19, F7-31 and F7-37). Direct sequencing of the 5'FR did not allow the detection of another sequence alteration. We then re-examined the three exons previously explored by

Table 2 Oligonucleotides used to amplify FVII exons, PCR and denaturing gradient gel electrophoresis conditions

Exon	*5′ oligomer (5′-3′ sequence)*	*3′ oligomer (5′-3′ sequence)*	*Product size (bp)*	*AT (°C)*	*DGGE running time*
5FR	tatttacatccacacccaag	tgcctgttgacattccccat	400	56	
1a	ccatccctctgtcacccttg	atttgcccactgcccttcca	210	58	
1b	[a]agtaggggtgtggcgtgag	tgggaggggaaggaggtgat	289	64	5 h
2	aggatgggcgaacggggtgg	ccgcagccaaagagacgcag	444	60	
3 and 4	cgttgggtgctctggtgaag	ccctgaatgcccggcctaca	386	62	
5	[a]agaacaccactgctgaccca	tcccacccgtcttttgtcca	323	60	6 h 30 min[b]
6	cctgccttccaccacccctg	gctgacctgcccatttttcccct	369	66	
7	gagggcgagtcatcagagaa	tgagacacttgagagctgcg	421	62	
8A	gtgaggtggcaggtggtgga	cgttcgggcaggcagagggg[a]	222	64	4 h[b]
8B	[a]gcaccaccaaccacgacatc	ccttgctgccatccgagtag	303	62	10 h
8C	[a]gcagcagtcacggaaggtgg	tgccctcctctaccccatta	391	60	11 h

A typical 100 μl PCR reaction contained, 1 μg genomic DNA, 20 pmol of each oligonucleotide primer, 200 μM of each desoxynucleotide triphosphate (Pharmacia, Uppsala, Sweden), 1.5 units of Taq DNA polymerase and its buffer (Ampli TaqR, Perkin-Elmer Cetus, Norwalk, CT, USA). For the PCR reactions of both exons 1b and 7, the buffer was replaced by a mix containing 16.6 mM ammonium sulphate, 67 mM Tris-Hcl pH 8.8, 6.7 mM magnesium chloride, 67 μM Na_2EDTA, 170 μg bovine serum albumin per ml and 10 mM β-mercaptoethanol. [a]Indicates the primer-end with the additional GC clamp (cgcccgccgcgccccgcgcccgtcccgccgcccccgcccc). AT: annealing temperature. [b]Indicates that a 1 h-run prior to loading was applied.

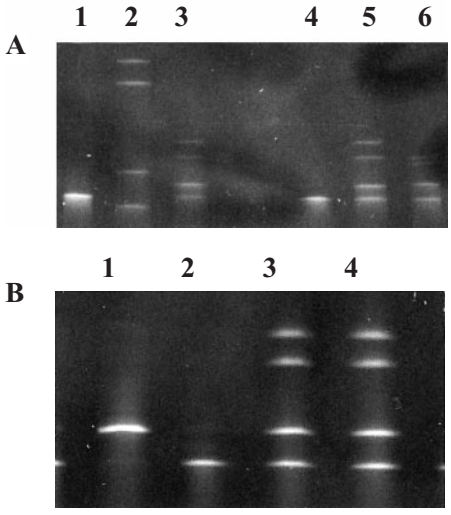


Figure 1 Denaturing gradient gel electrophoresis patterns of FVII mutations. (**A**) exon 5 DGGE pattern. Lane 1: Normal pattern (patient F7-34), lane 2: heterozygote for two mutations (Gln100→Arg and Gly97→Cys) and for the 115His dimorphism (patient F7-2), lane 3: heterozygote for the 115His dimorphism (patient F7-27), lane 4: normal pattern (patient F7-33), lane 5: heterozygote for the 115His dimorphism (patient F7-32), lane 6: heterozygote for the Cys102→Tyr mutation (patient F7-31). (**B**) exon 8B DGGE pattern. Lane 1: homozygote for the Arg304→Gln mutation (patient F7-22), lane 2: normal control, lanes 3 and 4: heterozygotes for the Arg304→Gln mutation (patients F7-24 and F7-34 respectively).

DGGE for these patients. However, direct sequencing of exons 1b, 5 and 8 failed to detect any sequence alteration.

FVII mutation spectrum of 37 unrelated patients with severe inherited FVII deficiency

Among the 68 identified mutated alleles, 40 different potential gene defects were detected. Twenty-two out of these 40 sequence alterations were mutations already described, while 18 had not been previously reported[1,4,6] (Table 1). More than a half of these sequence abnormalities (23/40) were located within both exons 2 and 8, whereas no mutation was found in exons 1b and 3. Only two mutations, affecting residues Cys and Arg at positions 135 and 152 respectively, were detected in exon 6 (Figure 2). The overwhelming majority of the identified lesions, except a short 15 base-pairs deletion, were single base substitutions.

Genotype distribution of FVII gene mutations (Table 1)

Twenty-five out of 37 patients were compound heterozygous for two FVII mutations, whereas 11 were found to be homozygotes. One patient still has an undetermined genotype. Among the 11 homozygous patients, six had a known family history of consanguinity (patients F7-1, -12, -14, -16, -23, -38). A seventh patient (F7-22) is also probably homozygous for the Arg304→Gln, a relatively frequent missense mutation. His parents could bear the same Arg304→Gln missense mutation at the heterozygous state by accident. As the parents' data were not available, the remaining four homozygous patients (F7-9, -25, -26, -40) can be either real homozygotes or compound heterozygotes for the identified mutation and a large deletion on the other allele. F7-10, F7-32 and F7-41 patients showed an unusual genotype including three different mutations (Table 1). The 11125 delC frameshift mutation has been shown to be frequently associated in *cis* with the Ala294→Val missense mutation in the Polish.[5] The DNA from these patient's families was not available to further characterise their genotype. However, we can assume that both 11125delC and Ala294→Val mutations probably lie on the same chromosome in these patients, whereas the third mutation represented the second mutated allele.

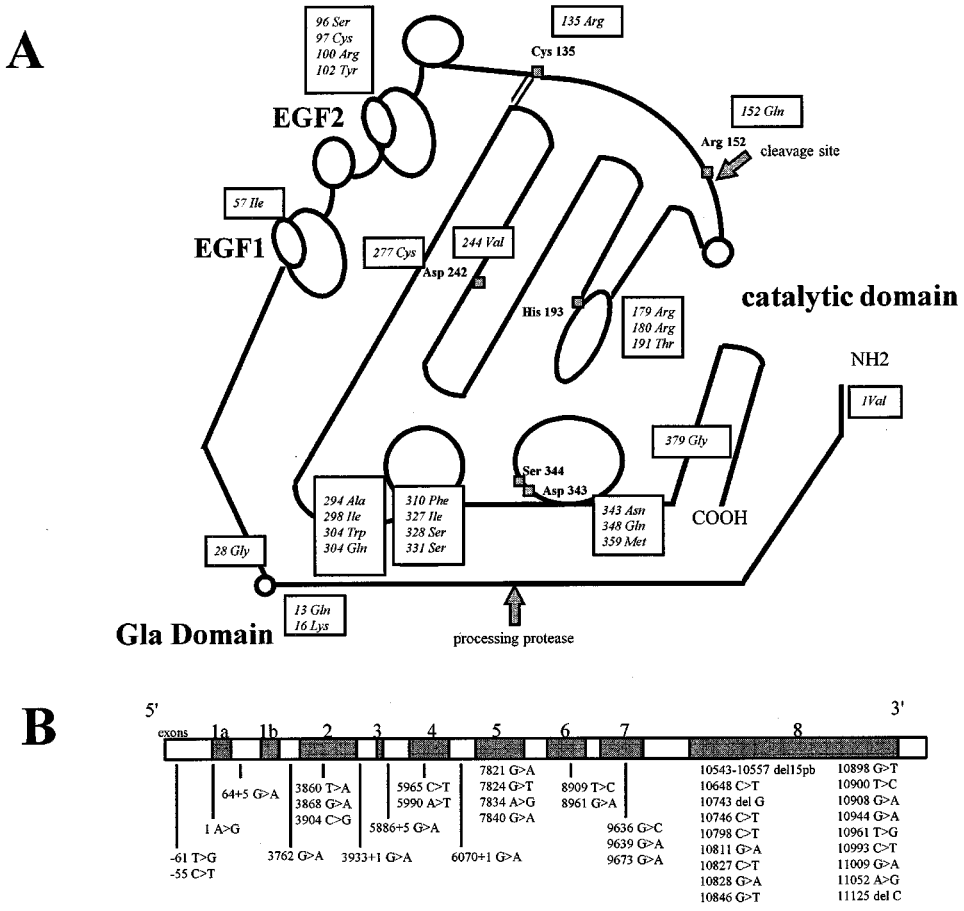


Figure 2 Mapping of the 40 FVII mutations that were identified in this study. (**A**) Mapping of the FVII missense mutations on the FVII molecule. The 28 FVII missense mutations are noted in italics. Critical residues of the FVII activation pathway and the typical residues of the catalytic triad are noted in bold type. (**B**) Location from the 5′ to the 3′ end of the FVII gene of the 40 different sequence alterations.

Polymorphisms and haplotypes associated with FVII mutations

The extensive analysis of the coding region and splice sites of FVII gene allowed us to detect eight sequence variations with apparently no disease causative effect. Six were already published common polymorphisms[10–13] whereas two were still undescribed. A Gln35 dimorphism was characterised in exon 2 at the heterozygous state in patient F7-33 and at the homozygous state in patient F7-40. Both patients F7-33 and F7-40 shared the same Cys310→Phe missense mutation. A C to T substitution at nucleotide 3954 in intron 2 was identified once at the homozygous state in patient F7-23 who is of Asian origins. This polymorphism has not been found in a 50 chromosomes control group. The most common polymorphisms were used to construct FVII haplotypes associated with each mutation when possible (Table 1).

Discussion

We have screened for mutations the entire coding regions and boundaries of FVII gene in 37 unrelated patients with inherited FVII deficiency. Among the 74 predicted FVII mutated alleles, 68 were characterised and corresponded to 32 complete FVII genotypes.

Strategy for the identification of FVII mutations

DGGE was chosen as the first screening step because of its reliability and of the large number of patients who can be simultaneously investigated. No mutation evaded detection in our study as far as we could check. DGGE could not be applied to the entire coding sequence because the base composition of FVII gene is GC rich with a 60% G+C content.[7] This characteristic is common to other vitamin K dependent factors such as factor X or Protein C.[7] This explains why gradients with high percentages of denaturant as well as long running times were necessary to accurately identify FVII mutations using DGGE. However, DGGE screening allowed the detection of 46 out of the 68 FVII mutated alleles (68%), corresponding to the complete genotypes of one third of the patients (13/37). DGGE is useful when studying large series of patients and should be first applied for the frequently mutated exons 8 and 5.

Furthermore, DGGE can be also performed as an individual screening tool, using a sequential loading on the same running gel.

FVII gene mutations spectrum in FVII deficiency

The overwhelming majority of the FVII mutations are single nucleotide variations according to the literature data. Short deletions (2–30 nucleotides) are rare on FVII gene. One was identified in this study, and another has been previously described.[1,14] Both are closely located, at the beginning of exon 8 which is a known region for nucleotide sequence repetitions. The single point mutations are distributed throughout the FVII gene suggesting that almost every domain of the molecule is essential for maintaining its overall structure and specific function. Only six gene defects could not be detected, despite an extensive screening. These missing mutations might occur within unexplored intronic regions and activate a competitive cryptic splice site. Further studies on illegitimate RNA transcripts would be useful to characterise the genetic mechanisms underlying the FVII deficiency, for these patients. Another possibility might be the existence of a large deletion or a gross gene rearrangement which would evade detection by DGGE or direct sequencing. Finally, the modification of an alternative locus which may control FVII levels has been suggested.[15]

Phenotype to genotype relationship in clinically severe FVII deficiency

Both FVII clotting activity and FVII antigen are known to be poorly correlated to the bleeding tendency in inherited FVII deficiency.[16] On the other hand, in many reports, severe mutations preventing synthesis of any amount of FVII protein have been shown to be associated with life-threatening haemorrhages.[16–18] This is in agreement with FVII gene knock out experiments in mice. These mice frequently die during the first day after birth because of fatal haemorrhages.[19] In our study, most of the genotypes of patients with severe bleeding diathesis, appeared to comprise on both alleles mutations preventing the production of a functional FVII molecule. Actually, in the clinically severe group C, 15 out of the 16 mutated allele can be considered as deleterious mutations on the basis of different mechanisms.

Alteration of the FVII activation pathway Three previously described missense mutations and one novel sequence-alteration occurred at residues implicated in the activation pathway of FVII. The Arg152→Gln occurs at the site that must be cleaved to generate activated FVII.[20] The rate of the 328Ser mutant activation by activated factor X was shown to be markedly reduced.[21] The 135Arg mutant[4] destroys the unique disulphide bridge, connecting the light and heavy chains of activated FVII. The novel Asp343→Asn mutation, involves a critical and highly conserved residue. After proteolytic activation of the factor VII zymogen, the β-carboxyl group of asparatic acid residue 343, adjacent to the active serine residue 344, forms the trypsin typical salt bridge with the newly exposed NH2-terminal alpha amino group of Ile 153. This salt bridge stabilises the active conformational state of the serine protease domain.[22] Thus, the residue Asp343 appears to be essential for the catalytic activity of the complex FVIIa/TF.

Secretion defect Both previously described 359Met and 348Gln mutants are sequestered in the protein secretory pathway.[23–24] In the same way, the FVII Arg100 mutant displays an abnormal conformation leading to a major, secretion defect.[25] The novel Asn57→Ile mutation lies within the N-terminal EGF like domain at an evolutionary conserved residue. A Asn57→Asp mutant has been reported by Leonard *et al*.[26] Because of the loss of an important intra-molecular hydrogen bond between Asn57 and Cys81, these mutations could alter the folding of the first EGF domain. In the Asn57→Asp model, this substitution resulted in a marked decrease in FVII mutant protein secretion with the loss of both procoagulant activity and TF binding.[26]

Generation of a truncated or elongated protein The frameshift 11125delC mutation has been reported at the homozygous state, in *cis* with the Ala294→Val substitution, in several subjects with moderately severe to severe bleeding diathesis.[5] The 6070+1 G→A transition occurs at the invariable dinucleotide splice site of intron 4.[17] Concurrently the newly described substitution of a C by a T at position 5965 generates a premature termination codon TAG at position 49 in the first EGF domain. The truncation of the EGF2 and catalytic domains, even if some amount of the mutant protein is still translated, would lead to an inactive polypeptide. The 5886+5 G→A transition occurred at the nucleotide position +5 at the 5′ splice site of intron 3. The frequency of a G at position +5 of the consensus splice site scored 0.84 whereas an A at the same position scored 0.05.[27] As an A is extremely rare at this position, the G to A transition may lead to the skipping of the corresponding exon or the activation of a cryptic splice site in the vicinity. A similar mutation in the FVII intron 7 (9726+5 G→A) has been found to disrupt the usual 5′ donor splice site.[28]

Expression defect Two mutations have been shown to alter FVII expression *in vitro*. Both −61 T→G and −55 C→T mutations occurred at the binding site of the transcription factor: hepatic nuclear factor 4 (HNF4).[29] The −61 T→G mutation completely abrogated gene expression in reporter gene assays,[18] whereas the −55 C→T mutation exhibited only 10% of expression level *in vitro*.[30]

Finally, the sixteenth Glu16→Lys mutated allele associated with a clinically severe phenotype, has an hypothetical severity. This mutation has a FIX counterpart (Glu17→Lys) causing severe haemophilia B,[31] but the phenotypic con-

sequences of the FVII 16Lys variant have to be further explored.

Thus in the severe group, both mutated alleles of eight out of nine patients could be related to severe FVII mutations, suggesting that the severe phenotypes could be related to the total absence of FVIIa. Conversely, this is not the rule in both mild or asymptomatic groups. In these groups, one severe mutation can be found occasionally but not on both alleles. In these cases, the other allele, that we could call 'mild', may allow the release of a minute amount of FVII protein that could be sufficient to trigger the haemostatic cascade. Conventional clotting assays may not be sensitive enough to detect the residual FVII activity. Whether a small release of FVII could be sufficient to prevent the expression of severe phenotypes needs further analyses. FVII variants from both A and B groups are currently under investigation to throw light on the structure-function relationships of FVII and on new prognostic parameters for bleeding tendency in severe inherited FVII deficiency.

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Population Genetic and Phylogenetic Evidence for Positive Selection on Regulatory Mutations at the *Factor VII* Locus in Humans

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ABSTRACT

The abundance of *cis*-regulatory polymorphisms in humans suggests that many may have been important in human evolution, but evidence for their role is relatively rare. Four common polymorphisms in the 5′ promoter region of *factor VII* (*F7*), a coagulation factor, have been shown to affect its transcription and protein abundance both *in vitro* and *in vivo*. Three of these polymorphisms have low-frequency alleles that decrease expression of *F7* and may provide protection against myocardial infarction (heart attacks). The fourth polymorphism has a minor allele that increases the level of transcription. To look for evidence of natural selection on the *cis*-regulatory variants flanking *F7*, we genotyped three of the polymorphisms in six Old World populations for which we also have data from a group of putatively neutral SNPs. Our population genetic analysis shows evidence for selection within humans; surprisingly, the strongest evidence is due to a large increase in frequency of the high-expression variant in Singaporean Chinese. Further characterization of a Japanese population shows that at least part of the increase in frequency of the high-expression allele is found in other East Asian populations. In addition, to examine interspecific patterns of selection we sequenced the homologous 5′ noncoding region in chimpanzees, bonobos, a gorilla, an orangutan, and a baboon. Analysis of these data reveals an excess of fixed differences within transcription factor binding sites along the human lineage. Our results thus further support the hypothesis that regulatory mutations have been important in human evolution.

CHANGE in transcriptional regulation is a crucial contributor to phenotypic evolution (Raff and Kaufman 1983; Gerhart and Kirschner 1997; Carroll *et al.* 2001; Davidson 2001; Wray *et al.* 2003). Genetic changes in *cis*-regulatory sequences controlling transcription can be as important to gene function as any change in coding sequences; indeed, it has been hypothesized that the changes distinguishing humans from our closest relatives reside in *cis*-regulatory regions (King and Wilson 1975; Wilson 1975). The ubiquity of *cis*-regulatory polymorphisms that have significant phenotypic effects in humans (Rockman and Wray 2002) suggests that much current phenotypic variation, as well as past evolutionary change, may be due to regulatory mutations. Whether the evolution of regulatory mutations in humans has been the result of genetic drift or adaptive natural selection, however, is known in only a few cases (Hamblin and Di Rienzo 2000; Bamshad *et al.* 2002; Sabeti *et al.* 2002; Rockman *et al.* 2003). Here we examine the patterns of variation and change over time in the well-characterized *cis*-regulatory region at the *factor VII* (*F7*) locus to tease apart the action of multiple evolutionary forces.

Factor VII is a vitamin K-dependent protease essential for coagulation. When blood vessel injury occurs, tissue factor is released and binds to circulating F7, allowing proteolysis of F7 to its active form and the initiation of blood coagulation through the production of thrombin and fibrin clots. The cause of most myocardial infarctions is thrombosis, the presence of a blood clot within a blood vessel (Fuster *et al.* 1992). Because of this, F7 was long suspected of playing a role in death due to coronary heart disease and myocardial infarction; multiple studies have now shown that high levels of plasma F7 are a predictor of death due to heart disease (*e.g.*, Meade *et al.* 1986; Heinrich *et al.* 1994; Redondo *et al.* 1999; Girelli *et al.* 2000). Genetic studies of the *F7* locus have found five common polymorphisms that explain at least 40% of the variance in levels of both active and inactive plasma F7 among healthy adults (Marchetti *et al.* 1993; Bernardi *et al.* 1996; Humphries *et al.* 1996; Pollak *et al.* 1996; van 't Hooft *et al.* 1999). Of these, four are in the 5′ *cis*-regulatory region and one is an amino acid variant. The four regulatory variants are associated with transcript and protein abundance of

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F7 in vivo, but because of linkage disequilibrium and epistasis the magnitude of the effect of each is not clear (Bernardi *et al.* 1996; Pollak *et al.* 1996; van 't Hooft *et al.* 1999; Di Castelnuovo *et al.* 2000; Girelli *et al.* 2000; Kudaravalli *et al.* 2002).

Three of the regulatory polymorphisms have minor alleles (defined by researchers on the basis of the relative frequencies in Europe) that individually decrease expression of *F7*: a single nucleotide polymorphism (SNP) at −401 (G/T) relative to the start of translation (based on the nomenclature of Pollak *et al.* 1996), an SNP at −122 (T/C), and a 10-bp insertion/deletion polymorphism at −324 (Bernardi *et al.* 1996; Pollak *et al.* 1996; van 't Hooft *et al.* 1999; Di Castelnuovo *et al.* 2000; Girelli *et al.* 2000). The indel polymorphism (dbSNP identifier rs5742910) has generally been ascribed to position −323. However, examination of the 5′ sequences generated by the Human Genome Project and by Pollak and colleagues (GenBank accession nos. NT_027140 and U40852, respectively) unambiguously puts the insertion allele between nucleotides −324 and −325. We therefore refer to this polymorphism as −324. The less-frequent allele of the common amino acid polymorphism at position 353 (R/Q) of the protein has also been shown to correlate with lower levels of plasma F7, but it is in strong linkage disequilibrium with the polymorphisms at −401, −324, and −122 (Bernardi *et al.* 1996; Humphries *et al.* 1996; Girelli *et al.* 2000). Lower levels of F7 may provide protection against myocardial infarction, with the frequencies of the low-expression alleles being found disproportionately in healthy individuals (Green *et al.* 1991; Iacoviello *et al.* 1998; Di Castelnuovo *et al.* 2000; Girelli *et al.* 2000). The fourth regulatory polymorphism, an SNP at −402 (G/A; dbSNP identifier rs762637), has a minor allele that leads to an increase in the level of transcription of *F7* (van 't Hooft *et al.* 1999). The alleles at sites −401 and −402 affect transcription through changes in transcription factor binding (van 't Hooft *et al.* 1999).

The association between allele frequencies and F7 activity has previously been examined in populations from Britain, Japan, India, Norway, Holland, France, Italy, and Greenland (Lane *et al.* 1992; Kario *et al.* 1995; Bernardi *et al.* 1997; de Maat *et al.* 1997). Populations differ significantly in the frequencies of the regulatory variants, even within Europe. Strong linkage disequilibrium among the regulatory indel (−324), a regulatory SNP (−401), and the amino acid variant was consistently observed. While extensive and careful study of these variants has shown that the regulatory polymorphisms all have effects on transcription, either independently or epistatically (*e.g.*, Kudaravalli *et al.* 2002), it is unknown whether natural selection acts on the polymorphisms independently.

To address the evolutionary history of functional variants affecting *F7* transcription we take both a phylogenetic and a population genetic approach. We first use sequences from the 5′ *cis*-regulatory region of *F7* from five chimpanzees, two bonobos, a gorilla, an orangutan, and a baboon to investigate the origin and interspecific patterns of selection on functional variants. We then present a population genetic survey of three regulatory variants with differing effects on F7 expression (−402, −401, and −324) from six locales: Italy, Cameroon, Ethiopia, China, India, and Papua New Guinea. Allele frequencies and population differentiation at each site were estimated, and comparison to 18 unlinked, putatively neutral SNPs in the same individuals allowed us to test for the action of natural selection. Our results demonstrate the action of adaptive natural selection in shaping the pattern of change over time in *F7* and in shaping patterns of variation in at least one of the regulatory polymorphisms. Thus, our results further support the hypothesis that regulatory mutations are important for human evolution.

MATERIALS AND METHODS

Subjects: DNA was obtained from 45 unrelated individuals in each of six focal populations: southern Italy, Cameroon, Ethiopia, China (Singaporean Chinese), India (Uttar Pradesh), and Papua New Guinea (Madang Coastal). In addition, we obtained DNA from 37 individuals from a population of Japanese. Human DNA samples were collected by the Goldstein lab with signed consent or were anonymous legacy collections provided to D. Goldstein by collaborators from other academic research universities. DNA from five chimpanzees (four *Pan troglodytes verus* and one *Pan troglodytes schweinfurthii*), two bonobos (*Pan paniscus*), and one baboon (*Papio papio*) was provided by A. Stone and D. Loisel. Gorilla (*Gorilla gorilla*) and orangutan (*Pongo pygmaeus*) DNA was purchased from Coriell Institute for Medical Research Cell Repositories.

Genotyping: A PCR-amplified 433-bp fragment of the 5′ regulatory region of *F7* was cloned and sequenced from all of the nonhuman primates using the primers 5′-ggctctgt ggctcacctaag-3′ and 5′-gactgacgggcaaagttctc-3′ (GenBank accession nos. AY493422–AY493433). Fragments were cloned into pGEM-T vectors (Promega, Madison, WI) and multiple clones were sequenced in each direction. All base-calls were confirmed in multiple identical clones, and the chimpanzee and bonobo products were also directly sequenced as a check on *Taq* error. For genotyping the six focal human populations we PCR amplified the same fragment using a 6-FAM labeled reverse primer. The −324 indel variant was genotyped by scoring the size fragment after running it out on an ABI 3700 capillary gel machine using the ABI Genescan/Genotyper software package. The −401 and −402 SNPs were genotyped by sequencing using only the forward primer. (The −401 polymorphism was submitted to dbSNP and given the identifier ss15737439.) The products were run out on the ABI 3700 and scored two independent times by the authors using Sequencher (Gene Codes, Ann Arbor, MI). For the Japanese population, all PCR products were sequenced in both directions with the forward and reverse primers.

Statistical analysis: All sequences were aligned in Clustal X (Thompson *et al.* 1994). Assignment of substitutions to branches of the primate phylogeny was done using HyPhy (S. Kosakovsky Pond and S. Muse; http://www.hyphy.org). Hardy-Weinberg equilibrium significance was tested by the random-permutation method implemented by the program GDA (P. Lewis and D. Zaykin; http://lewis.eeb.uconn.edu/lewishome/

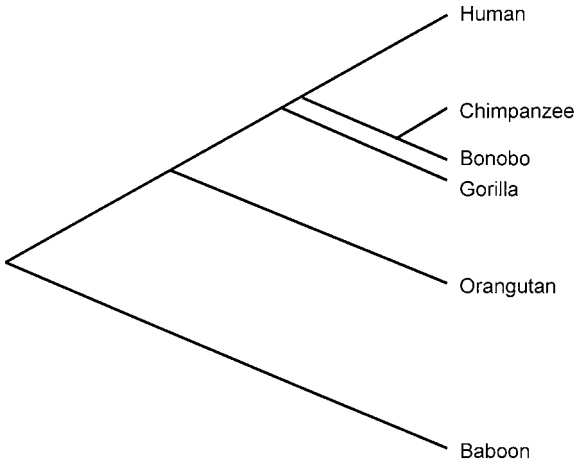


FIGURE 1.—Phylogeny of the great apes and baboon. Branch lengths are proportional to time (YI *et al.* 2002).

software.html). Linkage disequilibrium was assessed by Δ, a measure of composite genotypic disequilibrium that does not require a known phase of alleles (WEIR 1996), implemented in a perl program submitted to the Bioperl project (STAJICH *et al.* 2002). We corrected for multiple comparisons using the Dunn-Sidak method (SOKAL and ROHLF 1995, p. 239); this method is less conservative than a Bonferroni correction (SOKAL and ROHLF 1995). Wright's F_{ST} (WRIGHT 1951) was estimated using the diploid method of WEIR (1996, p. 178), also available through the Bioperl project (http://www.bioperl.org). All F_{ST} values <0 were set equal to 0 (negative values can occur when the true value is positive but very small). Significance of the difference between F_{ST} at *F7* −324, −401, and −402 and F_{ST} in 18 unlinked, neutral SNPs was estimated by comparison to a bootstrap-resampled distribution as follows: the difference between the single-locus F_{ST} of a randomly sampled neutral marker and the multilocus F_{ST} of 18 randomly resampled neutral markers was calculated 10,000 times. This resampling was performed for global and all pairwise F_{ST}'s. The observed difference between the functional variant F_{ST} value and the 18-locus estimate for the neutral loci was then compared to these distributions in a two-tailed test.

RESULTS

Phylogenetic patterns of F7 evolution: Analysis of a 433-bp alignment of the 5′ regulatory region (starting at −68 relative to the start of translation) in six primates revealed four fixed differences along the branch leading to humans (Figures 1 and 2). The level of divergence in the 5′ region along the human branch, as well as the substitution rate among all of these primates, is well within the average rate measured among 45 large, noncoding regions for this clade (YI *et al.* 2002). Three of the fixed differences in humans (positions −401, −220, and −108) are located in experimentally verified transcription factor binding sites (−220 and −108; POLLAK *et al.* 1996) or have been shown to have effects on transcription both *in vivo* and *in vitro* (−401; VAN 'T HOOFT *et al.* 1999). The fourth fixed difference, at −196, is not located within any known transcription factor binding site.

The binding site spanning −233 to −215 is an estrogen response element that binds estrogen receptor (DI BITONDO *et al.* 2002); mutation at −220 from the invariant A to a C has been shown to severely diminish F7 expression *in vitro* (DI BITONDO *et al.* 2002). While the change along the human branch at site −220 is from G to the consensus A (Figure 2), this site lies in the middle of the required sequence for binding of estrogen receptor and therefore represents a possible gain-of-function mutation in humans. The binding site spanning −108 to −84 is a transcription factor Sp1 binding site (POLLAK *et al.* 1996). A rare, naturally occurring mutation at −94 in this site disrupts Sp1 binding and leads to severe F7 deficiency (CAREW *et al.* 1998). We do not know the effect of the observed change at position −108 in this binding site. The two alleles observed at position −401 are both derived, and therefore we have counted this as a fixed difference. The ancestral C nucleotide has been replaced by a G/T polymorphism; this does not appear to be a CpG-related mutation. Polymorphisms for which both alleles are different from the presumed ancestral variant for hominids are rare because of both a short divergence time since the split from chimpanzees and bonobos and low levels of polymorphism within our species (KAESSMANN *et al.* 1999). The two alleles have different effects on transcription (see DISCUSSION).

The ancestral states of the three other polymorphic regulatory variants in *F7* present some equally complex relationships between derived states and effect on expression. It is clear from the alignment of noncoding DNA that a C at nucleotide position −122 is the derived state in humans (Figure 2). This derived mutation is the allele associated with lower expression of F7. At position −402, the derived A allele causes increased expression in F7. And at position −324, an invariant 10-bp sequence in nonhuman primates is now the low-frequency insertion allele of the insertion/deletion polymorphism. The derived deletion allele is responsible for higher levels of F7 expression. Derived variants in humans are thus found to be both increasing and decreasing levels of F7 expression relative to the ancestral alleles.

Although there are only four fixed differences in humans, there appears to be an excess of fixations within transcription factor binding sites along the human lineage. To determine whether three of the four fixed differences falling within binding sites is more than that expected by chance, we counted the total number of nucleotides in this region that comprise transcription factor binding sites; our conservative estimate was 74 (experimentally defined: −108 to −84, −233 to −215, 10-bp indel; estimates: 10 bp for −402/−401 binding site, 10 bp for −122 binding site). Note that most binding sites are 6–10 bp long (FAIRALL and SCHWABE 2001). A ratio of the substitutions per site in replacement sites (K_a) to synonymous sites (K_s) in coding regions has been

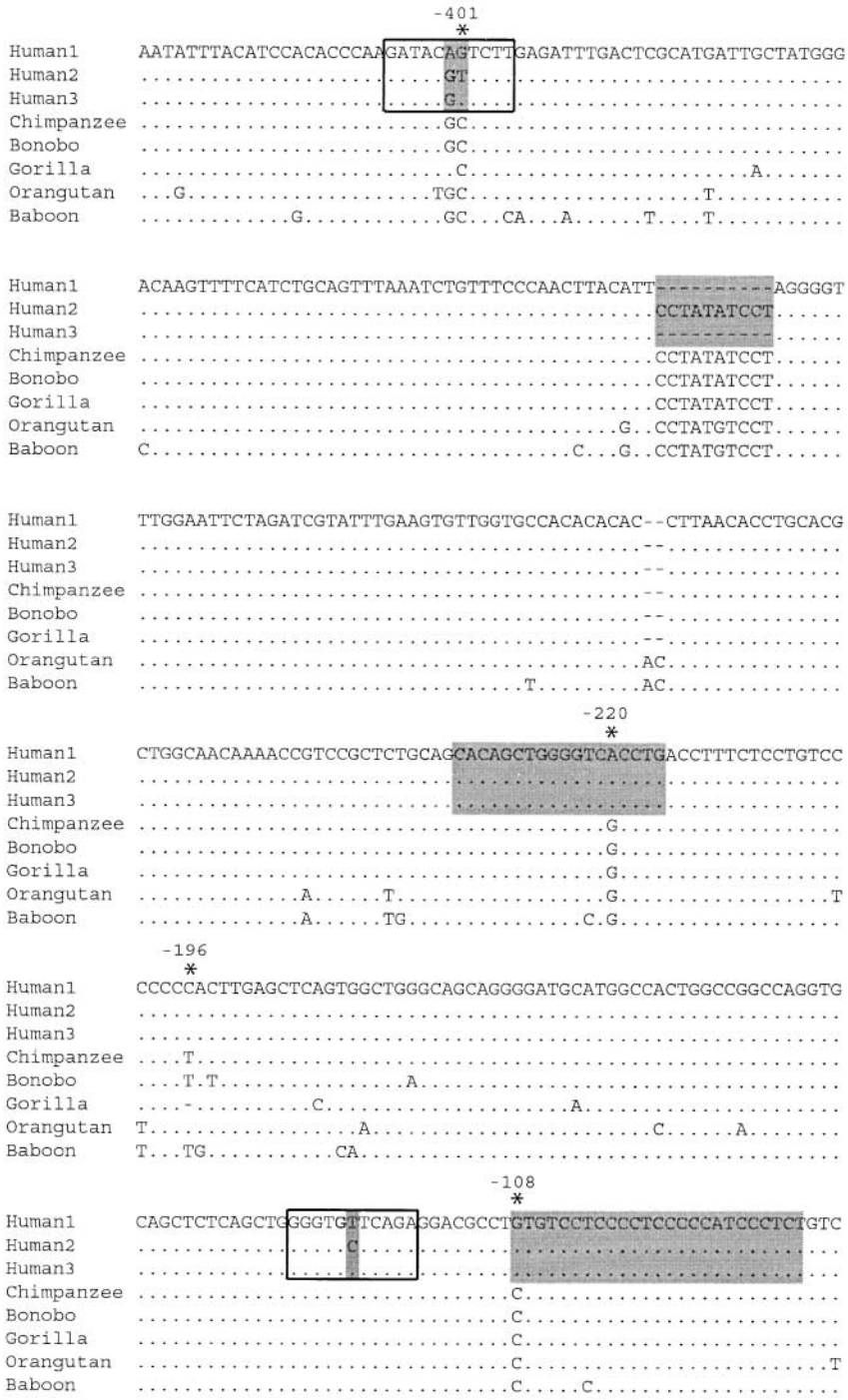


FIGURE 2.—Variation in the *F7* promoter region. Sequence alignment of part of the 5′ noncoding region of *F7* from −428 at the top left (based on numbering in humans), to −81 at the bottom right. The common human haplotypes (VAN 'T HOOFT *et al.* 1999) are listed separately. Experimentally verified transcription factor binding sites or nucleotides with effects on transcription are shaded, and presumed binding site nucleotides are boxed. Fixed differences along the human branch are noted with an asterisk and their nucleotide position.

used to quantify patterns of natural selection in proteins (KIMURA 1977). A K_a/K_s ratio <1 is consistent with a history of negative (purifying) selection, although it does not rule out positive selection, while a K_a/K_s ratio >1 indicates strong positive selection, although it does not mean that negative selection is not also acting (YANG and BIELAWSKI 2000). By analogy, we can measure the ratio of the substitutions per site in binding sites (K_b) to intervening, nonbinding nucleotide sites (K_i) in regulatory regions, with the same interpretation of results. Along the human branch of the primate tree, we find $K_b/K_i = 14.6$ [(3/74)/(1/359)]. Using a χ^2 statistic testing for an excess of fixed differences in binding sites relative to nonbinding sites gives a value of 9.2 ($P = 0.002$). This result suggests that positive selection may have been involved in the fixation of regulatory mutations in humans.

Population variation in human F7 regulatory mutations: Three of the functional regulatory polymorphisms at the *F7* locus were genotyped in humans: two SNPs (−402 and −401) and one indel (−324). The frequencies of the two alleles at each site were deter-

TABLE 1

Allele frequencies

Site (allele)	Cameroon	China	Ethiopia	India	Italy	Papua New Guinea
−324 (del)	0.65 (80)[a]	0.97 (88)	0.75 (88)	0.79 (86)	0.76 (88)	0.99 (88)
−401 (G)	0.56 (78)	0.90 (62)	0.74 (88)	0.70 (56)	0.75 (88)	0.99 (88)
−402 (A)	0.05 (78)	0.52 (62)	0.10 (88)	0.23 (56)	0.14 (88)	0.54 (88)

[a] The value in parentheses is the number of chromosomes sampled.

mined from six Old World human populations: southern Italy, Cameroon, Ethiopia, China (Singaporean Chinese), India (Uttar Pradesh), and Papua New Guinea (Madang Coastal). The frequencies of each allele in each of the populations are shown in Table 1. All populations were missing data for some individuals at all of the variants. The missing data ranged from 1 individual to 17 individuals of the 45 sampled in each population (Table 1). Tests for Hardy-Weinberg equilibrium in each population for each variant were nonsignificant in every case but the Ethiopian −402 data (Fisher's exact test, $P < 0.05$). The chromatograms from this population were rechecked for base-calling errors, but the significant disequilibrium remained. This result does not appear to affect any of our conclusions (see below). We found high levels of linkage disequilibrium between variants at −401 and −324 in every population ($P < 0.05$ in all comparisons; Table 3). This indicates that the low-expression, low-frequency variants were in positive linkage disequilibrium with each other. This pattern has been shown previously for *F7* in multiple human populations (*e.g.*, Bernardi *et al.* 1997; de Maat *et al.* 1997).

To measure the amount of population structure present in our data we calculated global and pairwise values for F_{ST} (Wright 1951) among all six populations. (F_{ST} can take on values between 0, no differentiation between populations, and 1, complete differentiation.) In the absence of selection, these values reflect divergence in allele frequencies due to genetic drift. To assess the possible role of natural selection in shaping the population differentiation in the functional SNPs, we compared F_{ST} at these variants to F_{ST} calculated for 18 SNPs genotyped in the same individuals and unlikely to be affected by selection (Rockman *et al.* 2003). These 18 SNPs were chosen to be >200 kb from any known gene and 50 kb from any mapped expressed sequence tag, unlinked (on different chromosomes), or at least 50 cM apart if on the same chromosome (on the basis of the UCSC April 2002 freeze, NCBI Build29). Alleles at each of the 18 unlinked loci are expected to have the same F_{ST}, but the stochastic nature of genetic drift creates a large variance in the distribution of values. We used the empirical distribution of the putatively neutral SNPs to identify variants with unusually high or low F_{ST}'s (*cf.* Bamshad *et al.* 2002; Fullerton *et al.* 2002; Hamblin *et al.* 2002). Significantly high F_{ST} values indicate positive selection driving changes in allele frequencies in individual populations, while significantly depressed values can indicate balancing selection maintaining allele frequencies between populations (Lewontin and Krakauer 1973). Statistical power to detect significantly depressed values in humans is low, as the average differentiation among continental populations is only ~0.12 (Akey *et al.* 2002).

Global F_{ST} values at sites −402, −401, and −324 were 0.228, 0.121, and 0.106, respectively; the mean of the neutral SNPs is 0.127. The value at position −402 is higher than that observed for 17 of the 18 neutral loci, but is not significantly different from the neutral distribution on the basis of bootstrap resampling of the difference between random single-locus and 18-locus F_{ST} values (see materials and methods). The global estimate of F_{ST} masks any population-specific processes by averaging over all populations. Therefore, we also calculated all the pairwise values for F_{ST} between populations for all variants (Table 2). Two consistent, significant patterns are seen. The first is that all of the functional variants show a significantly higher-than-expected F_{ST} in the China-India comparison. The neutral markers show extremely low levels of differentiation in this comparison, and thus even moderate F_{ST} values are far outside the neutral distribution. Previous studies of putatively neutral markers have also shown low levels of F_{ST} between East Asian and Indian populations (*e.g.*, Watkins *et al.* 2003). A second, striking pattern at position −402 is the significant pairwise comparisons between the Singaporean Chinese and Ethiopians, Indians, and Italians (Table 2). These significant values indicate that the derived A allele at −402 shows a greater-than-expected difference in frequency as compared to the neutral SNPs. Indeed, the F_{ST} values in these comparisons are higher than those measured for any of the neutral loci. Even though the Papua New Guineans show generally more differentiation from all human populations including the Chinese (Table 2), this comparison is not significant. Nevertheless, the large increase in frequency of the high-expression A allele at position −402 in Singaporean Chinese suggests the action of positive natural selection.

To ask whether the increase in frequency of the −402A allele in Singaporean Chinese represents a pop-

TABLE 2

Pairwise population differentiation (F_{ST})

	Population					
	Cameroon	China	Ethiopia	India	Italy	Papua New Guinea
Average of 18 neutral SNPs						
Cameroon		0.133	0.052	0.101	0.120	0.327
China			0.053	0.026	0.082	0.184
Ethiopia				0.018	0.041	0.251
India					0.033	0.222
Italy						0.302
Papua New Guinea						
−324						
Cameroon		0.276	0.012	0.037	0.019	0.327
China			0.165	0.125***	0.154	0.001
Ethiopia				0.000	0.000	0.213
India					0.000	0.173
Italy						0.203
Papua New Guinea						
−401						
Cameroon		0.234	0.053	0.021	0.063	0.418
China			0.069	0.112***	0.061	0.063
Ethiopia				0.000	0.000	0.225
India					0.000	0.315
Italy						0.215
Papua New Guinea						
−402						
Cameroon		0.430*	0.000	0.123	0.025	0.433
China			0.341***	0.142***	0.284***	0.000
Ethiopia				0.047	0.000	0.356
India					0.016	0.168
Italy						0.304
Papua New Guinea						

*$P < 0.1$; ***$P < 0.0001$.

ulation- or region-specific phenomenon, we also genotyped the *F7 cis*-regulatory region in a population of Japanese. Although we do not have data on the frequencies of the 18 neutral SNPs for this population, the frequency of the −402 alleles as well as alleles at −401, −324, and −122 provides us with data from another East Asian population. In a group of 37 individuals (74 chromosomes) sequenced, the −402A allele was present at a frequency of 36%. This value is midway between the frequency in the Singaporean Chinese (52%; Table 1) and the values in Cameroon, Ethiopia, India, and Italy (5–23%; Table 1). The frequencies of −401G (93%) and the −324 deletion (93%) in the Japanese are very similar to the values in the Singaporean Chinese (90 and 97%, respectively; Table 1). We were also able to score the C/T polymorphism at site −122 and found that the C was present at a frequency of 92%. The polymorphisms at −401, −324, and −122 were in almost perfect linkage disequilibrium among the Japanese: only one recombinant chromosome was detected. In contrast, nucleotide site −402 was not in linkage disequilibrium with any of these sites.

DISCUSSION

The estimated number of heterozygous functional *cis*-regulatory sites in humans is larger than the number of heterozygous amino acid sites (Rockman and Wray 2002). Regulatory polymorphisms are present at appreciable frequencies in all human populations, with an estimated 40% of all loci heterozygous for a functional regulatory mutation (Rockman and Wray 2002). With so much heritable variation available for selection to act upon, it behooves us to discover how natural selection has shaped and used *cis*-regulatory DNA in human evolution. The goal of our study was to uncover and characterize the patterns of selection, if any, in the *F7 cis*-regulatory region. Both our population genetic and phylogenetic sequence data showed patterns that dif-

TABLE 3

Linkage disequilibrium

Comparison	Cameroon	China	Ethiopia	India	Italy	Papua New Guinea
−324, −402	−0.01 (0.4)[a]	0.02 (1.0)	0.02 (0.4)	0.10 (8.1)	0.04 (3.4)	0.00 (0)
−324, −401	0.20 (25.4)*	0.04 (11.3)*	0.19 (40.5)*	0.19 (27.0)*	0.17 (40.2)*	0.01 (43.0)*
−401, −402	0.01 (0.1)	0.07 (4.7)	0.02 (0.6)	0.09 (7.0)	0.05 (3.7)	0.00 (0)

*$P < 0.05$ after Dunn-Sidak correction for multiple comparisons.
[a] First value is the composite genotypic disequilibria, Δ; χ^2 values are in parentheses.

fered from neutral expectations, with directional selection the most likely cause of these departures.

F7 is an important component of the blood-clotting pathway: levels of F7 protein in the bloodstream are strongly correlated with the risk of myocardial infarction. Expression is controlled by a well-characterized, TATA-less, *cis*-regulatory region 5′ of the start of transcription (Pollak *et al.* 1996). Four common regulatory polymorphisms have been shown to affect transcription and protein levels *in vivo*. Three of these polymorphisms have minor-frequency alleles that lower the level of F7 and, hence, lower the risk of heart attack, and one has a minor allele that raises the level of F7 (Bernardi *et al.* 1996; Pollak *et al.* 1996; van 't Hooft *et al.* 1999; Di Castelnuovo *et al.* 2000; Girelli *et al.* 2000). We studied the evolutionary history of these functional polymorphisms in humans and nonhuman primates.

Analysis of the 5′ *cis*-regulatory region among primates reveals the evolution of both high-expression and low-expression alleles in humans. At polymorphic position −122, the low-expression C allele is clearly derived in humans (Figure 2). At polymorphic positions −324 and −402, the high-expression alleles are derived. Position −401 segregates two derived alleles, one low expression and one high expression (the expression level of the ancestral allele is unknown). The sequencing of 10 chimpanzee and 4 bonobo chromosomes confirms our inferences of ancestral states of the four polymorphisms by showing that they are not segregating in these species. It is interesting to note that the low-expression derived allele at position −122 (C) is in positive linkage disequilibrium with the low-expression alleles at position −401 (T) and −324 (insertion; Table 3). Position −402 is not in linkage disequilibrium with any of these sites in our study, but was previously found to be in weak negative linkage disequilibrium with the low-expression alleles (van 't Hooft *et al.* 1999). The positive linkage disequilibrium between the low-expression variants found here and in previous studies (Bernardi *et al.* 1996; Humphries *et al.* 1996; Girelli *et al.* 2000) and the possible negative linkage disequilibrium between the high-expression allele at −402 and the low-expression alleles are important factors in studies that seek to diagnose the risk of heart attacks in patients with heart disease. The patterns of linkage between protective alleles in the seven human populations studied here indicate that only one or a few sites need to be genotyped to assess the complete haplotypic genotype of heart disease patients. This relatively low level of haplotype diversity is a promising development for large clinical studies of disease risk in multiple ethnic groups (Gabriel *et al.* 2002).

Combining information from previous biochemical studies of the *F7* polymorphisms with data on the ancestral states of these polymorphisms allows us to examine the evolution of transcription factor–DNA interactions. The derived, high-expression −402A allele has been shown to have a decreased binding affinity for an unknown nuclear protein (van 't Hooft *et al.* 1999). The low-frequency, low-expression −401T allele in the neighboring nucleotide position has been shown to have an *increased* binding affinity for the same unknown protein. This set of relationships between binding affinities and expression levels suggests that the unknown nuclear transcription factor acts as a repressor in the *F7* promoter: high levels of expression are associated with low binding affinities and low levels of expression are associated with high binding affinities. Therefore, we are able to infer the loss or severe lessening of a transcription factor-DNA interaction due to the derived −402A allele and an increased transcription factor-DNA interaction due to the −401T allele. Also, −402A shows a decreased binding affinity for a second, unknown nuclear protein, while −402T shows a decreased binding affinity for this protein relative to the more common G allele (van 't Hooft *et al.* 1999). These two SNPs therefore both change the binding affinities of multiple transcription factors that share the same or overlapping binding sites. Pleiotropic and epistatic interactions like these may be relatively common among regulatory mutations (Horan *et al.* 2003; Wray *et al.* 2003). No biochemical evidence is available for the transcription factor-DNA interactions at the other polymorphisms in this region.

There are four fixed differences along the human lineage in the 433 bp of 5′ noncoding sequence that we have collected. By analogy with the ratio of K_a to K_s, the ratio of nonsynonymous to synonymous substitutions per site, we can measure the ratio of the substitutions per site in binding sites (K_b) to intervening, nonbinding nucleotide sites (K_i) to show an excess of binding site substitutions along the human lineage (see

results). We believe that this is the first example of a case of positive selection in a *cis*-regulatory region detected by an analysis of K_b/K_i. A number of authors have previously used a modified McDonald-Kreitman test (McDonald and Kreitman 1991; Ludwig and Kreitman 1995) to demonstrate the action of positive selection in *cis*-regulatory regions (Jenkins *et al.* 1995; Jordan and McDonald 1998; Crawford *et al.* 1999). This modified test uses a comparison of the ratio of polymorphism to fixed differences of binding site and nonbinding site mutations (the original McDonald-Kreitman test compares nonsynonymous and synonymous mutations).

While the McDonald-Kreitman test requires a random sample of within-species haplotypes, the K_b/K_i ratio requires only single sequences from different species. Genotype data on previously ascertained polymorphisms, as we have collected, are not appropriate for use in a McDonald-Kreitman-type test. Indeed, it is exactly the previous ascertainment of functional regulatory polymorphisms that led us to study this region; however, no previous data on fixed differences existed. The previous ascertainment of functional polymorphisms in the *F7* *cis*-regulatory region raises the possibility that our study was biased toward common variants and thus that the differentiation among populations was biased at these sites. However, both the fact that overall differentiation among our functional polymorphisms was not different from the neutral differentiation and the fact that the high differentiation that we did find was in a population different from the ones used to ascertain the functional polymorphisms suggests that there was little or no effect of ascertainment bias. Our study of fixations along the human lineage should not have been affected by any ascertainment bias as we did not simply use one human haplotype in this comparison, but rather took full advantage of knowledge about polymorphism. It should also be noted that both types of tests of selection described here have been applied to promoter regions whose binding sites have been characterized in only one or a very few cell types. Sequences identified as nonbinding, therefore, may have some as yet unknown role in transcription factor binding. However, the proportion of binding site nucleotides identified in the *F7* *cis*-regulatory region (17%) agrees with the 10–20% range found in many other eukaryotic promoters (Wray *et al.* 2003).

To study the effects of natural selection, if any, in shaping the standing regulatory variation at *F7*, we genotyped three of the functional, phenotypically relevant promoter polymorphisms (−402, −401, and −324) in our six focal human populations. The amount of differentiation expected between populations in the absence of natural selection is a function of the time since divergence and the effective population size. As such, there is no one value of F_{ST} that indicates whether natural selection has acted between various populations without knowledge of the expected variation due to drift. To assess the role of natural selection in causing the differences in frequency between the human populations studied here, we compare the observed F_{ST} at the functional regulatory sites to a distribution of putatively neutral SNPs genotyped in the same individuals (Rockman *et al.* 2003). Comparison of loci of interest to an empirical neutral distribution of F_{ST}'s has been previously shown to be a powerful method for detecting selection on specific genes (Karl and Avise 1992; Taylor *et al.* 1995; Bamshad *et al.* 2002; Fullerton *et al.* 2002; Hamblin *et al.* 2002). If multiple polymorphisms within a single locus are not in linkage disequilibrium, then this method also offers the opportunity to detect selection on individual mutations contributing to differences in fitness.

A comparison of data from the three functional promoter polymorphisms examined here to the neutral distribution shows that the Singaporean Chinese population has diverged at position −402 much more than is expected under neutrality, consistent with a pattern of positive directional selection in this population (Table 2). Neither of the other two sites shows a signature of natural selection in any single population, although they both do show significantly high F_{ST}'s in the China-India comparison. Directional selection on the −402A allele is consistent with either an ongoing or an incomplete selective sweep in the Chinese population; it may be that an intermediate frequency is the evolutionary optimum for this polymorphism. Linkage disequilibrium between site −402 and other sites showing extreme patterns of differentiation could lead us to ascribe this selection to the incorrect polymorphism. Although the −401 and −324 polymorphisms show patterns of positive linkage disequilibrium with each other and several other polymorphisms, −402 does not show linkage disequilibrium with any known functional polymorphisms in *F7* in this study and only weak negative disequilibrium with −401 and −324 in a previous study (van 't Hooft *et al.* 1999). Because of this, we believe that the −402A allele is the mutation that contributes to differences in fitness and is therefore under positive selection.

Human populations have not diverged according to a strict branching process: migration between populations has almost certainly led to gene flow and the homogenization of genetic differences between populations (Goldstein and Chikhi 2002). Therefore, the effects of shared ancestry and migration will both cause populations to have similar allele frequencies. By looking at divergence in allele frequencies at many loci throughout the genome in the same individuals, we have attempted to control for phylogenetic and demographic effects. These effects are expected to act throughout the genome; our results show that the divergence in allele frequency at site −402 goes well beyond divergence at any of the neutral loci or even the other, closely linked functional polymorphisms. Our results using the neutral loci do not, however, tell us whether the increase in frequency of −402A found in Singaporean Chinese

is found in all East Asian populations or whether this pattern may actually be due to selection and shared ancestry with another East Asian population. To address this question we further characterized the allele frequencies of the functional *cis*-regulatory polymorphisms in a Japanese population. The frequencies of alleles −401G (93%) and the −324 deletion (93%) in the Japanese were almost exactly the same as those found in the Singaporean Chinese (90 and 97%; Table 1). But the frequency of −402A in the Chinese (52%) is still significantly higher than that found in the Japanese (36%); the Japanese are much closer in frequency to populations in Cameroon, Ethiopia, India, and Italy (Table 1). This result suggests two main scenarios for the slight increase in frequency of the −402A allele in the Japanese population. The first explanation is that there was a very large increase in frequency of this allele due to directional selection only in a Chinese population and that subsequently migration has been slowly homogenizing East Asian populations. The second main explanation is that there was some combination of genetic drift and directional selection in the ancestor of the Japanese and Chinese populations and that then selection continued only in the Chinese. Obviously, many other combinations of selection, demography, and phylogenetic history could explain the frequencies of the −402A allele; what we have argued here is simply that natural selection almost certainly had a role in East Asia.

Our study does not provide complete evidence for or against selection on the regulatory polymorphism at site −122 or the amino acid variant segregating at residue 353. In the Japanese population we have found that the polymorphism at −122 is in significant, strong positive linkage disequilibrium with sites −324 and −401, the two polymorphisms that we have found to be behaving neutrally; other studies have also consistently shown that sites −122 and residue 353 are both in linkage disequilibrium with these two polymorphisms (Lane *et al.* 1992; Kario *et al.* 1995; Bernardi *et al.* 1997; de Maat *et al.* 1997). Further studies comparing the amount of differentiation at residue 353 and nucleotide −122 to the neutral markers may definitively show whether or not selection has acted on them. While our results cannot exclude the possibility of either of these polymorphisms being under selection in an unknown population, neither does the lack of data contradict the conclusion that the *cis*-regulatory polymorphism at −402 is under positive selection.

Association studies and cell culture assays have both shown that the −402A allele confers increased transcriptional activity and increased levels of F7 in blood plasma (van 't Hooft *et al.* 1999). Results from long-term studies of heart disease, however, have shown that high levels of F7 are correlated with the probability of heart attack and death (*e.g.*, Meade *et al.* 1986; Heinrich *et al.* 1994; Redondo *et al.* 1999; Girelli *et al.* 2000). Given the above facts, is our finding of positive selection for an apparently deleterious allele unusual or even contradictory? We think that it is not. The allele frequencies in current populations are the result of drift and selection over many generations. Consequently, current patterns of population differentiation will be due to drift and selection acting across many generations of human history. Heart disease may be a relatively recent problem in human health, not common before 1900 (Fuster *et al.* 1992); it is also rare in other primates (Schmidt 1978). Heart disease also generally affects individuals after their reproductive years and so may not be an important component of fitness. It is therefore unlikely to have been a strong selective agent in our past. The "thrifty-genotype" hypothesis (Neel 1962) posits that humans are adapted to past conditions and that previously advantageous traits may now be associated with diseases such as diabetes. If alleles currently associated with disease are at frequencies that reflect past positive selection, then population genetic models must take this into account. The common disease/common variant hypothesis (Lander 1996) states that the genetic risk for common diseases will generally be associated with one or a few common alleles. The allelic spectra expected for disease-associated loci, however, have been predicted under the assumption of weak negative selection against disease alleles (Pritchard 2001; Reich and Lander 2001). Our results, as well as the thrifty-genotype hypothesis, would seem to violate these assumptions.

We do not know the selective forces responsible for the large increase in frequency of the high-expression allele at site −402. However, it is interesting to note that the other high-expression alleles show no signatures of natural selection. Simple assays of F7 levels in the bloodstream or transcription levels in only one or a few cell types may be missing a host of complex regulatory interactions or even whole transcriptional domains that might explain differences between "high-expression" alleles. Indeed, Wilcox *et al.* (2003) found that *F7* was expressed in multiple unanticipated tissues and therefore may have a role in multiple unknown cellular functions. Further research into the specific effects of individual *F7* alleles in multiple genetic backgrounds and in multiple cell lines is needed. Population genetic studies in humans showing positive directional selection within a population (this study; Sabeti *et al.* 2002), balancing selection between disease and protective states within and among populations (Hamblin and Di Rienzo 2000; Bamshad *et al.* 2002; Rockman *et al.* 2003), epistatic interactions between variants (Rockman and Wray 2002; Horan *et al.* 2003), and varying selective effects among polymorphisms (this study) argue against a simplistic view of *cis*-regulatory population dynamics. Transcriptional regulation is complex, idiosyncratic, and context dependent (Wray *et al.* 2003); understand-

ing the evolutionary forces shaping regulatory variation will be commensurately difficult.

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Compound heterozygous mutations in severe factor VII deficiency including a novel nonsense mutation

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A 26-year-old man who had a history of recurrent spontaneous nasal bleeding was admitted to our hospital with uncontrolled traumatic subdural hemorrhage. His plasma factor VII activity was less than 10% of normal. DNA sequence analysis revealed that the proband had a compound heterozygote for a novel nonsense mutation (*F7* NM_000131.2 c.345C>A; p.Cys115X) in exon 4 encoding the EGF1 domain and a known missense mutation (*F7* NM_000131.2 c.1027G>A; p.Gly343Ser) in exon 8 encoding the serine protease domain of *F7*. The same *F7* Gly343Ser mutation was present in the asymptomatic father, who exhibited a modest reduction in the plasma level of factor VII activity (48%). *Blood Coagul Fibrinolysis*

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Introduction

Factor VII (FVII) deficiency is a rare inherited disorder, usually transmitted in an autosomal recessive manner with an incidence of one per 500 000 in the general population [1]. In affected patients, factor activity levels are lower than normal but clinical features can be highly variable from asymptomatic patients to patients with severe bleeding tendencies [2]. The FVII gene (*F7*) is located on 13q34-qter and contains nine exons. Approximately 136 mutations responsible for FVII deficiency have been described to date (FVII mutation database, http://europium.csc.mrc.ac.uk [3]). Although mutations are scattered throughout the gene, the majority of the mutations are missense mutations and are located within the catalytic domain of FVII [1]. We have identified a novel nonsense mutation involving exon 4 (Cys115X) in a severe FVII-deficient patient with subdural hemorrhages. A known missense mutation in exon 8 (Gly343Ser) encoding the catalytic domain was also detected in the patient and his father, with the nucleotide and the protein numbering using accession numbers NM_000131.2 and NP_000122.1, respectively.

Case report

A 26-year-old man attended our hospital emergency room with uncontrolled traumatic subdural hemorrhage. He had a history of recurrent spontaneous nasal bleeding since his childhood and had required transfusions occasionally. At admission, a complete blood count showed a white cell count of 8.94×10^9/l, a platelet count of 296×10^9/l and a hemoglobin level of 4.8 g/dl. The prothrombin time was markedly prolonged (36.3 s; International Normalized Ratio, 3.76) in contrast to an activated partial thromboplastin time of 40.6 s (normal range 30–44 s). The prolonged prothrombin time was corrected in a 50 : 50 mix with normal plasma. FVII coagulant activity was less than 10% of normal (STAGO DEFICIENT VII; Diagnostica Stago, Asnières, France). The factor IX activity was 33% of normal, and other factors (factor II, factor X, factor XI) showed normal activity in the functional assay.

For genetic analysis, the DNA of the patient and his father was extracted using a Wizard Genomic DNA purification Kit (Promega, Madison, Wisconsin, USA). The entire coding regions and the promoter region of *F7* and were amplified by polymerase chain reaction with the specific primers for the individual exon presented in Table 1. The polymerase chain reaction conditions were available in the FVII mutation database [3]. For sequencing each exon of *F7*, the sequence of polymerase chain reaction products was determined using the ABI Prism BigDye Terminator Sequencing Kit (version 3.1; Applied Biosystems, Foster City, California, USA) in an ABI Prism 3730XL genetic analyzer (Applied Biosystems). Each sequencing was performed in duplicate using forward and reverse primers for each exon. The result showed that the sequence of the patient had a novel nonsense mutation in exon 4 encoding the EGF1 domain of FVII (*F7* NM_000131.2 c.345C > A; p.Cys115X) and a known missense mutation in exon 8 encoding the serine protease domain of FVII (*F7* NM_000131.2 c.1027G > A; p.Gly343Ser) (Fig. 1). QUANTA (Accelrys, San Diego, California, USA) was used for the structure analysis and

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Table 1 Primers used in the polymerase chain reaction for the entire coding region and promoter region of the factor VII gene (*F7*)

Exon	Sequences	Product size (base pairs)
Promoter	5′-CCTGGTCTGGAGGCTCTC-3′	352
	5′-GGGGAGGACACAGGCGT-3′	
1a	5′-CTTGGAGGCAGAGAACTTTG-3′	311
	5′-CCTGGATGCTGGTTTCTAGA-3′	
1b	5′-GGGTGGGCTGTGAGGGAC-3′	220
	5′-GAGGGGAAGGAGGTGATGTT-3′	
2	5′-GAGGCACTGGGCGGGGCACG-3′	261
	5′-CCGCCGCCGTGCAGTGTCCG-3′	
3 and 4	5′-TGTGTCCAGTGCTTACCGT-3′	336
	5′-CTCCACCAGCTCCCCA-3′	
5	5′-CTGACCCCCAGAAGCCCCTC-3′	240
	5′-CTAGTGGGACAGGGACTGGT-3′	
6	5′-GGCACGTTCATCCCTCA-3′	257
	5′-ACCTGCCCATTTTCCC-3′	
7	5′-ATGACAGCAATGTGACTTCC-3′	439
	5′-GTCTGTGGAAGTGACAGCAC-3′	
8	5′-GGCAGGTGGTGGAAAGGGCC-3′	660
	5′-CCACAGGCCAGGGCTGCTGG-3′	

modeling study with the coordinate of the crystal structure of activated FVII (PDB entry 1W8B) [4].

The same *F7* Gly343Ser mutation was present in the phenotypically asymptomatic father, who exhibited a modest reduction (48%) in the plasma level of FVII coagulant activity. We could not identify whether the nonsense mutation was inherited from the mother or was *de novo* because the mother had been divorced from the father. After treatment with recombinant activated FVII for 20 days, the patient was discharged with improved laboratory results (hemoglobin, 11.0 g/dl; prothrombin time International Normalized Ratio, 1.55).

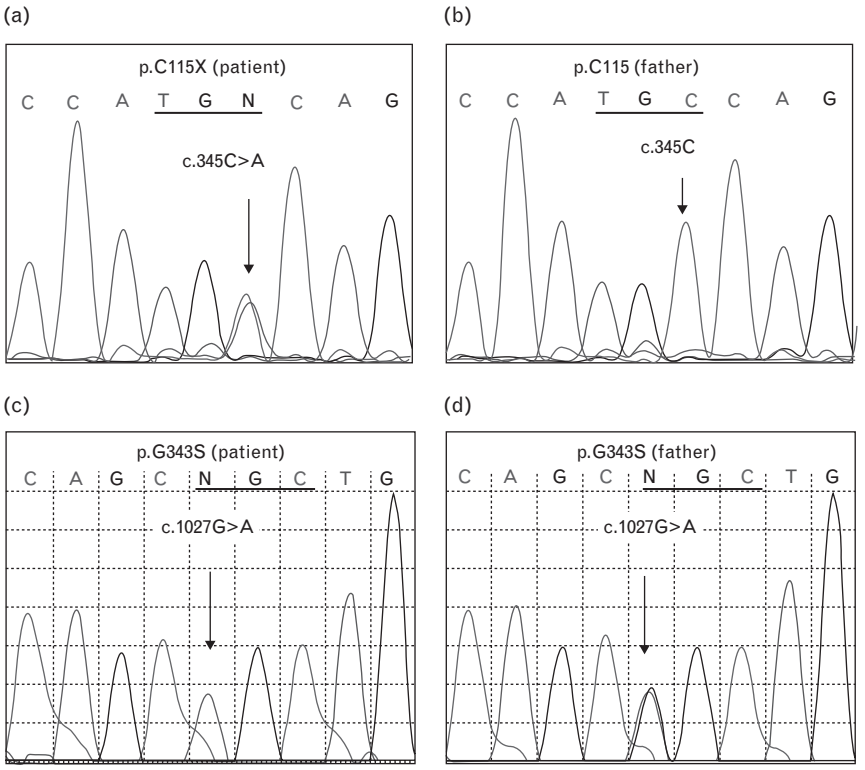
Discussion

Our patient was found to have a double heterozygous mutation with a novel nonsense mutation of exon 4 (c.345C > A) and a known missense mutation of exon 8 (c.1027G > A) encoding the catalytic domain.

The novel nonsense mutation in exon 4 leading to a truncated protein could be more responsible for the bleeding diathesis of the patient. Skipping of exon 4 by a mutation in the splice site of intron 4 of *F7* was reported previously to cause lethal FVII deficiency in a family [5]. Moreover, alternations of the EGF1 domain could affect the Ca^{2+} binding and therefore the tissue factor affinity [6].

The catalytic domain on which the majority of the missense mutations were located has a high-affinity calcium binding site and is thought to be related to the formation

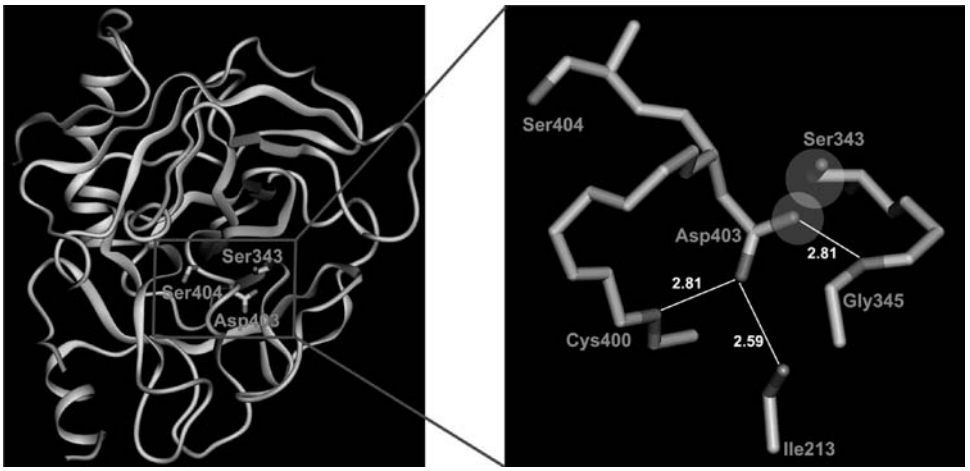
Fig. 1



(a) Patient showing a compound heterozygote for a novel nonsense mutation (*F7* NM_000131.2 c.345C > A; p.Cys115X) in exon 4 encoding the EGF1 domain; (b) father does not show the mutation. Both the (c) patient and (d) father had a known missense mutation (*F7* NM_000131.2 c.1027G > A; p.Gly343Ser).

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Fig. 2



Effect of the Gly343Ser mutation on Ser404 of the catalytic triad of the serine protease. Close-up view of the protease domain shows that the mutation, Gly343Ser, would cause a steric collision with Asp403. The collision is depicted as the overlap of the semitransparent circles. The hydrogen bonds of Asp403 are depicted as white lines and their bond distances are labeled (Å).

of the complex of FVII and tissue factor, the primary event in the initiation of blood coagulation *in vivo* [7]. Conformational analysis of the crystal structure of activated FVII indicated the missense mutation in this case also leads to reduction of the intrinsic activity of the protease domain. Activated FVII is a serine protease with a catalytic triad consisting of serine, histidine, and aspartic acid (Fig. 2). The arrangement of these three residues by hydrogen bonds leads to the high reactivity of the serine residue. From the inspection of the crystal structure of activated FVII, the missense mutation, Gly343Ser, can reduce the catalytic activity of the serine protease by changing the position of Ser404, which is the key residue of the catalytic triad. The loop of the active site containing Ser404 adapts its optimal conformation by the hydrogen bonds of Asp403 with the backbone amide NH of Ile213, Gly345, and Cys400 (Fig. 2). The Gly343-Ser mutation results in the replacement of a hydrogen atom with a larger side chain, however, and thus causes a severe collision with the side chain of Asp403 with a distance of 1.8 Å between the carboxyl group of Asp403 and C-beta of Ser343. The conformational change of Asp403 is therefore inevitable in order to minimize the collision, and this change would reduce the hydrogen bonds of Asp403. The reduced hydrogen bond strength will change the conformation or stability of the loop containing Ser404; thereby Ser404 cannot be well positioned for catalytic activity. The catalytic triad of the mutated activated FVII would be distorted by the subtle change of the position of Ser404, leading to the loss of protease activity.

In conclusion, we have described a patient with severe symptomatic FVII deficiency as a result of compound heterozygous mutations – a nonsense mutation on exon 4 and a missense mutation on exon 8. This is the first report on genetically and biochemically confirmed FVII deficiency in Korea with compound heterozygous mutations including a novel nonsense mutation.

Acknowledgements

The authors are grateful to Hyunjung Jang for technical support. This work was supported by Pusan National University Hospital Research Grant 2007.

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American Journal of Hematology 77:90–91 (2004)

A Rare Inherited Coagulation Disorder: Combined Homozygous Factor VII and Factor X Deficiency

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The combined presence in the homozygous state of more than one recessively transmitted coagulation defect may rarely occur in countries with a high rate of consanguinity. In an Iranian family consisting of two parents (second cousins) and two affected siblings, initial phenotypic analysis led to a diagnosis of mild FX deficiency (10–19% FX activity, 42–54% FX:Ag), and genotyping revealed a new homozygous missense mutation in the corresponding gene (Ser3Cys). As both of the sibs had a severe bleeding history that was not compatible with mild deficiency of FX, further phenotypic analysis revealed the additional presence of severe FVII deficiency (<1% FVII activity; 63–111% FVII:Ag) associated with the homozygous missense gene mutation Cys310Phe. In this kindred, lack of identification of the double coagulation defect might have led not only to incomplete understanding of the clinical phenotype but also to an incorrect prenatal diagnosis. Am. J. Hematol. 77:90–91, 2004. © 2004 Wiley-Liss, Inc.

Key words: factor VII deficiency; factor X deficiency; mutational analysis; prenatal diagnosis

INTRODUCTION

Factor VII (FVII) and factor X (FX) are vitamin K-dependent serine proteases synthesized in the liver. The former, in its activated form, plays a key role in the initiation of coagulation, while the latter occupies a central position in the coagulation cascade as the first pro-enzyme of the common pathway of fibrin formation. Isolated FVII deficiency, the most common recessively inherited coagulation disorder (1:500,000) [1], is characterized by prolonged prothrombin time (PT) and normal activated partial prothrombin time (APTT). FX deficiency, with a prevalence of 1:1,000,000 in the general population [2], is characterized by a prolongation of both the PT and the APTT. Both the FX and FVII genes map on the long arm of chromosome 13 [3], and until now, the few reports of combined deficiency of FVII and FX were due only to gross abnormalities of this chromosome [4,5], not to the co-segregation of two separate gene defects.

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CASE REPORT

An Iranian family consisted of two consanguineous parents (second cousins) and two siblings: a 38-year-old man and a 24-year-old woman. The man had severe lifelong bleeding symptoms, including muscle hematomas and frequent hemorrhages in the gastrointestinal tract that led him to receive several blood transfusions. His sister had recurrent episodes of

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TABLE I. Results of Phenotype and Genotype Analyses

Patient	FX:Ag (%)	FX:C (%)	Ser3Cys TCC→TGC	FVII:Ag (%)	FVII:C (%)	Cys310Phe TGC→TTC
Male proband	42	10	Homozygous	63	< 1	Homozygous
Female proband	54	19	Homozygous	111	< 1	Homozygous
Mother	83	75	Heterozygous	130	75	Heterozygous
Father	67	56	Heterozygous	68	39	Heterozygous

epistaxis and menorrhagia that led her to use a combined estrogen–progesterone preparation. She received blood transfusions on several occasions. Both the patients had prolonged PT and APTT that, on the assumption that they were due to the defect of a single coagulation factor, prompted us to assay the factors of the final common pathway of fibrin formation (factor X, factor V, prothrombin, and fibrinogen). While the latter were in the normal range, both sibs had low of levels of FX coagulant activity (FX:C) (10–19%, respectively) and FX antigen (FX:Ag) (42–54%). DNA analysis was carried out to detect the FX gene mutation underlying the deficiency. Direct sequencing (ABI Prism^{TM} Genetic Analyzer, PE Applied Biosystems, Monza, Italy) of all coding regions, intron/exon boundaries, and the 3′ and 5′ untranslated regions (UTR) of the FX gene identified a new missense mutation, TCC to TGC, in the homozygous state in both the sibs. The C to G transition leads to the replacement of a serine by a cysteine at codon 3 in exon 2. The presence of the Ser3Cys mutation was confirmed by repeating sequencing and by endonuclease restriction of the PCR fragment using *Mun*I enzyme (New England Biolabs, Celbio, Milan, Italy), as the mutation creates a restriction site. This genetic variant is unlikely to be a frequent polymorphism, because it was not found in 106 alleles in an Iranian control population.

As the intermediate phenotype (a mild FX deficiency) and the genotype (a missense mutation in homozygosity) were not totally compatible with the clinical phenotype (severe bleeding symptoms in both siblings), further phenotypic studies were carried out. They revealed the additional presence of FVII deficiency with plasma FVII:C levels of less than 1% and normal levels of FVII:Ag (Table I). FVII mutational analysis identified in both probands the presence, in the homozygote state, of the previously reported missense mutation Cys310Phe [6,7].

DISCUSSION

The results obtained in this kindred emphasize the importance of a comprehensive and critical analysis of laboratory results, clinical phenotype, and genotype in patients with recessively inherited coagulation disorders originating from populations characterized by a high rate of consanguinity and genetic homogeneity. For instance, in a previously studied kindred we observed a predominant transmission of factor VII deficiency from an affected homozygous mother to her child [8]. This unusual pattern of inheritance was due to heterozygosity of the unaffected parent, which was unrelated to the mother but was originating, like her, from the restricted community of Iranian Jews. In the consanguineous Iranian kindred described herein, homozygosity for two different abnormal traits in the two sibs and heterozygosity in their parents indicated that both the mutations were on a single allele. Diagnosing these cases as isolated FX deficiency and missing the FVII deficiency and the associated gene mutations could have led to an incorrect molecular diagnosis.

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Prevention of β Strand Movement into a Zymogen-like Position Does Not Confer Higher Activity to Coagulation Factor VIIa

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ABSTRACT: Coagulation factor VIIa (FVIIa) belongs to the chymotrypsin family of S1 peptidases, whose members require the cleavage of at least one internal peptide bond to attain an active conformation. FVIIa also requires association with tissue factor (TF) to attain full catalytic competency. Without this, FVIIa has very low activity toward peptide and physiologic substrates. Reregistration of β strands has been suggested to play a part in the activation of FVII, and their positioning is possibly important for the active conformation of FVIIa. To scrutinize this hypothesis, we have designed FVIIa variants which prevent β strand movement and lock FVIIa in the alleged active conformation. The V299M mutation, alone or combined with the L280I mutation, was introduced to alter the first of two Leu-X-Val motifs in β strand B2 and thereby prevent reregistration. Along the same line, C164V/V299C-FVIIa has a new disulfide which would keep β strand B2 in the registration of active FVIIa. The amidolytic and proteolytic activities of V299M-, L280I/V299M-, and C164V/V299C-FVIIa were indistinguishable from or lower than those of wild-type FVIIa, and none of the mutants displayed an altered exposure of the N-terminal amino group of the protease domain. Moreover, the affinities of mutant and native FVIIa for TF increased to a similar extent upon incorporation of an active site inhibitor, and the enzymatic activities were equally stimulated by TF. In conclusion, we found no evidence that the mutants were in a more active state than native FVIIa. Thus, the proposed β strand reregistration, if part of the regulatory mechanism governing FVIIa activity, apparently does not suffice for the transformation of FVIIa into an enzymatically active conformation. Our data raise the possibility that the structural differences between enzymatically latent (zymogen-like) and active FVIIa resemble those between trypsinogen and trypsin.

After vascular damage, factor VIIa (FVIIa)[1] associates with tissue factor (TF) to initiate the blood coagulation cascade (*1*). This complex formation is required to render FVIIa biologically active by dramatically enhancing the catalytic efficiency (*2*), which is readily measured as an increased rate of peptidic substrate hydrolysis (*3*). The allosteric mechanism by which TF stimulates the activity of FVIIa is under intense investigation.

FVIIa belongs to the chymotrypsin family of serine proteases, requiring specific endoproteolytic processing to attain the active conformation. Following zymogen activation, the amino terminus of the protease domain (Ile-153)[2] typically enters the so-called activation pocket to form a salt bridge with Asp-343. The activation pocket is part of the activation domain which has been shown to become ordered during trypsinogen activation (decreased crystallographic *B* factors) (*4*).The conformational change observed by X-ray crystallography when comparing trypsinogen to trypsin reveals a maturation of the S1 pocket as well as generation of the oxyanion hole that is important for catalytic competency (*4*, *5*). The insertion of the N-terminal amino group also protects it from chemical modification. However, the amino terminus of the protease domain of free FVIIa is still susceptible to chemical modification, indicative of retained solvent accessibility and a zymogen-like conformation. Binding of TF stabilizes the insertion of the N-terminus and promotes the active conformation (*6*). Thus, FVII is incompletely converted from zymogen to enzyme upon cleavage after Arg-152.

Considerable progress derived from functional and mutational studies has helped elucidate details of the allosteric regulation of FVIIa (*7*). The findings suggest that the regulation is not mediated by a simple, single conformational transition. The inherent flexibility of the activation region is employed to allosterically regulate the protease activity. It appears that three regions of the FVIIa protease domain are important for the allosteric regulation and function (*7*), i.e., the TF binding site, the active site region, and the macromolecular substrate exosite which also comprises the activation pocket (Figure 1A). Cross talk, in the form of

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[1] Abbreviations: FVII(a), coagulation factor VII(a); FX(a), coagulation factor X(a); SPR, surface plasmon resonance; sTF, soluble tissue factor (residues 1−219); TF, tissue factor.

[2] Chymotrypsinogen numbering is as follows: Arg-152, 15; Ile-153, 16; Lys-157, 20; Cys-159, 22; Pro-160, 23; Glu-163, 26; Cys-164, 27; Pro-165, 28; Trp-166, 29; Leu-280, 137; Ala-294, 152; Glu-296, 154; Leu-297, 155; Met-298, 156; Val-299, 157; Leu-300, 158; Val-302, 160; Arg-304, 162; Met-306, 164; Gln-313, 170A; Asp-343, 194; Trp-356, 207; Tyr-377, 228.

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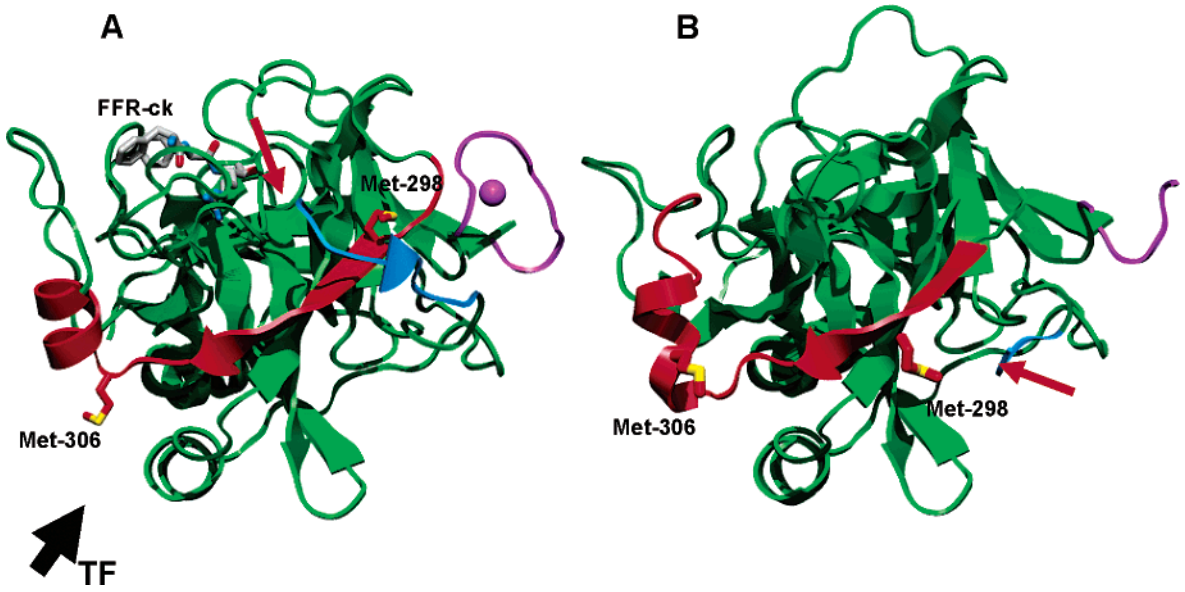


FIGURE 1: Protease domain of the active FVIIa conformation (A) (PDB entry 1dan) and zymogen FVII conformation (B) (PDB entry 1bju). Residues 294−313 subjected to reregistration are colored red. The side chain of Met-298 is shifted from panel A to B. In panel A, the calcium loop and the calcium ion are colored magenta. This part of the zymogen structure is poorly resolved in the structure in panel B. The N-termini are colored blue (red arrows). In panel A, the covalently bound inhibitor FFR-ck is shown as a stick model in light green and the black arrow marked TF indicates the direction in which the cofactor tentatively approaches and binds the protease domain. The three regions of the FVIIa protease domain important for function are the TF binding site (the region in the vicinity of Met-306), the active site region (the region close to the FFR-ck inhibitor), and the macromolecular substrate exosite (the region in the vicinity of Met-298). These regions communicate along unknown paths in response to binding of an allosteric regulator.

allosteric signals, is transmitted between these regions. Alanine scanning of FVIIa has identified, by loss of function, residues in the protease domain that are important for TF binding and catalytic function (*8*). It has also served as an inspiration for site-specific mutagenesis efforts aimed at elucidating the nature of the allostery or creating FVIIa analogues with enhanced activity (*9*−*15*). These gain-of-function variants have provided additional insights into the allosteric regulation. In contrast, no clues are offered by the three-dimensional structure of the protease domain of free FVIIa because it is, apart from certain loop regions, virtually identical to that of thrombin and other constitutively active serine proteases. In addition, the available structures of free (*16*−*18*) and TF-bound FVIIa (*19*, *20*) are very similar, perhaps due to the presence of an active site inhibitor in all structures. In ref *18*, a structure without an inhibitor in the active site was obtained by removing the inhibitor from a preformed crystal. However, the possibility that there are restrictions in the allowed conformational adjustments caused by the existing crystal cannot be ruled out. A structure of noninhibited FVIIa, formed in the absence of an inhibitor, is needed to reveal the potential structural differences between the zymogen-like and enzymatically active conformations of FVIIa, especially considering that the active site inhibitor exerts allosteric effects manifested as an influence on the affinity for TF (*21*, *22*). Nonetheless, information possibly pertaining to the zymogen-like conformation of free FVIIa has been provided by the solution of the crystal structure of zymogen FVII (*23*). The zymogen, however, was crystallized in complex with an exosite-binding inhibitory peptide. The structure reveals a new registration of two β strands (Figure 1), resulting in large differences in the region binding TF as well as in the macromolecular substrate exosite region compared to previous structures of FVIIa. TF binding is hypothesized to reorganize the macromolecular substrate exosite, resulting in the insertion of the N-terminal Ile-153 into the activation pocket, and to induce the shift of a β strand by three amino acid residues to attain the active FVIIa conformation (*23*). The FVII(a) sequence contains a duplicated Leu-X-Val repeat in positions 297−302 which is believed to facilitate β strand reregistration.

In the chymotrypsin family, comprising proteases with closely related enzyme structures, at present two substantially different classes of zymogen conformations appear to exist. In trypsinogen, the major conformational differences from trypsin are confined to the activation domain. This observation also holds for other members of the family. However, the zymogen FVII structure infers unexpected conformational differences. One is the large reorganization in the TF binding region resulting from the β strand reregistration. We set out to investigate whether the FVII zymogen structure represents the zymogen-like (latent) conformation of FVIIa, and whether the β strand hypothesis could be exploited to enhance the intrinsic activity of FVIIa. We constructed two types of FVIIa analogues designed to prevent β strand movement so that they thereby would be locked in the enzymatically active conformation. First, the first Leu-X-Val motif was modified by the introduction of Met in the third position (299). Second, a new disulfide bridge was introduced between Cys-159 and an introduced Cys at position 299 (C164V/V299C-FVIIa). If successfully locked in an active conformation (similar to that when bound to TF), the mutants would be expected to process the chromogenic substrate used approximately 50 times more efficiently than in wild-type FVIIa. The absence of an increased efficiency for any of the FVIIa variants, supported by the similar TF binding and cofactor-induced stimulation and unrestricted freedom of the N-terminus of the protease

domain, suggests that the conformational changes pertinent for the conversion of latent FVIIa to an active enzyme are confined to the activation domain.

EXPERIMENTAL PROCEDURES

Reagents and Standard Methods. Wild-type FVIIa and soluble TF (sTF) were obtained as described previously (*24*, *25*), and the concentrations of FVII(a) and sTF were determined by an ELISA and absorbance measurement at 280 nm, respectively (*26*). Factor X (FX) was from Enzyme Research Laboratories (South Bend, IN). D-Phe-Phe-Arg-chloromethyl ketone was from Calbiochem (La Jolla, CA). The chromogenic substrates S-2288 and S-2765 from Chromogenix (Mölndal, Sweden), and potassium cyanate was from Merck. SDS−polyacrylamide gel electrophoresis was carried out on a 4−12% NuPAGE Novex Bis-Tris gel (Invitrogen, Carlsbad, CA).

Mutagenesis and FVIIa Mutant Production. Wild-type FVII expression plasmid pLN174 (*27*) was the template for site-directed mutagenesis using the QuikChange kit (Stratagene, La Jolla, CA). The following primers (only sense primers shown) were used to introduce the mutations, with the base substitutions in italics and the affected codons underlined: V299M, GCCCTGGAGCTCATG*ATG*CTCAACGTGCCCCGG; L280I, CGTGCCTTCTCA*ATC*GTCAGCGGCTGGGG; C164V, CCCAAAGGGGAG*GTA*CCATGGCAGGTCC; and V299C, GCCCTGGAGCTCATG*TGC*CTCAACGTGCCC. Plasmids were prepared using the plasmid midi kit and QIA filter (Qiagen, Valencia, CA). The entire coding sequences were verified by sequencing to confirm the identities of the mutants. Baby hamster kidney cell transfection and selection and mutant expression, purification, and autoactivation were carried out as described previously (*12*, *26*).

Mass Spectrometry. Mass spectrometric peptide mapping of C164V/V299C-FVIIa was performed as follows. Wild-type and mutant FVIIa (1 μg/μL in 50 mM NH_4HCO_3) were digested in parallel with trypsin (2%, w/w) for 2 h at 37 °C, and 1 μL of the digest was applied to a MALDI target using α-cyanocinnamic acid as a matrix. A Bruker Ultraflex MALDI-TOF/TOF instrument was used to record the mass spectra. The instrument was operated in standard reflectron mode. The resulting spectra were externally calibrated using a mixture of known standard peptides. All masses are monoisotopic.

FVIIa Activity Assays. All assays were performed in 50 mM Hepes (pH 7.4) containing 0.1 M NaCl, 5 mM $CaCl_2$, and 1 mg/mL bovine serum albumin. The amidolytic activity of free enzyme was measured using 100 nM mutant or wild-type FVIIa, whereas the amidolytic activity when bound to sTF was measured using 10 nM FVIIa and 100 nM sTF. The substrate was S-2288 at concentrations ranging from 0.1 to 10 mM. The FX activation catalyzed by wild-type or mutant FVIIa was measured by incubating 10 nM FVIIa with 0.8 μM FX for 15 min. The reaction was terminated by the addition of assay buffer containing 20 mM EDTA, followed by the addition of S-2765 (final concentration of 0.5 mM) for quantification of FXa. The effect of potassium cyanate on the activity of mutant and wild-type FVIIa was studied by incubation of 1 μM enzyme with 0.2 M cyanate in assay buffer without albumin followed by measurement of the residual amidolytic activity as described previously (*12*). All activity measurements were carried out at 405 nm using a SpectraMax 190 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA).

Surface Plasmon Resonance (SPR) Measurements. Mutant and wild-type FVIIa or FFR-FVIIa, in 50 mM Hepes (pH 7.4) containing 0.1 M NaCl, 5 mM $CaCl_2$, and 0.02% Tween 80, were injected for 7 min at a flow rate of 5 μL/min and a concentration of 30 or 60 nM over a CM5 sensor chip coated with 650 resonance units of amine-coupled sTF. The subsequent dissociation phase lasted for 10 min. The instrumentation, regeneration between runs, and data evaluation were as described previously (*28*, *29*).

Structural Analyses and Molecular Modeling. Molecular analyses (and figure preparations) were performed within the frameworks of the molecular modeling package Quanta 2000 and the molecular mechanics force field CHARMm 27 (Accelrys Inc., San Diego, CA). The models of the introduced mutations have been created by mutating the side chains using the "mutation facility" within the protein modeling module in Quanta 2000. The conformations of the side chains were then adjusted using a main chain conformation-dependent rotamer library (based on high-resolution X-ray structures), followed by energy minimization [250 steps of conjugated gradient (CONJ) minimization in CHARMm].

RESULTS

Isolation of FVIIa Variants and Verification of C164V/V299C-FVIIa. The variants V299M-FVIIa, L280I/V299M-FVIIa, and C164V/V299C-FVIIa were generated by site-directed mutagenesis and stably expressed in baby hamster kidney cells. After purification from the cell culture supernatant and autoactivation, the variants were apparently homogeneous and indistinguishable from wild-type FVIIa as judged by SDS−PAGE (not shown). Mass spectrometry was employed to confirm that the mutations in C164V/V299C-FVIIa had been introduced and yielded the intended, new disulfide bond between Cys-159 and Cys-299. Figure 2A shows the MALDI-TOF spectrum of trypsin-digested FVIIa. The presence of the native disulfide link between the peptides containing residues 158−161 and 162−197 (theoretical mass of 4296.2 amu) is verified, as is the presence of peptide 291−304 (theoretical mass of 1482.8 amu). The corresponding spectrum of trypsin-digested C164V/V299C-FVIIa is shown in Figure 2B. If peptide 291−304, after introduction of the V299C mutation, was not disulfide-linked, a mass of 1486.8 amu would be detected. Alternatively, if the introduced Cys residue at position 299 was involved in a disulfide with glutathione, a mass of 1738.1 amu would be detected. Neither of these peaks is found; instead, we find a mass corresponding to peptide 291−304 disulfide-linked to peptide 158−161 (theoretical mass of 1930.0 amu). In addition, the signals corresponding to 1482.8 and 4296.2 amu are absent. This shows that the new disulfide indeed has been formed and that it is formed in all the C164V/V299C-FVIIa molecules.

Enzymatic Activity of FVIIa Variants. The amidolytic activity was measured to investigate the direct influence of the mutations on the active site functionality, whereas the proteolytic activity (activation of FX) also probes macro-

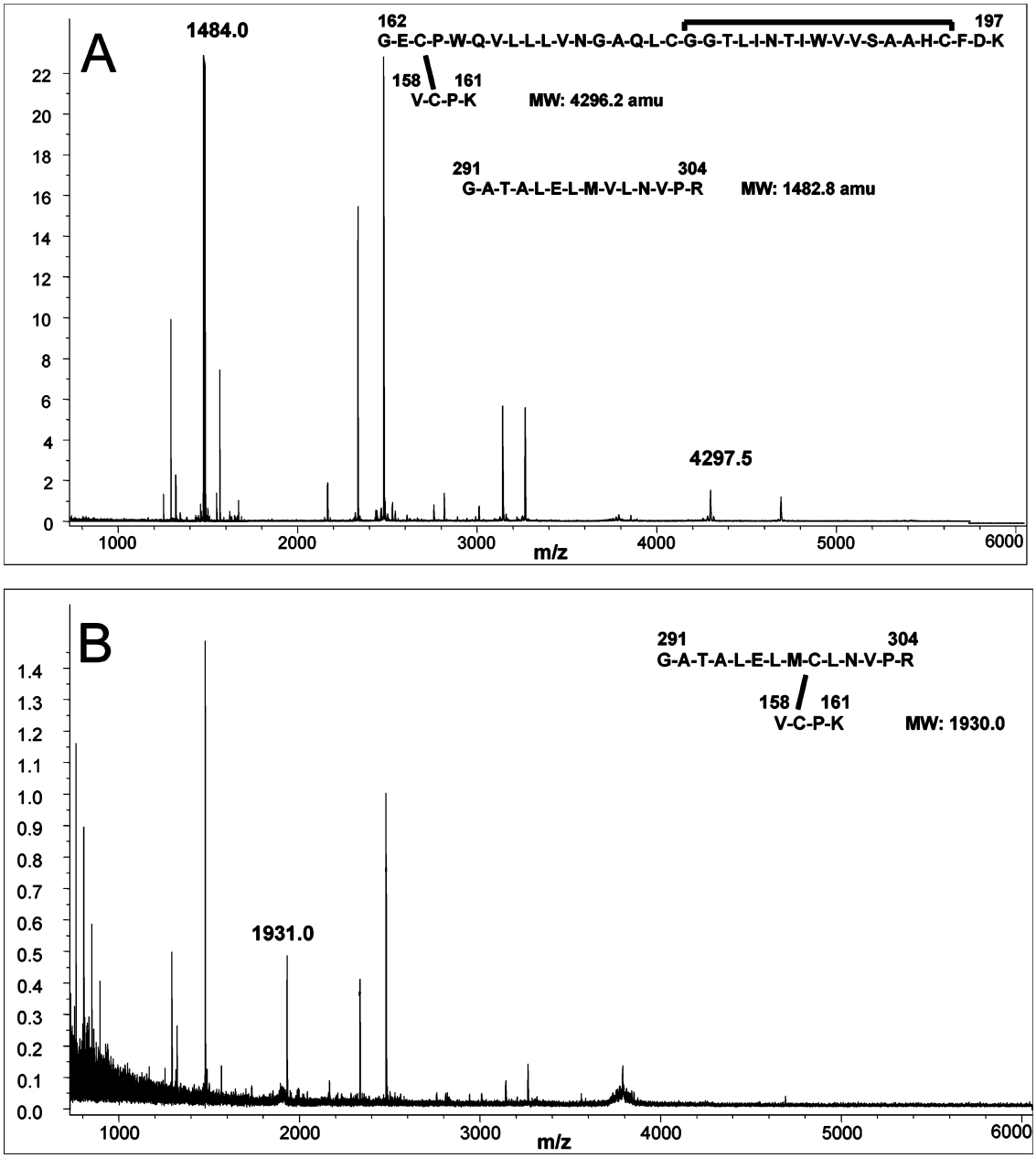


FIGURE 2: MALDI-TOF spectrum of trypsin-digested wild-type FVIIa (A) and trypsin-digested C164V/V299C-FVIIa (B). In panel A, the signals of disulfide-linked peptides 158−161 and 162−197 (theoretical mass of 4296.2 amu) and of free peptide 291−304 (theoretical mass of 1482.8 amu) are marked. In panel B, the presence of the signal of the new disulfide link connecting peptides 158−161 and 291−304 (theoretical mass of 1930.0 amu) is indicated. Notice the absence of the signals corresponding to 1482.8 and 4296.2 amu as well as the absence of signals corresponding to 1486.8 and 1738.1 amu (see the text). The dominant peak at 1477.8 amu corresponds to tryptic peptide 278−290 (theoretical mass of 1476.8 amu) and is as expected present in both panels.

molecular substrate exosite alterations. The activities of mutant and wild-type FVIIa in the absence of TF are shown in Table 1. When the parameters of the amidolytic activity were investigated, there was no significant difference between FVIIa and V299M-FVIIa in terms of k_{cat} or K_m values. The additional substitution of Ile for Leu in position 280 if anything lowered the K_m value slightly, and the double mutant L280I/V299M-FVIIa had normal kinetic parameters. If real, the slightly decreased efficiency of V299M-FVIIa may be ascribed to an unfavorable interaction of the introduced Met side chain with the surrounding protein, particularly the neighboring disulfide bridge (Cys-159−Cys-164), resulting in a slight misalignment of N-terminal insertion. The introduction of the new disulfide bridge in C164V/V299C-FVIIa decreased considerably the amidolytic activity (~2.5-fold), exclusively due to a decreased k_{cat} value.

Table 1: Chromogenic Substrate Hydrolysis and FX Activation by Free FVIIa Variants

enzyme	K_m (mM)	v_{max} (mOD/min)	v_{max}/K_m (mOD mM^{-1} min^{-1})	FX activation[a]
FVIIa	8.0 ± 0.6	69 ± 3	8.6 ± 1.0	1.00
V299M-FVIIa	9.8 ± 0.6	60 ± 2	6.1 ± 0.6	1.05 ± 0.04
L280I/V299M-FVIIa	6.6 ± 0.5	68 ± 3	10.3 ± 1.2	1.07 ± 0.02
C164V/V299C-FVIIa	9.2 ± 0.7	33 ± 1	3.6 ± 0.4	1.15 ± 0.04

[a] Rate of activation compared with that of wild-type FVIIa which is given an arbitrary value of 1. The FX concentration was 0.8 μM. Values are means ± the standard error of the mean ($n = 2$).

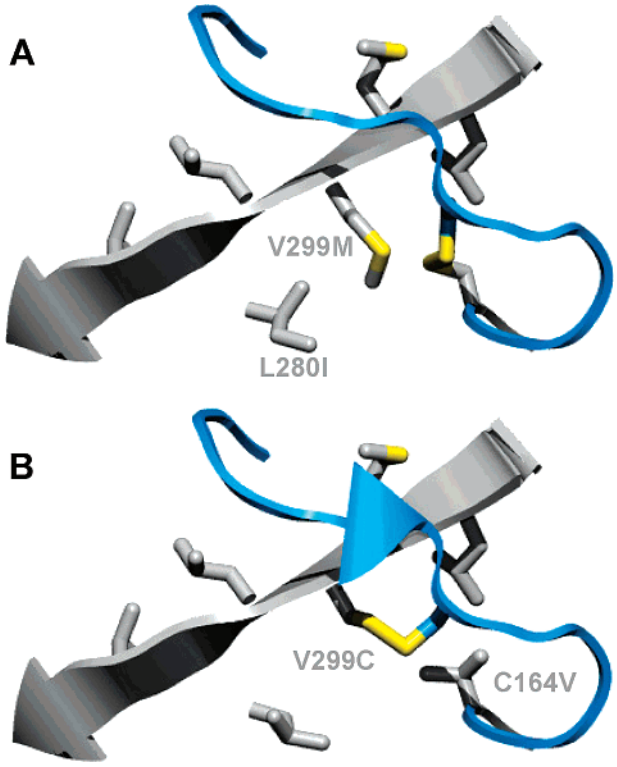


FIGURE 3: Models of mutants L280I/V299M-FVIIa (A) and C164V/V299C-FVIIa (B). β strand B2 in the active FVIIa conformation, residues 296−304, is colored gray, and N-terminal residues 153−165 are colored blue. The model in panel A has been generated by mutating the side chains *in silico* and adjusting the conformations by consulting rotamer libraries, followed by energy minimization to relieve steric clashes. In panel B, mutations C164V and V299C were introduced *in silico* to mimic the situation in bovine trypsin or human kallikrein. Formation of the new disulfide bridge, followed by application of rotamer libraries and energy minimization, resulted in the depicted model. The conformation of the disulfide is almost identical to those observed in the X-ray structures of bovine trypsin and human kallikrein.

This reduction in activity is most likely due to a minor rearrangement of the N-terminus which weakens the insertion. The mutant constructs are illustrated in Figure 3, and the proximity of the mutated positions to the N-terminus is clear. Interestingly, the proteolytic activity of all variants, including C164V/V299C-FVIIa which displayed a lower amidolytic activity, was normal or possibly marginally increased compared to that of FVIIa (Table 1). The activity measurements suggest that the modifications in V299M-, L280I/V299M-, and C164V/V299C-FVIIa are tolerated and leave FVIIa in a functionally native state, apart from a slightly lower amidolytic activity in one case. We also investigated whether the mutations affect the exposure of the N-terminus of the protease domain. The susceptibility to chemical modification (carbamylation) by potassium cyanate, measured as a reduction in amidolytic activity, was similar in L280I/V299M-FVIIa, C164V/V299C-FVIIa, and wild-type FVIIa. All three molecular species retained between 33 and 40% of the activity following incubation for 1 h. This indicates that the mutations do not alter the propensity of the N-terminal amino group to form a salt bridge with the side chain of Asp-343.

FVIIa·TF Interaction and Allostery. The functional state of the variants appeared to be unaltered as judged from the enzymatic activity measurements and as inferred from the carbamylation data. To further corroborate that the mutants were in a conformation, or functional state, similar to that of wild-type FVIIa, we studied TF binding, the ability of sTF to stimulate the amidolytic activity, and the effect of active site inhibitor incorporation on the affinity for sTF. After complex formation with sTF, all three FVIIa variants achieved an activity similar to that of wild-type FVIIa (Table 2). This suggests that sTF binding is able to correct for the slight functional impairment of free C164V/V299C-FVIIa. As measured by SPR, L280I/V299M-FVIIa and C164V/V299C-FVIIa bound sTF with K_d values of 2.6 and 4.2 nM, respectively. These values are similar to that obtained with wild-type FVIIa (3.4 nM), indicating that the TF binding region is indistinguishable in the three forms of FVIIa and neither perturbed nor improved by the mutations. Incorporation of active site inhibitor FFR-ck is known to increase the affinity of FVIIa for sTF, lowering K_d to <1 nM (*21*, *22*). We here find that the interaction between FFR-FVIIa and sTF is characterized by a K_d of 0.5 nM, whereas L280I/V299M-FFR-FVIIa and C164V/V299C-FFR-FVIIa bind sTF with K_d values of 0.8 and 0.9 nM, respectively. The indistinguishable activities in complex with sTF and similar affinities for sTF after incorporation of FFR-ck strongly suggest that the same allostery occurs in all three species of FVIIa.

Table 2: Chromogenic Substrate Hydrolysis by FVIIa Variants in the Presence of sTF

enzyme	K_m (mM)	v_{max} (mOD/min)[a]	v_{max}/K_m (mOD mM^{-1} min^{-1})
FVIIa	1.5 ± 0.1	41 ± 1	27.3 ± 2.5
V299M-FVIIa	2.3 ± 0.2	50 ± 2	21.7 ± 2.8
L280I/V299M-FVIIa	2.0 ± 0.2	47 ± 2	23.5 ± 3.3
C164V/V299C-FVIIa	2.2 ± 0.2	39 ± 2	17.7 ± 2.5

[a] Values are means ± the standard error of the mean (n = 2).

DISCUSSION

Enzymatically active FVIIa adopts a conformation which is virtually identical to that of trypsin (*18*). Hence, the structure of the activation domain and the active site region, as well as the overall topology, are conserved as judged from crystal structures. In contrast, large differences are observed between the structures of zymogen FVII (*23*) and trypsinogen (*4*). The activation domain of FVII is quite different from that of trypsinogen, and in particular, large differences are observed in the TF binding region resulting from the reregistration of β strand B2. On the basis of the structure of FVII, it was suggested that binding of TF to FVIIa promotes a three-residue shift of β strand B2 for attainment of the enzymatically active conformation (*23*). The Leu-X-Val repeats were hypothesized to favor the reregistration (Figure 4). One remaining question is whether the structure of FVII resembles or corresponds to the latent structure of FVIIa.

If β strand reregistration is an integral part of the transition between the zymogen-like and enzymatically active conformations, locking β strand B2 in the "active position" should result in FVIIa activity enhancement. To investigate this possibility, appropriate mutations aimed at locking the protease domain in the conformation seen in active site-inhibited FVIIa complexed with TF were introduced (*18*).

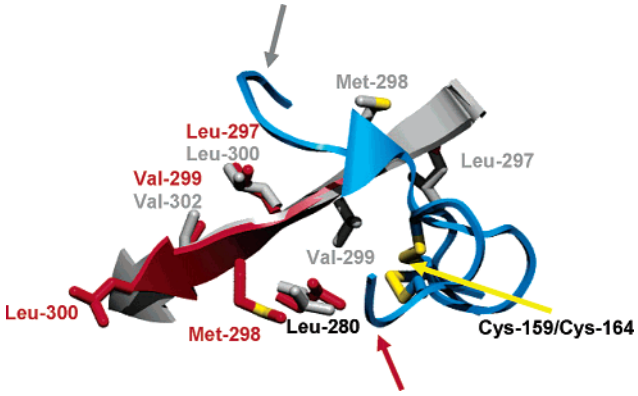


FIGURE 4: Reregistration of β strand B2. Superposition of residues 296−304 in gray comprising β strand B2 of the active FVIIa conformation and residues 294−300 in red comprising β strand B2 of the zymogen FVII conformation. Shown in blue are parts of the N-termini: residues 153 (gray arrow) through 165 of active FVIIa and residues 157 (red arrow) through 165 of zymogen FVII. The position of the disulfide bridge between Cys-159 and Cys-164 is virtually identical in the two structures (yellow arrow). As a consequence of the reregistration, the position of the Cα atom of Met-298 in the active FVIIa conformation (gray) has shifted 11.5 Å relative to the position in the zymogen conformation (red). The same shift is observed for the leucine and valine residues in the Leu-Met-Val-Leu-Asn-Val (residues 297−302) motifs.

The effects of the mutations on the intrinsic properties of FVIIa were assessed in activity assays, TF binding, and N-terminus accessibility measurements capable of capturing the impact on the active site, the macromolecular substrate exosite, and the TF-interactive region. The influence of the mutations on the allosteric effects of TF and active site occupancy were also studied in terms of cofactor-mediated activity enhancement and an inhibitor-induced TF affinity increase.

The first attempt to stabilize the enzymatically active FVIIa conformation by mutagenesis was based on the idea that disruption of the first of two Leu-X-Val repeats would prevent the transition from an active to a zymogen-like conformation. To accomplish this, valine in position 299 was replaced with methionine. Methionine would presumably experience a steric clash if the β strand shifted by three residues, in particular to the side chains constituting the cavity surrounding Val-302 in the active form of FVIIa (*18*). To test this hypothesis, the V299M mutation was introduced *in silico* into the zymogen structure (see Experimental Procedures). Because of the bulkiness of the methionine, several neighboring main chain and side chain atoms moved relative to their positions in the native zymogen structure, e.g., main chain atoms in the loop of residues 331−333 (1.4 Å) and side chain atoms of Leu-297 and Tyr-377 (1.5 Å). The hydrogen bonds stabilizing the β sheet were however intact. The *in silico* V299M mutation led to much smaller structural changes in the enzymatically active conformation; i.e., only close contacts with Leu-280 were observed. Hence, the observation that V299M disturbs the zymogen structure but only marginally changes the active conformation points to the conclusion that the V299M mutation favors the latter conformation. In this context, it is worth mentioning that a comparison of the two native structures reveals that the solvent exposure of nonpolar side chains is much larger in the zymogen structure than in the active structure. Likewise, polar side chains tend to be less solvent exposed in the zymogen structure than in the enzymatically competent structure (*23*). Thus, the active conformation seems to be favored which points to the suggestion that the dramatic changes in the sTF region of the zymogen structure compared to the active structure are promoted by the presence of the allosteric inhibitor. The amidolytic activity of free and TF-bound V299M-FVIIa, as well as its intrinsic proteolytic activity, was found to be similar to that of wild-type FVIIa. It is conceivable that the (only) normal activity was due to a clash between the introduced Met in position 299, when in the "active position", and Leu-280 and/or the disulfide bridge between Cys-159 and Cys-164, resulting in a slight misalignment of the insertion of the N-terminus into the activation pocket (Figure 3). To rule out this possibility, the L280I mutation was also introduced. The double mutant L280I/V299M-FVIIa resembles, for instance, FXa in these two positions. However, L280I/V299M-FVIIa was not significantly different from FVIIa and V299M-FVIIa in terms of proteolytic and amidolytic activity. Moreover, the rate of N-terminal carbamylation is indistinguishable from that of FVIIa. Our results suggest that the free forms of wild-type FVIIa, V299M-FVIIa, and L280I/V299M-FVIIa are in the same conformational, and consequently activity, state and that TF exerts the same allosteric stimulatory effect. In addition, the finding that TF has the same affinity for FVIIa and L280I/V299M-FVIIa, both before and after chloromethyl ketone inhibition, strongly suggests that the TF binding region is unaltered in the mutant and does not lend support to differences in relative β strand positions. In other words, the disruption of the first Leu-X-Val repeat does not appear to affect the TF binding region. This can be explained in two ways. Either the β strand reregistration occurs despite the V299M mutation, or the β strand shift is not an integral part of the transition between the active and zymogen-like conformations of FVIIa. It is noteworthy that a comparison of available FVII sequences from various species shows that only human FVII contains two consecutive Leu-X-Val repeats. In the third position of the first repeat, one occasionally finds serine or alanine, while the first position of the second repeat sometimes is occupied by isoleucine or valine. Even though this observation can support both explanations, it is likely that FVIIa in all species employs the same activation mechanism, suggesting that the three-residue shift does not play a role in the transition to and from a more active conformation.

To corroborate our findings regarding the relevance of the β strand shift, we decided to construct a FVIIa variant with a dramatically restricted neighborhood around the β strand. Cys-159 participates in two different disulfide bridge arrangements in the trypsin family of serine proteases. FVIIa (and FXa) contains the Cys-159−Cys-164 disulfide, while the others (e.g., trypsin) contain a bridge between Cys-159 and Cys-299. On the basis of the notion that the different patterns do exist, as well as on the basis of modeling of the latter disulfide pattern into the FVIIa structure (Figure 3), we constructed C164V/V299C-FVIIa. Importantly, the position of the Cys-159−Cys-164 disulfide is quite similar in the zymogen FVII structure and in the enzymatically active structure of FVIIa (Figure 4). Hence, the arrangement of hydrophobic side chains (in particular, Pro-165 and Trp-166) surrounding the Cys-159−Cys-164 disulfide is conserved in the two structures. The buried side chains of Pro-165 and

Trp-166 are stabilized via hydrophobic interactions to structurally conserved light chain residues Ile-138 and Pro-139 and to Trp-356 in the protease domain. The aliphatic part of the Glu-163 side chain in the enzymatically competent structure interacts with light chain residues Ile-140 and Leu-141. Because of coiling of the N-terminus in the zymogen structure, this interaction is taken up by Pro-160 which stabilizes the zymogen conformation. Again, modeling experiments were performed to elucidate the impact of the C164V and V299C mutations on the zymogen structure. The altered disulfide pattern has consequences for key interactions seen in the zymogen FVII structure. First, the Leu-X-Val repeat has been more profoundly affected than in the L280I/V299M mutant because in the alleged latent conformation the disulfide bridge and parts of the N-terminal tail have to fit the cavity occupied by Val-302 in the active conformation. Second, the hydrogen bonds involving the carboxylate of Glu-296, which were suggested to be the major reason for the stabilization of the zymogen structure (*23*), are no longer feasible because the donor atoms (the main chain amide nitrogens of Cys-159 and Pro-160) have been translated 10 Å due to the β strand shift. Similarly, the remaining part of Pro-160 must translate at least 10 Å due to the β strand shift, thereby losing the stabilizing interaction with Ile-140 and Leu-141. However, the most dramatic effect of the new disulfide is that buried residues Pro-165 and Trp-166 also must translate to maintain the β strand shift. The notion that the structural arrangement of these buried residues is conserved in the two structures makes us suggest that the β strand shift is highly unlikely to occur in C164V/V299C-FVIIa. The formation of the new disulfide was verified by means of mass spectroscopy, and the intrinsic amidolytic activity of C164V/V299C-FVIIa was approximately half of that of wild-type FVIIa, whereas the proteolytic activity was normal. Remodeling of a macromolecular substrate exosite has perhaps compensated for a less mature active site. In addition, sTF largely restored the amidolytic activity to normal (Tables 1 and 2). The observed reduction in amidolytic activity is most likely due to a minor rearrangement of the N-terminus which affects its insertion. However, this is barely supported by the carbamylation data. The fact that sTF binds with unaltered affinity to the mutant, also after incorporation of an active site inhibitor, and restores the activity points to the conclusion that the TF binding region is unperturbed. In a recent study (*30*), the binding of a panel of 12 sTF mutants to zymogen FVII and FVIIa was evaluated. Albeit altered, the affinities for FVII and FVIIa were comparable, and it was concluded that zymogen FVII and FVIIa interact with sTF in a nearly identical fashion. These data can be interpreted in two ways; either the zymogen FVII can readily adopt a FVIIa-like conformation (to bind sTF), or the structural difference between zymogen FVII and active FVIIa is subtle.

The body of data obtained with C164V/V299C-FVIIa points to the same conclusion as for L280I/V299M-FVIIa, namely, that the equilibrium between the zymogen-like and active conformations of the mutant is indistinguishable from that of FVIIa. In particular, the mutants showed no signs of increased intrinsic activity as anticipated if the β strand positioning is important for the activity. These experimental findings, in combination with the structural constraints introduced by the mutations preventing β strand reregistration, make us conclude that the dramatic structural differences in terms of β strand positioning between FVII and FVIIa (TF-bound and/or active site-inhibited) are not involved (or do not suffice) in the mechanism that governs the activity state of FVIIa. In contrast, our data raise the possibility that the structural differences between the latent (zymogen-like) and active structures of FVIIa resemble those between trypsinogen and trypsin. Hence, we expect the structural differences between the two conformations of FVIIa to be confined to the activation domain, including the N-terminal insertion pocket. We hypothesize that the allosteric effects of TF only materialize in subtle conformational changes. Small structural changes with a dramatic impact on enzymatic activity have been elucidated for thrombin (*31*), where concerted movements of the Cys-168c−Cys-182c (c for chymotrypsinogen numbering) disulfide bridge and the neighboring side chains of Phe-227c, Trp-215c, and Trp-60Dc constitute an allosteric switch which opens and closes the substrate binding cleft. The corresponding region of FVIIa is in the vicinity of the TF binding region, and an allosteric mechanism similar to that observed for thrombin can be responsible for the transmission of the signal from TF to the active site region of FVIIa.

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Association of pharmacokinetic (*CYP2C9*) and pharmacodynamic (*factors II, VII, IX,* and *X*; *proteins S* and *C*; and *γ-glutamyl carboxylase*) gene variants with warfarin sensitivity

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We analyzed mutations of 7 vitamin K–dependent protein and cytochrome P450 2C9 genes in 45 patients and investigated whether any contribute to the large interpatient variability in the warfarin dose-effect relationship. Total clearance and daily dose, INR and INR/Cp, were used as pharmacokinetic and pharmacodynamic indexes, respectively. Patients were grouped by genotype based on a single polymorphism and combinations of polymorphisms. Among the 30 sequence variants identified, *CYP2C9*3*, *165Thr → Met* of the *factor II* gene, *−402G → A*, *(37-bp repeat)$_n$*, and *−746T → C* of the *factor VII* gene, and *(CAA repeat)$_n$* of the *γ-glutamyl carboxylase* gene were selected as candidate polymorphisms. As the analysis of single polymorphisms implied, the highest INR/Cp mean values and the lowest warfarin maintenance doses were observed in patients homozygous for the *165Met*, *−402G*, *(37-bp repeat)$_6$* and *−746T* alleles. Multiple regression analysis revealed that warfarin sensitivity was independently associated with *−402G → A*, *(CAA repeat)$_n$*, *CYP2C9*3*, and *165Thr → Met*, which accounted for 50% of variance. These results suggest that part of the considerable interpatient variation is attributable to genetic variation, and the combined genotyping of *CYP2C9* and certain vitamin K–dependent protein genes is useful for predicting anticoagulant responses. (Blood. 2004;103: 2630-2635)

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Introduction

Warfarin is the most widely prescribed anticoagulant drug for the prevention and treatment of arterial and venous thromboembolic disorders.[1-3] Because of large interpatient variability in the dose-anticoagulant effect relationship and a narrow therapeutic index,[4] careful dosage adjustment based on INR (prothrombin time expressed as international normalized ratio) is essential. Warfarin is available as a racemic mixture of 2 enantiomers, (S)- and (R)-warfarin. In contrast to (R)-warfarin, which is metabolized by multiple cytochrome P450s (CYPs), including CYP1A2 and CYP3A4, (S)-warfarin is predominantly metabolized to 7-hydroxywarfarin by polymorphic CYP2C9.[5,6] Since the potency of (S)-warfarin is much higher than that of (R)-warfarin, about 3- to 5-fold, any change in the activity of CYP2C9 is likely to have a significant influence on the anticoagulant response.[4,7] Previous in vitro and in vivo findings revealed that certain variants in the *CYP2C9* gene are associated with large interindividual differences in the pharmacokinetic and pharmacodynamic outcomes of warfarin therapy.[8-11] Three major alleles have been found to date in humans: *Arg144/Ile359*, *Cys144/Ile359*, and *Arg144/Leu359*, which have been designated *CYP2C9*1* (wild-type or reference allele), *CYP2C9*2*, and *CYP2C9*3*, respectively, by the Nomenclature Committee.[7] Patients having at least one *CYP2C9*3* allele require a lower dose of warfarin on average than homozygotes for the *CYP2C9*1* allele due to an impaired metabolic capacity.[4,7] However, the *CYP2C9*3* allele provides important genetic information for the use of warfarin: the frequency of the allele is extremely low (~2%) in Japanese subjects, and large interpatient variability in the dose-anticoagulant effect relationship remains even when the pharmacogenetic concept of *CYP2C9* is introduced into the clinical setting. These findings suggest that additional factor(s) contribute to the relationship.

Warfarin functions as a vitamin K antagonist, decreasing the activities of the vitamin K–dependent procoagulant components.[12] These proteins include factor II (prothrombin), VII, IX, and X as well as the coagulation inhibitors protein C and protein S. In addition, γ-glutamyl carboxylase (GGC) is a key enzyme in the production of γ-carboxyglutamate, which has a profound influence on the normal function of vitamin K–dependent proteins.[12,13] Thus, it is hypothesized that functional polymorphisms of the genes encoding these components are associated with the large variability in the warfarin dose-effect relationship.

Despite the identification of variants of vitamin K–dependent protein genes that cause bleeding disorders, such as hereditary deficiencies of proteins,[14-17] no study has examined the contributions of variants to the warfarin dose-effect relationship. The initial aim of this study was the identification of polymorphisms of 7 genes of interest (those for *factors II, VII, IX,* and *X*; *proteins C* and *S*; and *GGC*). The second and major aim of this study was to evaluate the impact of variants in 8 genes (including *CYP2C9*) on the pharmacokinetic and pharmacodynamic outcomes of warfarin therapy.

Patients, materials, and methods

The investigation was approved by the Review Board of Tottori University Hospital, and all subjects gave informed consent to participate in this study.

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Patients

Forty-five unrelated patients (27 males and 18 females; mean age, 63.1 years; age range, 35 to 82 years) treated at the Department of Second Surgery, Tottori University Hospital, participated. They received warfarin therapy for the prevention or treatment of thromboemboric diseases (valve replacements, atrial fibrillation, and ischemic heart failure) with fixed maintenance doses for at least 2 months before the study. The average warfarin dose and INR were 3.8 mg/d (range, 1 to 8.5 mg/d) and 2.32 (range, 1.52 to 3.84), respectively. Biochemical and hematologic tests performed before the study revealed no evidence of hepatic or renal impairments. Because the patients were elderly, some of them also were taking other drugs; however, none of the patients were taking any medications that might have interfered with the pharmacokinetics or pharmacodynamics of warfarin.[18,19]

Study protocol and clinical data collection

A single nonfasting blood sample (approximately 10 mL) was obtained from each patient, just before the morning dose of warfarin. Each blood sample was divided into 3 portions: (1) for measuring concentrations of warfarin enantiomers in plasma, (2) for determining INR, and (3) for extracting DNA for genotyping.

Data collection consisted of a review of inpatient and outpatient medical records. We used the electronic medical database available in the hospital to obtain precise information on the INR value, the warfarin daily dose, type of prescription drugs, and bleeding events. We collected these data prospectively for each patient for at least 7 months from the day the sample was collected.

Assay of (S)- and (R)-warfarin plasma concentrations

(S)- and (R)-warfarin concentrations in serum were measured by high performance liquid chromatography (HPLC).[20] Internal standard [diclofenac, 100 μL of a 10 μg/mL solution in a 10/90 (vol/vol) mixture of isopropanol-hexan solution], 1 mL of HCL (2 N), and 5 mL of ether were added to a 0.5-mL plasma sample. The mixture was shaken for 10 minutes and then centrifuged at 1500*g* for 10 minutes. A 4-mL aliquot of the organic layer was transferred to a glass tube and evaporated to dryness under a stream of nitrogen. The residue was reconstituted with 200 μL of 10% (vol/vol) isopropanol in hexane, and 40 μL of the solution was then injected into the HPLC system. The sensitivity of the assay of both compounds was 20 ng/mL. The coefficient of the intra- and interassay variation was within 10%. Recovery of both compounds ranged from 95% to 102%. A Shimadzu LC-10A system (Shimadzu, Kyoto, Japan) equipped with an LC-10AS pump and an ultraviolet detector (SPD-10A) was used. A Chiralcel OD analytical column (10 μm, 250Å~4.6 mm ID, Daicel, Tokyo, Japan) coupled with a Chiralcel OD guard column (50 × 4.6 mm ID) was used with a mobile phase that consisted of a 18/0.5/81.5 (vol/vol) mixture of isopropanol, acetic acid, and hexane delivered at a flow rate of 1.2 mL/min. The column temperature was maintained at 25°C.

Identification of variants in vitamin K–dependent proteins, proteins C and S, and GGC genes

Genomic deoxyribonucleic acid (DNA) was isolated from blood samples using the Toyobo blood kit on a Toyobo HMX-2000 robot (Toyobo, Osaka, Japan). The primer design was based on the sequence of the 5′-flanking region and/or the intron/exon junction of the 7 genes of interest. Almost all of the primers were designed to divide all 14 exons of the *factor II* gene, the promoter region, and 8 exons of the *factor VII* gene, 8 exons of the *factor IX* gene, 8 exons of the *factor X* gene, 15 exons and intron 6 of the *GGC* gene, 15 exons of the *protein S* gene, and the promoter region and 9 exons of the *protein C* gene into fragments of approximately 350 bp, for the screening of mutations by subsequent single-strand conformation polymorphism (SSCP) analysis. Fragments over 350 bp in length were digested with appropriate restriction enzymes prior to SSCP analysis.

SSCP analysis was performed using the GenePhor system (Amersham Pharmacia Biotech AB, Uppsala, Sweden) as recommended by the manufacturer. The polymerase chain reaction (PCR) product (3-6 μL) was mixed with 5 μL of 20 mM EDTA (ethylenediaminetetraacetic acid), 95% formamide, and 0.05% bromophenol blue, and this mixture was heated at 95°C for 5 minutes and then quick-chilled in an ice-water bath. The resulting single-stranded DNA (5.5 μL) was then loaded on a 12.5% polyacrylamide gel (GeneGel excel 12.5/24 kit; Amersham Pharmacia Biotech AB). Electrophoresis was carried out at 450V of constant power at 15°C for 2 to 4 hours, depending on the fragment size. After electrophoresis, gels were stained using an automated gel stainer with PlusOne (Amersham Pharmacia Biotech AB).

DNA sequence

PCR products were sequenced either directly or after subcloning on an ABI 3100 automatic sequencer (Applied Biosystems, Foster City, CA) using a Big-Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). If the direct sequencing was incomplete, each amplified PCR product was subcloned into the vector pGEM (Promega, Madison, WI) and introduced into competent JM109 cells (Promega). Prior to the sequencing, reaction mixtures were purified with a DyeEx Spin kit (QUIAGEN GmbH, Hilden, Germany). The sequencing primers were those used in the PCR amplifications.

Genotyping

Genotyping of *CYP2C9* was performed with the Sequence Detection System (ABI PRISM 7000; Applied Biosystems) using a commercially available Taqman probe and primer set (Applied Biosystems).

Pharmacokinetic analysis

Apparent oral clearance (CL, mL/kg/min) of the respective warfarin enantiomers was used as an index for pharmacokinetic evaluation and was calculated as follows: dose/2 (mg/kg)/[Plasma (S)– or (R)–warfarin concentration (Cp, μg/mL) × 1000/1440], in which dose/2 is the daily dose of respective warfarin enantiomers. We also calculated the INR response per warfarin plasma concentration, termed the warfarin sensitivity index (INR/Cp).

Statistical analysis

All data are presented as the mean ± standard deviation (SD). The statistical differences between 2 groups were evaluated using Student *t* test and between more than 2 groups using ANOVA (with Tukey-Kramer multiple comparison test), as appropriate. A χ^2 test was used to compare the allele frequency of each variant with that expected for a population in Hardy-Weinberg equilibrium. Stepwise multiple regression analysis was used to determine the relative effects of candidate gene polymorphisms on warfarin sensitivity. Each mutation was treated as a categorical value (eg, homozygote for the reference allele = 0; heterozygote for the mutant allele = 1; and homozygote for the mutant allele = 2). A *P* value less than .05 was taken to be the minimum level of statistical significance.

Results

Variants of vitamin K–dependent protein genes

Before the functional characterization, variants in 7 genes of interest were identified by SSCP analysis. Altogether, over 90 PCRs were performed for each patient. The results are summarized in Table 1. Of the 29 sequence variants identified, 26 were single nucleotide polymorphisms (SNPs) and 3 were insertions: the decanucleotide insertion *[(cctatatcct)$_{0\rightarrow1}$]* at position −323 (promoter region) and *(37-base pair repeat)$_{6\rightarrow7}$* in intron 7 of the *factor VII* gene, and *(CAA repeat)$_{10\rightarrow11\ or\ 13}$* in intron 6 of the *GGC* gene. Allele frequencies of these insertions were as follows: *−323(cctatatcct)$_{0/1}$* (0.94/0.06), *(37-bp repeat)$_{6/7}$* (0.80/0.20), and *(CAA repeat)$_{10/11/13}$* (0.62/0.34/0.03). The frequency of the *(37-bp repeat)$_{6\rightarrow7}$* of the *factor VII* gene deviated significantly from the

Table 1. Genetic variants of the 7 vitamin K-dependent proteins in our study population

Gene	Location	Position*	Nucleotide sequence		Amino acid substitution	Genotype			Allele frequency	
			Reference (Re)	Mutant (Mu)		Re/Re	Re/Mu	Mu/Mu	Re	Mu
Factor II										
(Prothrombin)	intron 4	+36	acatGgagg	acatAgagg	—	36	9	0	0.90	0.10
	intron 5	+90	tcctCttcc	tcctGttcc	—	3	19	23	0.28	0.72
	exon 6	494	accaCggga	accaTggga	*Thr165Met*	8	18	19	0.38	0.62
	intron 7	−69	ggaaCtggg	ggaaTtggg	—	19	18	8	0.62	0.38
	exon 12	1602	ggccGgtct	ggccAgtct	*Pro534Pro*	36	9	0	0.90	0.10
	intron 13	+88	gcagAggaa	gcagGggaa	—	1	8	36	0.11	0.89
Factor VII	promoter	−402	atacGgtct	atacAgtct	—	14	22	9	0.56	0.44
	promoter	−401	tacgGtctt	tacgTtctt	—	40	5	0	0.94	0.06
	promoter	−323	$(cctatatcct)_0$	$(cctatatcct)_1$	—	40	5	0	0.94	0.06
	promoter	−122	ggtgTtcag	ggtgCtcag	—	40	5	0	0.94	0.06
	intron 4	−58	ggggGctgg	ggggActgg	—	40	5	0	0.94	0.06
	exon 5	525	gccaCgagg	gccaTgagg	*His115His*	40	5	0	0.94	0.06
	exon 5	549	cagaCgggg	cagaTgggg	*Asp123Asp*	44	1	0	0.99	0.01
	intron 7	−788	$(37\text{-bp repeat})_6$	$(37\text{-bp repeat})_7$	—	36	0	9	0.80	0.20
	intron 7	−746	ctctTccct	ctctCccct	—	16	20	9	0.58	0.42
	intron 7	−206	ctccGctgt	ctccActgt	—	41	1	3	0.92	0.08
	intron 7	−20	ctgaGgggg	ctgaAgggg	—	41	4	0	0.96	0.04
	exon 8	1238	taccGgggc	taccAgggc	*Arg353Gln*	41	4	0	0.96	0.04
Factor IX	intron 8	+1401	ctttTtgtg	ctttCtgtg	—	40	3	2	0.92	0.08
Factor X	exon 7	792	gaacCattc	gaacTattc	*Thr264Thr*	3	21	21	0.30	0.70
Protein C	exon 9	891	ccgaCaatg	ccgaTaatg	*Asp297Asp*	43	2	0	0.98	0.02
Protein S	intron 12	+3	gtaaTagat	gtaaCagat	—	32	12	1	0.84	0.16
	exon 15	2001	gtccAtcag	gtccGtcag	*Pro667Pro*	7	20	18	0.38	0.62
GGC	intron 2	−67	gtgcAgtga	gtgcTgtga	—	21	23	1	0.72	0.28
	intron 2	−33	ccccGcaca	ccccAcaca	—	31	14	0	0.84	0.16
	intron 6	−152	$(CAA)_{10}$	$(CAA)_{11}$	—	15	24	3	0.62	0.34
		−152		$(CAA)_{13}$	—		3	0		0.03
	exon 9	1218	cccgTtccc	cccgCtccc	*Arg406Arg*	4	21	20	0.32	0.68
	exon 9	1242	tcacCtacc	tcacTtacc	*Thr414Thr*	40	5	0	0.94	0.06
	exon 10	1378	taatGtcac	taatAtcac	*Val460Ile*	43	2	0	0.98	0.02

The reference allele for each gene had the following GenBank accession number; M17262 for *factor II*; J02933 for *factor VII*; K02402 for *factor IX*; AH002727 for *factor X*; M11228 for *protein C*; NT_022625 and M57853 for *protein S*; U65896 for *GGC*. — indicates synonymous mutation.

*Position is relative to the ATG start site except for the *factor VII* gene. In the *factor VII* gene, residue +1 is the first amino acid of the mature protein.[33]

expected Hardy-Weinberg distribution ($P < .05$). Interestingly, 3 mutations in the promoter region of the *factor VII* gene (*−401G → T*, *−323(cctatatcct)$_{0/1}$*, and *−122T → C*) occurred simultaneously, indicating a haplotype; *−401G-323(cctatatcct)$_0$-122T* (haplotype A1) and *−401T-323(cctatatcct)$_1$-122C* (haplotype A2). Of the 26 SNPs, 15 were located in noncoding regions, 8 were synonymous mutations, and 3 were nonsynonymous mutations. Three nonsynonymous mutations, a cytosine-to-thymine substitution at nucleotide 494 *(494C → T)* in the *factor II* gene, *1238G → A* in the *factor VII* gene, and *1378G → A* in the *GGC* gene, resulted in an amino acid change from threonine to methionine at codon 165 *(165Thr → Met)*, *353Arg → Gln*, and *460Val → Ile*, with an allele frequency of 0.62, 0.04, and 0.02, respectively. By the screening of databases, *460Val → Ile* was identified as a novel variant.

Effect of genetic polymorphisms on the pharmacokinetic and pharmacodynamic outcomes of warfarin therapy

CYP2C9 *gene.* Among the 45 patients, 2 were heterozygous carriers for the *CYP2C9*3* allele. Allele frequencies for *CYP2C9*1* and *CYP2C9*3* were 0.978 and 0.022, respectively, and were consistent with those of previous studies.[4,21] As shown in Figure 1A, although the time course of change in mean INR values did not differ between the 2 genotype groups, the mean warfarin daily dose was 40% lower in the *CYP2C9*3* group than in the reference *CYP2C9*1* group throughout the observation period. When pharma-

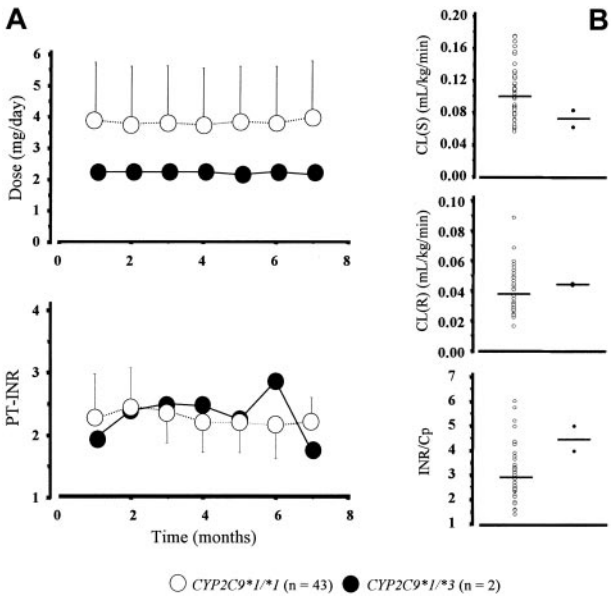


Figure 1. Polymorphisms of the *CYP2C9* gene and in vivo pharmacodynamic and pharmacokinetic outcomes of warfarin. (A) Time course of changes in warfarin daily dose and INR values during the observation period (7 months) in patients with and without the *CYP2C9*3* mutant allele. (B) Comparisons of warfarin clearance and INR/Cp between the 2 *CYP2C9* genotypic groups. Bars represent the mean.

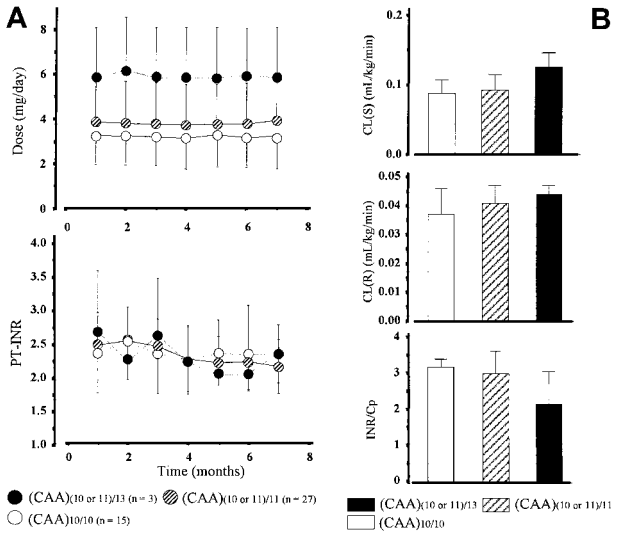


Figure 2. Polymorphisms of the *γ-glutamylcarboxylase* gene (*CAA repeats* in intron 6) and in vivo pharmacodynamic and pharmacokinetic outcomes of warfarin. (A) Time course of changes in warfarin daily dose and INR values during the observation period (7 months) in patients with various patterns of the microsatellite *(CAA repeat)* in the *GGC* gene. (B) Comparisons of warfarin clearance and INR/Cp among the 3 *GGC* genotypic groups. Bars represent the mean ± SD.

cokinetic backgrounds were compared between the 2 groups, mean plasma (S)-warfarin clearance was found to be 33% lower in the *CYP2C9*3* group. On the other hand, the mean INR/Cp value was 43% higher in the *CYP2C9*3* group (Figure 1B).

GGC *gene.* We divided patients into 3 groups with regard to the number of microsatellites in intron 6: $(CAA)_{10/10}$ (n = 15), $(CAA)_{(10\ or\ 11)/11}$ (n = 27), and $(CAA)_{(10\ or\ 11)/13}$ (n = 3). The mean daily dose of warfarin increased together with the number of microsatellites at comparable mean INR values in the 3 genotype groups (Figure 2A). However, the increase did not reflect an elevation in plasma warfarin clearance, because these values did not differ significantly among the 3 groups. The mean INR/Cp value was almost 32% lower in patients with a $(CAA)_{13}$ allele than those homozygous for $(CAA)_{10}$, a reference allele, and patients heterozygous for the $(CAA)_{11}$ allele having an intermediate value (Figure 2B).

Other genes. The contributions of the mutations in other vitamin K–dependent protein genes to mean INR/Cp values are shown in Table 2. In the *factor II* gene, homozygotes for the nonsynonymous mutant allele (*494T*, tentatively designated the *M2* allele, *M2/M2* genotype) had significantly higher mean INR/Cp values than patients having a reference allele (*494C*, *M1* allele) with homozygosity (*M1/M1* genotype). In contrast, homozygous carriers of the *−402A* allele (*B2* allele, *B2/B2* genotype) of the factor VII gene had significantly lower mean INR/Cp values than those of the reference allele (*−402G*, *B1* allele, *B1/B1* genotype), and heterozygotes (*B1/B2* genotype) had values intermediate between those of the 2 homozygous groups. Similarly, homozygous carriers for the $(37\ bp\ repeat)_7$ (*b/b* genotype) or *−746C* allele (*N2/N2* genotype) in the *factor VII* gene had lower mean INR/Cp values than those for the $(37\ bp\ repeat)_6$ (*a/a* genotype) or *−746T* allele (*N1/N1* genotype). Taking these findings into consideration, all of the mutations in the vitamin K–dependent protein genes except *494C → T* in *factor II* were associated with a lower INR/Cp value, suggesting reduced warfarin sensitivity. In contrast to the INR/Cp values, there were no significant differences in the warfarin daily doses for each genotype.

Further analysis was performed with these 4 mutations combined. Combining the 4 polymorphisms allowed around 60% of the population to be grouped in 5 genotypes (I through V in Table 2), each found in at least 3 subjects. As the analysis of single polymorphisms implies, the highest INR/Cp mean values were observed in patients homozygous for the *M2*, *B1*, and *N1* alleles (genotype IV), whereas the lowest values were associated with the combined genotypes that include homozygous *M1*, *B2*, and *N2* alleles (genotype I). Patients with genotype IV had mean INR/Cp values (4.48 ± 0.83) 2 times as high as patients with genotype I (2.14 ± 0.31). Furthermore, patients with genotype IV had the lowest mean warfarin daily dose (2.3 ± 1.4) among the 5 genotypic groups: the mean warfarin dose was 1.3 to 2.3 times lower in patients with genotype IV than patients in other groups.

In order to assess the contributions of all selected candidate gene polymorphisms to warfarin sensitivity, a stepwise multiple regression analysis was carried out. In the analysis, the only variables that were significantly and independently associated with warfarin sensitivity were *−402G → A* in the *factor VII* gene

Table 2. Mean INR/Cp values and warfarin daily dose in various genotypes determined from single and multiple gene polymorphisms

Gene	Allele	Genotype	n	INR/Cp	Warfarin dose, mg (range)
Factor II	*494C (M1)*	*M1/M1*	8	2.62 ± 0.54	3.3 ± 1.1 (1.5-5.0)
	494T (M2)	*M1/M2*	18	3.00 ± 0.39	3.9 ± 1.8 (1.5-7.5)
		M2/M2	19	3.26 ± 0.18*	3.8 ± 2.1 (1.0-8.5)
Factor VII	*−402G (B1)*	*B1/B1*	14	3.42 ± 0.82	3.5 ± 2.0 (1.5-8.0)
	−402A (B2)	*B1/B2*	22	3.09 ± 0.43†	3.9 ± 1.9 (1.0-8.5)
		B2/B2	9	2.39 ± 0.18*	3.8 ± 1.3 (1.5-6.0)
	(37-bp repeat)$_6$ (a)	*a/a*	36	3.14 ± 0.55	3.8 ± 1.9 (1.0-8.5)
	(37-bp repeat)$_7$ (b)	*b/b*	9	2.58 ± 0.21	3.5 ± 1.3 (1.5-5.0)
	−746T (N1)	*N1/N1*	16	3.34 ± 0.66	3.6 ± 1.9 (1.5-8.0)
	−746C (N2)	*N1/N2*	20	3.00 ± 0.51	4.0 ± 2.0 (1.0-8.5)
		N2/N2	9	2.58 ± 0.21*	4.0 ± 1.3 (1.5-5.0)
Combination	I	*(M1/M1)(B2/B2)(b/b)(N2/N2)*	5	2.14 ± 0.31	3.6 ± 1.4 (1.5-5.0)
	II	*(M1/M2)(B1/B1)(a/a)(N1/N1)*	7	3.56 ± 0.41	3.0 ± 1.3 (1.5-4.5)
	III	*(M1/M2)(B1/B2)(a/a)(N1/N2)*	3	2.63 ± 0.72	5.3 ± 2.6 (2.5-7.5)
	IV	*(M2/M2)(B1/B1)(a/a)(N1/N1)*	3	4.48 ± 0.83	2.3 ± 1.4 (1.5-4.0)
	V	*(M2/M2)(B1/B2)(a/a)(N1/N2)*	9	2.93 ± 0.41	4.3 ± 1.9 (2.5-8.5)

*$P < .05$ versus homozygotes for the reference allele.

†$P < .05$ versus homozygotes for the mutant allele.

Other genotypes of patients in groups I through V were fixed as follows: *CYP2C9*1/*1*, haplotype A1 in the *factor VII* gene, and $(CAA\ repeat)_{10\ or\ 11}$ in the *GGC* gene.

(partial $r^2 = 0.35$), *(CAA repeat)$_n$* in the *GGC* gene (partial $r^2 = 0.09$), *CYP2C9*3* (partial $r^2 = 0.04$), and *494C → T* in the *factor II* gene (partial $r^2 = 0.01$), together accounting for approximately 50% of variance (multiple $r = 0.70$). The other 2 mutations in the *factor VII* gene, *(37-bp repeat)$_n$* and *−746T → C*, had poor explanatory capacities compared with the *−402G → A* mutation (data not shown), suggesting that *−402G → A* was the main predictor for warfarin sensitivity among the 3 mutations.

Discussion

In the present study, we analyzed mutations in the genes of 7 vitamin K–dependent coagulation proteins (factor II, VII, IX, and X; protein C and S; and GGC) in 45 patients, and then investigated whether these mutations contribute to the large interpatient variability in the dose-anticoagulant effect relationship. In addition to *CYP2C9*3*, a variant associated with a decrease in hepatic metabolism and an increase in sensitivity to warfarin, *494C → T (165thr → Met)* of the *factor II* gene, *−402G → A*, *(37-bp repeat)$_n$*, and *−746T → C* of the *factor VII* gene, and *(CAA repeat)$_n$* of the *GGC* gene were selected as candidate polymorphisms.

Several studies have shown that certain mutations in the *factor VII* gene contribute to the variability in factor VII plasma activity; most of the data available are on the *−402G → A*, *353Arg → Gln*, and *(37-bp repeat)$_n$* polymorphisms.[22-25] Recent study revealed that the *−401T* allele was responsible for decreased transcriptional activity, whereas the *−402A* allele was associated with an increased rate of transcription thus leading to higher factor VII levels.[22] Because of high linkage disequilibrium among certain mutations, the net influence of *(37-bp repeat)$_n$* on factor VII levels might be partially masked. In order to estimate the independent contribution of the *(37-bp repeat)$_n$* polymorphism, Pinotti et al established eukaryotic cells generating various numbers of *(37-bp repeat)* and demonstrated that higher numbers of repeats were associated with higher mRNA expression levels.[25] Since warfarin is a competitive antagonist of vitamin K in the processes leading to the synthesis of factor VII (maintained in the inactive form), and since high levels of factor VII may be related to a hypercoagulable state, less sensitivity to warfarin therapy would be expected in patients with mutations leading to high factor VII levels. In contrast to these 2 polymorphisms, the *353Arg → Gln* variant in the catalytic domain was reported to be associated with an approximately 25% reduction in factor VII coagulant activity in heterozygous carriers and a 50% reduction in homozygous carriers.[26] However, no contribution of this variant to warfarin sensitivity was observed in the present study. The lack of any apparent effect may be explained by the fact that all our patients were heterozygotes.

Among various mutations identified in the present study, only the *(37-bp repeat)$_{6 \to 7}$* insertion polymorphism in the *factor VII* gene was not in Hardy-Weinberg equilibrium because of a deficit of heterozygotes. The sequence of this portion was confirmed for all patients by at least 2 independent PCR amplifications to ensure that the polymorphisms identified were not PCR-based artifacts. These results were in contrast to recent findings by Shimokata et al, who reported that approximately 42% of 380 unrelated Japanese individuals were heterozygous carriers for the *(37-bp repeat)$_7$* allele.[27] In the present study, there were no clinical differences (eg, disease state and biochemical tests) between the 2 homozygous groups (data not shown). Thus, the significance of this deviation was not clear, and further genetic analysis will be necessary to confirm this finding; however, since most mutations in the present study were in Hardy-Weinberg equilibrium, the findings obtained should be valid for the general population.

In the *GGC* gene, one novel nonsynonymous variant and 3 patterns of *(CAA) repeats* were observed. To date, at least 2 nonsynonymous mutations, *394Arg → Leu* and *501Trp → Ser*, leading to a combined congenital deficiency of all vitamin K–dependent coagulation factors, have been reported.[15,16,28] These mutations were responsible for weak procoagulant activity, and oral vitamin K_1 administration was effective in resolving the clinical symptoms, indicating that carboxylase activity plays a critical role in the warfarin dose-effect relationship. As shown in Figure 2A, the mean daily dose of warfarin increased as the number of microsatellites increased even when mean INR values were comparable among the 3 genotype groups. Patients with 13 repeats *[(CAA)$_{13}$]* had the lowest mean INR/Cp values, suggesting the possibility of reduced sensitivity to warfarin in these patients. Theoretically, the large dose of warfarin necessary to reach the target INR value in these patients may, at least partially, be explained by increased GGC activity. However, there is no data to support this hypothesis, and more in vivo and in vitro experiments are required to elucidate the role of the microsatellites in carboxylation capability.

As shown in Table 2, patients with different genotypes in conjunction with 4 polymorphisms from the 2 genes showed up to 2-fold differences in mean INR/Cp values, thus part of the considerable interpatient variation in the dose-effect relationship was attributable to genetic variation, which may be of assistance in the interpretation of INR/Cp levels on an individual basis. It is worth noting that patients with genotype IV had the highest mean INR/Cp values among the 5 groups, suggesting high sensitivity to warfarin. Moreover, an intergenotypic difference was observed in the maintenance dose of warfarin; the mean dose was 1.3 to 2.3 times lower in patients with genotype IV than those in other genotype groups. These results were also supportive of a contribution of individual genetic status to the relationship.

Among various candidate gene polymorphisms, it is of interest which mutation(s) contributed most to, and to what extent, the overall variation in the INR/Cp values. In order to address these issues, we carried out a stepwise multiple regression analysis. Four functional polymorphisms (ie, *CYP2C9*3*, *494C → T*, *−402G → A*, and *[CAA repeat]$_n$*) together accounted for 50% of overall between-patient variability in warfarin sensitivity. In contrast to the extensive contribution of *−402G → A* and *(CAA repeat)$_n$*, *CYP2C9*3* and *494C → T* explained only approximately 5% of the variance. However, although the *CYP2C9*3* allele is extremely rare in Japanese populations (ie, 2%), it accounted for 4% of the variance in warfarin sensitivity. Since the frequency of the *3 allele is known to be higher in Caucasian subjects than in Japanese subjects,[4] the *3 allele is expected to be the major factor responsible for the between-patient variability in warfarin sensitivity in Caucasian patients. Prothrombin (factor II) is composed of 3 structural regions as follows: fragment 1, which contains the γ-carboxyglutamic acid (Gla) domain (residues 1 to 40) and kringle 1 domain (residues 41 to 155); and fragment 2, which contains mainly the kringle 2 domain (residues 156 to 271); and a serine protease precursor domain (residues 272 to 597).[29,30] Previous studies showed that the kringle 2 domain plays an important role in the binding of prothrombin to factor Xa and factor Va.[31,32] A cytosine to thymine transition at nucleotide 494 in the *factor II* gene, resulting in the substitution at codon 165 of an uncharged polar side chain amino acid "threonine" with a nonpolar side chain amino acid "methionine," is located in the kringle 2 domain of prothrombin.

The limitations of our study need to be addressed. First, we calculated the warfarin sensitivity index (INR/Cp) based on the total plasma concentration (bound plus unbound). Both warfarin enantiomers are extensively bound to plasma protein (ie, 99%), and no enantioselective differences in the binding have been observed.[4] Since only the unbound fraction can reach and/or be equilibrated with that at the site of action (eg, γ-glutamyl carboxylase), one must be careful that the interindividual variability in INR/Cp is dependent on the status of the plasma protein binding of the drug. Most studies with regard to phenotypic and genotypic correlations measured various vitamin K–dependent protein plasma levels. Unfortunately, we did not measure plasma antigenic levels and so were unable to assess the changes in protein levels due to genetic polymorphisms. An extended large population study that combines the genotyping of the 4 candidate polymorphisms with corresponding phenotype indexes should be conducted to confirm the present findings.

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Factor VII mutant V154G models a zymogen-like form of Factor VIIa

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Proteolytic cleavage of the peptide bond between Arg^{152} and Ile^{153} converts the procoagulant protein Factor VII (FVII) to an activated two-chain form (FVIIa). The formation of a salt bridge between Ile^{153} and Asp^{343} drives the conversion of FVIIa from being zymogen-like to the active form. In the present paper, we describe the novel FVII mutant V154G ($Val^{154} \rightarrow Gly$ mutation; residue 17 in the chymotrypsin numbering system), found in three FVII-deficient patients, which models a zymogen-like form of FVIIa. Recombinant V154G FVIIa, although normally cleaved, shows markedly reduced activity towards peptidyl substrate and undetectable activity towards macromolecular substrates. Susceptibility of Ile^{153} to chemical modification, in either the presence or the absence of tissue factor (TF), suggests that the reduced V154G FVIIa activity is caused by impaired salt-bridge formation, thus resulting in a zymogen-like FVIIa form. The TF-mediated protection from chemical modification of V154A indicated that Gly^{154} is responsible for this peculiar feature, and suggests that this region, proximal to the heavy chain N-terminus, is directly involved in the conversion of FVII into FVIIa. V154G FVII was exploited to study the FVII–TF interaction, together with three additional FVII variants that were expressed to serve as models for different FVII forms. The comparison of binding affinities of full-length TF after relipidation in L-α-phosphatidylcholine for the zymogen FVII ($Arg^{152} \rightarrow Gln$, $K_d = 1.04 \pm 0.27$ nM), inactive FVIIa ($Ser^{344} \rightarrow Ala$, $K_d = 0.27 \pm 0.06$ nM) and a zymogen-like FVIIa (V154G, $K_d = 1.15 \pm 0.16$ nM) supports the hypothesis that preferential binding of TF to active FVIIa is insufficient to drive the 10^5-fold enhancement of FVIIa activity. In addition, the inability of V154G FVIIa to accommodate an inhibitor in the active site, indicating an improperly shaped specificity pocket, would explain the low activity of the zymogen-like form of FVIIa, which is predominant in the absence of TF.

Key words: mutation, salt bridge, tissue factor, zymogenicity.

INTRODUCTION

The enzymic complex formed between the procoagulant serine-protease-activated Factor VII (FVIIa) and the integral membrane protein tissue factor (TF) triggers the initiation of blood clotting [1]. The action of the FVIIa–TF complex generates a burst of activated Factors IX (FIXa) and X (FXa) ultimately leading to formation of a stable fibrin clot [2,3]. *In vivo*, only approx. 1% of Factor VII (FVII) exists as FVIIa [4]. At sites of vascular injury, FVIIa interacts with TF resulting in the rapid formation of the extrinsic complex [5].

Steps involved in the conversion of FVII to a catalytically active FVIIa–TF complex are only partially understood. The initial event, cleavage of the peptide bond between Arg^{152} and Ile^{153}, converts the zymogen FVII into a two-chain serine protease that exists in equilibrium between a predominant zymogen-like form and an active FVIIa [6–9]. In the zymogen-like conformation, the new N-terminus of Ile^{153} is solvent-exposed and accessible to modification, whereas in the active FVIIa, a salt bridge forms between the α-amino group of Ile^{153} and the β-carboxyl group of Asp^{343}, stabilizing the insertion of Ile^{153} into the protein [10]. As inferred from studies on trypsinogen, the formation of this salt bridge orders the activation domain and shapes the S1 specificity pocket [11,12] (nomenclature of Schechter and Berger [13]). However, it is not well understood whether the exposure of extended macromolecular interaction sites (exosites) that are necessary for substrate recognition depends upon this rearrangement. Two-chain FVIIa shows poor activity towards both macromolecular and peptidyl substrates [14,15], possibly due to a predominant zymogen-like conformation in the absence of TF. TF dramatically enhances FVIIa activity (10^5-fold) either by preferentially binding to the active form of FVIIa or by directly driving the conversion of zymogen-like FVIIa into active FVIIa when bound to the inactive form [7,16]. TF may enhance FVIIa activity by exploiting a combination of these mechanisms.

Understanding the mechanism by which TF can boost FVIIa catalytic efficiency may provide novel insights into designing alternative molecules for therapeutic aims in haemophilia and other bleeding disorders [17,18]. Previous experimental assays used to study the binding of FVII to TF have been limited by the susceptibility of the zymogen FVII to autoactivation in the presence of TF and by inhibitors in the FVIIa active site that are known to constrain FVIIa in the active conformation [7,19].

In the present paper, we characterize a naturally occurring mutation, the substitution of glycine for Val^{154} (V154G) at the P2′ residue in the serine-protease domain of FVIIa (residue 17 in the chymotrypsin numbering system [20]). This substitution creates a peculiar model of zymogen-like FVIIa, since V154G FVIIa cannot reach the active conformation even in the presence of TF. V154G FVIIa therefore represents a 'stable' form of the FVIIa intermediate, which was exploited to study its interaction with TF compared with other FVII/FVIIa forms. The V154G FVIIa has also allowed us to analyse the competence of the

Abbreviations used: FVII, Factor VII; FVIIa, activated FVII; FVII:Ag, FVII antigen; FVII:C, FVII coagulant activity; FIX, Factor IX; FIXa, activated FIX; FX, Factor X; FXa, activated FX; ITS, insulin/transferrin/sodium selenite supplement; PC, L-α-phosphatidylcholine; PS, L-α-phosphatidylserine; PCPS, 75% (w/w) PC and 25% (w/w) PS; PEG 8000, poly(ethylene glycol) with a mean molecular mass of 8000 Da; TAP, tick anticoagulant peptide; TF, tissue factor; TF/PC, TF after relipidation in PC.

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specificity pocket in a zymogen-like form of FVIIa, and suggests the importance of the region proximal to the heavy chain N-terminus in the conversion from zymogen-like FVIIa into the active form. This aspect was further investigated by expressing and characterizing an alternate substitution of alanine for Val^{154} (V154A) in FVII.

EXPERIMENTAL

Materials

Hepes, poly(ethylene glycol) of a mean molecular mass of 8000 Da (PEG 8000) and Nunc cell factories were from Fisher Scientific (Pittsburgh, PA). L-α-Phosphatidylcholine (PC), L-α-phosphatidylserine (PS) and benzamidine were from Sigma (St. Louis, MO). *N*-Octyl β-D-glucopyranoside was from Calbiochem (La Jolla, CA) and Q Sepharose Fast Flow was from Pharmacia (Piscataway, NJ).

The chromogenic substrates methoxycarbonyl-D-cyclohexylglycyl-glycyl-arginine *p*-nitroanilide acetate (Spectrozyme fXa), methanesulphonyl-D-cyclohexylalanyl-butyl-arginine *p*-nitroanilide acetate (Spectrozyme fVIIa), methylsulphonyl-D-cyclohexylglycyl-glycyl-arginine *p*-nitroanilide acetate (Spectrozyme fIXa) and the IMUBIND® Factor VIIa ELISA kit were from American Diagnostica (Greenwich, CT). Stock solutions (approx. 5 mM) of these substrates were prepared in water and concentrations were determined using a molar absorption coefficient, ϵ_{342}, of 8270 $M^{-1} \cdot cm^{-1}$ [21]. Insulin/transferrin/sodium selenite supplement (ITS) was purchased from Roche Diagnostics (Indianapolis, IN). The reaction buffer consisted of 20 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM $CaCl_2$ and 0.1 % (w/v) PEG 8000.

Phospholipid vesicles, composed of 75 % (w/w) PC and 25 % (w/w) PS (PCPS) were prepared as described in [22] and their concentration was determined by colorimetric phosphate analysis [22].

Proteins

Plasma-derived human FVII, Factor IX (FIX) and Factor (FX), and human FIXa were from Haematologic Technologies Inc. (Essex Junction, VT). Plasma-derived FXa, soluble human TF (amino acid residues 1–219) and tick anticoagulant peptide (TAP), expressed in *Pichia pastoris*, were generously given by Dr Sai Kumar Buddai and Dr Sriram Krishnaswamy from the Children's Hospital of Philadelphia, PA. Recombinant Novoseven™ FVIIa was from Novo-Nordisk (Gentofte, Denmark). The ELISA kit to detect FVII was from Enzyme Research Laboratories (South Bend, IN). Full-length human TF made in insect cells was purchased from American Diagnostica (Greenwich, CT). Full-length and soluble human TF (amino acid residues 1–219), both produced in *Escherichia coli*, were kindly provided by Dr James H. Morrissey of the University of Illinois College of Medicine, Urbana-Champaign, IL. Full length human TF (25 μg) was reconstituted in 100 % (w/w) PC (1.4 mg) vesicles using 80 mM *N*-octyl β-D-glucopyranoside, as described in [23], for reconstitution of TF in PCPS. The effective concentration of TF after relipidation in PC (TF/PC) was assumed to correspond to 50 % of the total concentration present due to the random orientation of TF during reconstitution [24]. This TF concentration was validated by titration experiments. Reconstituted TF was stored in the dark at 22 °C and used in experiments within 4 days of preparation. Ca^{2+}-dependent anti-FVII monoclonal antibody was generously given by Dr Ulla Hedner of Novo-Nordisk, Gentofte, Denmark.

The γ-carboxyglutamic acid content of purified protein, which was determined by alkaline hydrolysis performed at Commonwealth Biotechnologies (Richmond, VA), verified full carboxylation of recombinant proteins.

Genomic studies and site-directed mutagenesis

FVII-exon and splicing-junction scanning was performed by direct sequencing as described in [25]. Population screening for the $T^{8967} \rightarrow G$ mutation, which results in the FVII mutant V154G, was performed by restriction analysis of an amplified genome fragment. Amplification of patient genomic DNA using the oligonucleotide primer 5′-AAACCCCAAGGCCGAACTG-3′ (corresponding to residues 8948–8966) created a new *Bsr*I restriction site exclusively in the mutated sequence that results in a different pattern of enzymic digestion in the presence of the mutation from that in its absence (nucleotide numbering is according to FVII DNA sequence published by O'Hara et al. [26]).

The substitutions $Arg^{152} \rightarrow Gln$ (R152Q), V154G, V154A, $Asp^{343} \rightarrow His$ (D343H) and $Ser^{344} \rightarrow Ala$ (S344A) were introduced in FVII cDNA cloned in pCDNA3.1 vector (Invitrogen, San Diego, CA) using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The presence of the point mutation was confirmed by DNA-sequence analysis of the entire FVII cDNA.

In vitro expression and purification of FVII recombinant proteins

HEK-293 cells were transfected using FuGene™ 6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN) or Lipofectamine 2000 (Gibco, Gaithersburg, MD) following the manufacturers' instructions. Cells were grown in Dulbecco's modified Eagle's medium/F12 in the presence of 10 % (v/v) foetal bovine serum, 5 μg/ml vitamin K, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Selection was performed as described in [27]. The highest producers (1.5–2 μg/ml per 10^6 cells over 24 h) were selected by ELISA, following the manufacturer's instructions, and further expanded to a cell-factory system using medium containing 10 μg/ml ITS. After 72 h, conditioned medium was collected at 24 h intervals. Benzamidine (10 mM) was added before filtering the medium through a 0.45 μm cellulose nitrate filter and the filtrate was frozen at −20 °C.

Purification of FVII from conditioned medium was carried out as described in [28]. The homogeneity of all protein preparations was evaluated by SDS/4–12 % PAGE [29]. FVII concentration was determined using a molecular mass of 50000 kDa and a molar absorption coefficient, ϵ, of 69500 $M^{-1} \cdot cm^{-1}$.

FVII cleavage and functional assays

Recombinant FVII derivatives (2 μM) were incubated for 30 min in reaction buffer at 37 °C with 400 μM PCPS and 20 nM human FXa. TAP, at a concentration of 2 μM, was used to specifically inhibit FXa. The formation of two-chain FVIIa was evaluated by SDS/PAGE analysis after Coomassie Blue staining.

Peptidyl substrate cleavage

FVIIa (10 nM) was incubated in reaction buffer at 22 °C in the presence or absence of 50 nM soluble TF and 200 μM PCPS. Spectrozyme fVIIa was added at concentrations ranging from 0 to 1 mM immediately before monitoring the A_{405} as a function of time using a VMax plate reader (Molecular Devices, Menlo Park, CA). Initial rates were expressed in concentration terms using a molar absorption coefficient, ϵ_{405}, of 9650 $M^{-1} \cdot cm^{-1}$ and an

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effective path length of 0.59 cm calculated for a 200 μl reaction volume. Due to their markedly reduced activity, K_m values of V154G and V154A FVIIa for the chromogenic substrate were evaluated using 200 nM or 40 nM FVIIa in the presence or absence of 1 μM or 200 nM soluble TF respectively. Amidolytic activity of R152Q FVII, D343H FVIIa and S344A FVIIa was tested using 10 nM, 200 nM and 500 nM FVII/FVIIa in the presence of 200 μM PCPS and 50 nM, 400 nM and 1 μM soluble TF respectively.

Macromolecular substrate cleavage

Activity towards FX

FVIIa (1 nM) was incubated in reaction buffer at 37 °C with 10 nM TF and 60 μM PC, or with 10 nM soluble TF and 100 μM PCPS; the reaction was initiated by the addition of 100 nM human FX. Aliquots of the reaction were quenched at several time points up to 3.5 min in 20 mM Hepes, pH 7.4, 150 mM NaCl, 50 mM EDTA and 0.1 % (w/v) PEG 8000. The FXa chromogenic substrate (Spectrozyme fXa) was added at a final concentration of 100 μM. FXa activity was monitored by measuring the A_{405} at 22 °C. The concentration of FXa formed as a function of time was determined by interpolation from the linear dependence of the initial rate of Spectrozyme fXa hydrolysis on known concentrations of FXa that were determined separately. The initial steady-state rate of FXa formation was determined from the slope of plots that documented the linear appearance of FXa with time. The same experiment was performed using different concentrations of FVIIa (1 or 10 nM) in the presence of soluble TF (10 or 100 nM respectively).

Activity towards FIX

FVIIa (1 nM) was incubated at 37 °C in reaction buffer with 200 nM soluble TF and 500 μM PCPS. The reaction was initiated by the addition of 400 nM human FIX. Aliquots of the reaction were quenched at several time points within 2 min in 30 mM EDTA and 60 % (v/v) ethylene glycol. FIXa activity was monitored at 405 nm after the addition of the chromogenic substrate Spectrozyme fIXa (500 μM). The concentration of FIXa that formed as a function of time was determined by interpolation from the linear dependence of the initial rate of Spectrozyme fIXa hydrolysis on known concentrations of FIXa that were determined separately. The initial steady-state rate of FIXa formation was determined from the slope of plots that documented the linear appearance of FIXa with time.

Carbamylation of FVIIa

FVIIa (4 μM), in the presence or absence of 20 μM soluble TF, was incubated with 0.2 M NaNCO at 22°C in reaction buffer, pH 7.2. Aliquots from the reaction were taken at 0, 15, 30, 60, 90, 120, 150 and 180 min. Residual activity of FVIIa towards Spectrozyme fVIIa (800 μM) was measured using 20 nM FVIIa, 100 nM soluble TF for wild-type; 200 nM FVIIa, 1μM soluble TF for V154G FVIIa and 60 nM FVIIa, 300 nM soluble TF for V154A FVIIa.

In order to rule out the possibility that carbamylation affects TF cofactor activity, 2 μM soluble TF was incubated at 22°C in the absence or presence of 0.2 M NaNCO in reaction buffer, pH 7.2. After 0, 1 and 2 h time intervals, TF cofactor activity was tested using 100 nM soluble TF in the presence of 20 nM FVIIa, 100 μM PCPS in Reaction Buffer. The amidolytic activity of the soluble TF–FVIIa complex was assayed adding Spectrozyme fVIIa (375 μM).

FVII interaction with an active-site inhibitor

The ability of wild-type and mutated FVIIa to accommodate an inhibitor in the active site was tested using IMUBIND® Factor VIIa ELISA kit that employs a biotinylated enzyme inhibitor of FVIIa and an anti-FVII/FVIIa monoclonal antibody as the capture antibody. The assay was performed following instructions of the manufacturer. Wild-type and Novoseven™ FVIIa concentrations ranged from 0.031 nM to 2 nM, whereas V154G FVIIa concentrations ranged from 0.5 nM to 8 nM. The active-site inhibitor provided was a Phe-Pro-Arg chloromethylketone.

TF-binding competition assay for recombinant proteins

Novoseven™ FVIIa (10 nM) was incubated in Reaction Buffer and 100 μM PCPS, with different fixed concentrations of soluble TF (5, 10 and 20 nM) in order to form a functional FVIIa–TF complex. Novoseven™ FVIIa was displaced from the complex by adding increasing concentrations of each of the inactive FVII/FVIIa mutants (R152Q uncleavable FVII, V154G FVIIa, D343H zymogen-like FVIIa or S344A inactive FVIIa) ranging from 0 to 1 μM, during a 30 min incubation at 37 °C. Residual amidolytic activity of soluble TF–FVIIa complex was assayed adding Spectrozyme fVIIa. Figure 3(C) refers to the experiment using the different FVII mutants in the presence of 10nM soluble TF. In order to rule out a possible difference in dissociation constant values among FVII forms due to the presence of phospholipid vesicles, the same competition experiment was carried out in the absence of membrane using R152Q FVII as competitor. Binding of FVII/FVIIa to full length TF reconstituted in PC was performed under the same conditions but in the presence of 5 nM Novoseven™ FVIIa, and different fixed concentrations of full length TF (3, 5 and 15 nM TF relipidated in 12, 20 and 60 μM PC, respectively). FVII/FVIIa mutants ranged from 0 to 200 nM.

FVII autoactivation

The time course of wild-type FVII autoactivation was performed in reaction buffer using concentrations of the soluble TF–FVII complex above (100 nM FVII and 100 nM soluble TF in the presence of 2.5 nM FVIIa, either with or without 430 μM PCPS) and below (32 nM FVII and 30 nM soluble TF in the presence or absence of 2.5 nM FVIIa, either with or without 430 μM PCPS) the K_d value we calculated in the present study. Aliquots of the reactions were tested for the amidolytic activity of the soluble TF–FVIIa complex at 0, 10, 20, 40, 60, 90 and 120 min by adding Spectrozyme fVIIa (375 μM).

Data analysis

Steady-state kinetic constants for synthetic substrate hydrolysis were determined from duplicate measurements of the initial velocity that were obtained using different substrate concentrations. Initial velocity data were fit to the Michaelis–Menten equation [30] by nonlinear least-squares regression analysis using the Marquardt algorithm [31]. When saturation of enzyme by substrate was not achieved ($K_m > [S]$), catalytic efficiency (k_{cat}/K_m) was extracted with reasonable accuracy from the slope of the linear dependence of initial velocity on substrate concentration.

For competition experiments with the inactive FVII/FVIIa mutants, titration curves with different concentrations of TF were simultaneously analysed by fitting the initial velocity of synthetic peptide hydrolysis to the cubic Equation 17 of Olson et al. [32] as described in [33]. Nonlinear least-squares regression

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yielded values of ΔV, V_t, K_d, and n, which described the binding of Novoseven™ FVIIa to TF, and K_{dcomp} and n_{comp}, which described the equivalent interaction of inactive mutant FVIIa with TF. In these analyses ΔV is the decrease in velocity of substrate cleavage when competitor FVIIa concentration saturates the fixed concentration of TF; V_t is the velocity at infinite concentration of competitor, equal to the activity of the fixed concentration of Novoseven™ FVIIa in the absence of TF; K_d is the equilibrium dissociation constant for the FVIIa–TF interaction, and n represents the number of moles of FVIIa bound per mole of TF at saturation. According to previous results, in this analysis n was set at 1 [15].

Reduction in activity after carbamylation was fitted to first-order exponential decay through nonlinear least-squares regression analysis.

RESULTS

Identification and characterization of mutant FVII

Two asymptomatic patients from Northern Italy were referred for a prolonged prothrombin time (> 2 S.D. above the prothrombin time of the reference plasma) detected in pre-surgery screening. FVII antigen (FVII:Ag) and coagulant activity (FVII:C) levels were 125%, and 60% for patient 1, and 128% and 51% for patient 2, compared with pooled normal plasma respectively. A third patient, also of Italian descent, was referred for a mild bleeding tendency from childhood including recurrent epistaxis. His FVII:Ag and FVII:C values were 42% and < 1% respectively. Genomic DNA was isolated, after obtaining informed consent, from leucocytes of the patients. Sequence analysis of exon 6 showed heterozygosity for a $T^{8967} \rightarrow G$ substitution in the three patients. This point mutation causes the V154G mutation. No additional mutations were found in the other FVII exons or promoter region. Patient 3 was also heterozygous for the substitution G to A at the fifth nucleotide of intron 7 (IVS7+5, FVII Lazio mutation) [25]. This mutation, which affects the donor splice site in intron 7 of FVII, determines the insertion of 37 bp in the mature mRNA. The frameshift, caused by the abnormal splicing, results in a premature termination of translation at residue 231, thus abolishing most of the C-terminal catalytic domain [34]. Population screening using a mutagenized primer that produced a *Bsr*I site excluded the presence of the $T^{8967} \rightarrow G$ change, which is responsible for the V154G substitution, in 100 normal Italian subjects, thus indicating that this transversion is not a common polymorphism.

FVII mutants

The substitution V154A was expressed and characterized to test whether Gly^{154} was responsible for the impaired salt-bridge formation in V154G FVIIa.

In order to compare the interaction of TF with V154G and different FVII forms, we expressed three additional representative FVII mutants, R152Q, D343H and S344A. R152Q FVII results in a molecule that cannot be cleaved, thus providing a stable zymogen form [27]; D343H FVII is a naturally occurring substitution of the aspartic residue that is involved in salt-bridge formation, as reported in [35], but is not yet characterized; S344A FVII, owing to the substitution of the serine residue in the catalytic triad, leads to an inactive molecule that is properly cleaved and has normal TF-binding properties [16,36].

FVII proteolytic activation and FVIIa activity

Proteolytic activation of purified recombinant proteins was tested using human FXa. The cleavage pattern and rate of activation of

Table 1 Catalytic efficiency of FVIIa for the hydrolysis of the peptidyl substrate (Spectrozyme fVIIa)

The experiment was performed in reaction buffer in the presence of 200 μM PCPS with FVIIa and TF as indicated. Spectrozyme fVIIa ranged from 0 to 1 mM.

	FV11a (nM)	Soluble TF (nM)	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
Wild-type	10	50	2.5×10^4
V154G	200	1000	5×10^2
V154A	40	200	2.5×10^3

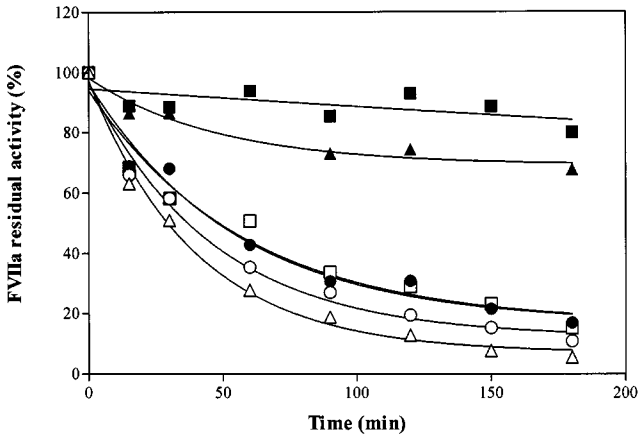


Figure 1 Effect of carbamylation on FVIIa activity

Residual activity of FVIIa after carbamylation of the α-amino group of Ile^{153}. NaNCO (0.2 M) was used to carbamylate 4 μM wild-type FVIIa (triangles), V154G FVIIa (circles) or V154A FVIIa (squares) in the presence (filled symbols) or absence (open symbols) of 20 μM soluble TF, at 22 °C in reaction buffer.

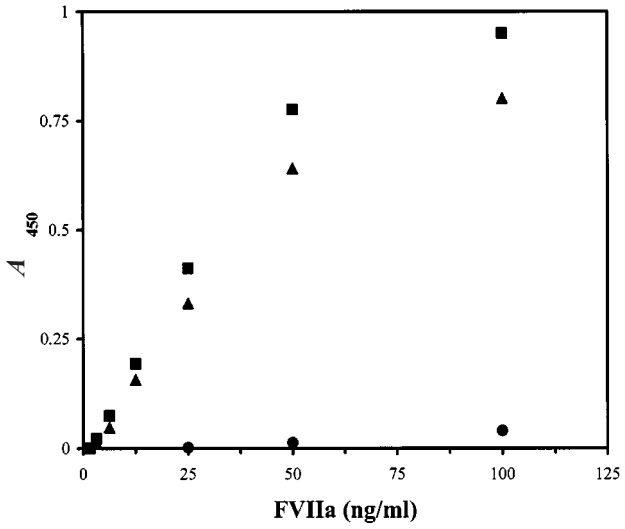


Figure 2 Binding of FVIIa to an active-site inhibitor

Interaction of FVIIa with a Phe-Pro-Arg chloromethylketone active-site inhibitor, detected by a horseradish peroxidase conjugated to streptavidin that recognized the biotinylated inhibitor–FVIIa complex (IMUBIND® Factor VIIa ELISA). FVIIa concentrations ranged from 0.031 to 2 nM for Novoseven™ (■) and wild-type (▲) FVIIa, whereas V154G FVIIa (●) concentrations ranged from 0.5 to 2 nM.

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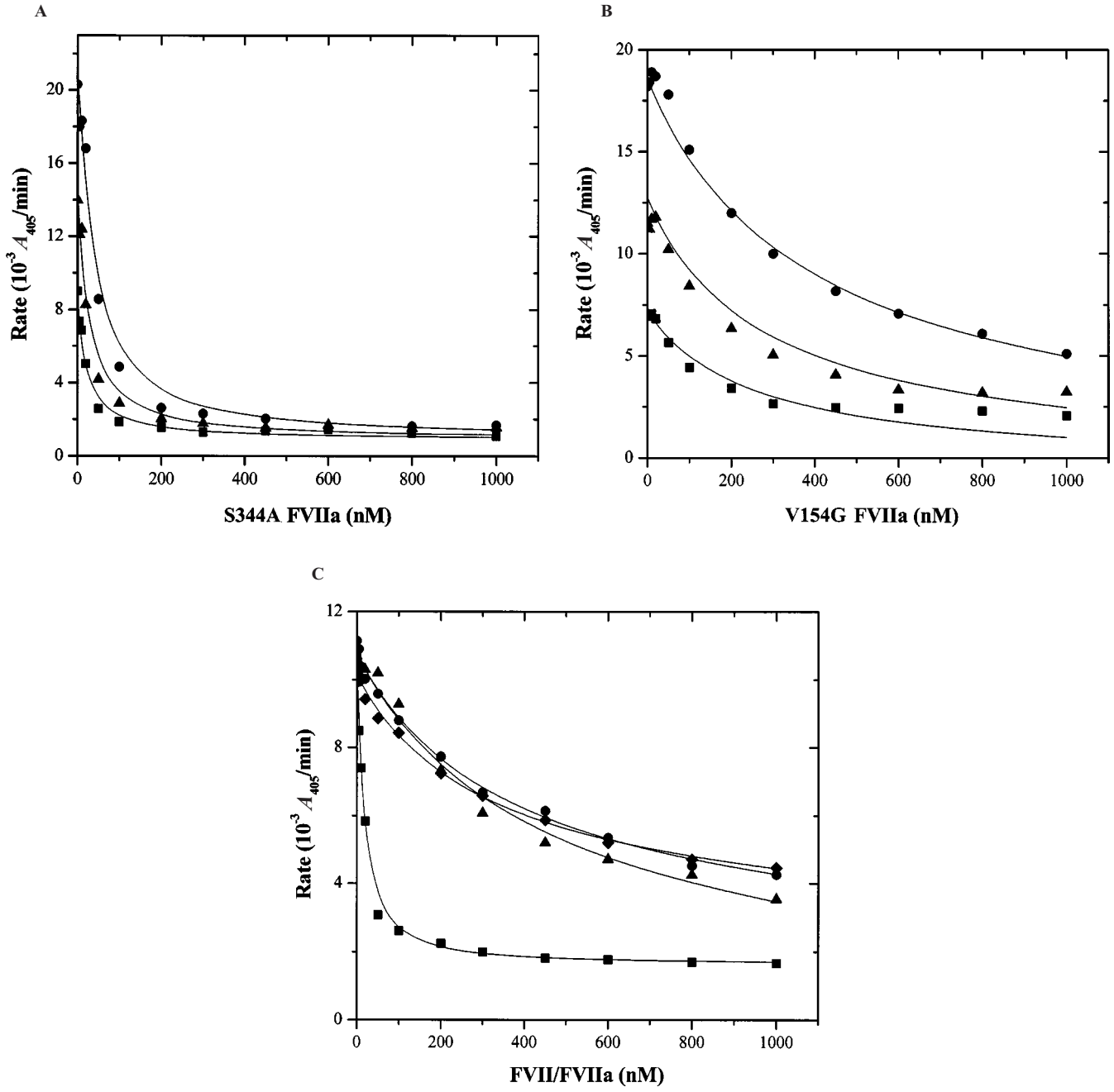


Figure 3 Binding of FVII variants to soluble TF

Binding of FVII mutants to soluble TF was performed in reaction buffer by incubating 10 nM Novoseven™ FVIIa in the presence of 100 μM PCPS with different fixed concentrations of soluble TF, at 5 (■), 10 (▲) and 20 nM (●). S344A FVIIa (**A**) and V154G FVIIa (**B**) were used as competitors at concentrations ranging from 0 to 1000 nM. After 30 min of incubation at 37 °C, the residual activity of the FVIIa–TF complex was assayed using Spectrozyme fVIIa (375 μM). (**C**) Competition assay using 10 nM soluble TF and 10 nM Novoseven™ FVIIa using increasing concentrations of functional FVIIa competitors, R152Q FVII (●), V154G FVIIa (◆), D343H FVIIa (▲) and S344A FVIIa (■).

wild-type, V154G, V154A, D343H and S344A FVII were indistinguishable, whereas R152Q was not cleaved after Arg^{152}, even after a prolonged incubation for 4 h.

The resulting activated products were characterized with respect to their activity towards the FVIIa peptidyl substrate, Spectrozyme fVIIa, in the presence of soluble TF. The kinetic constants that were calculated for wild-type FVIIa were $K_m = 0.67 \pm 0.05$ mM and $k_{cat} = 17$. Whereas the catalytic efficiency parameters for wild-type, V154G and V154A FVIIa are shown in Table 1, R152Q FVII, D343H FVIIa and S344A FVIIa did not show any activity at concentrations of 10 nM, 200 nM and 500 nM.

Recombinant wild-type FVIIa showed similar activity towards FX as compared with Novoseven™ FVIIa (33.8 and 19.6 nM FXa generated/min for wild-type and Novoseven™, respectively) and FIX (24.9 and 21.4 nM FIXa generated/min for wild-type and Novoseven™, respectively). R152Q FVII, V154G FVIIa, D343H FVIIa and S344A FVIIa, tested under different sets of conditions,

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Table 2 Dissociation constant (K_d) for soluble TF, or TF/PC, and FVII/FVIIa

Values (means ± S.D.) were calculated from curves of TF binding to different forms of FVII, simultaneously fitting the data to equation 17 in [32]. N.M., not measured.

	K_d (nM)	
FVII/FVIIa type	TF–FVII/FVIIa	TF/PC–FVII/FVIIa
R152Q FVII	65.2 ± 5.4	1.04 ± 0.27
V154G FVIIa	75.8 ± 6.4	1.15 ± 0.16
D343H FVIIa	68.1 ± 8.9	N.M.
S344A FVIIa	4.8 ± 1.6	0.27 ± 0.06

were characterized by undetectable activity towards FIX and FX. V154A FVIIa showed reduced proteolytic activity towards FX (1.4 nM FXa generated/min) as compared with wild-type FVIIa, and undetectable activity towards FIX, most probably owing to the low sensitivity of the chromogenic substrate for FIXa.

Carbamylation of Ile^{153} in FVIIa

Previous studies have demonstrated that free FVIIa is susceptible to chemical modification of the exposed α-amino group of Ile^{153} by cyanate ions [7,10]. In the presence of TF, structural rearrangements protect the FVIIa Ile^{153} N-terminus from carbamylation. Since carbamylation of Ile^{153} results in a loss of activity, the rate of modification of the N-terminus of FVIIa can be used to estimate the formation and the stability of the Ile^{153}–Asp^{343} salt bridge [7].

Cyanate ions did not perturb TF-cofactor activity, as indicated by the unaltered capability of soluble TF to enhance FVIIa activity after carbamylation (results not shown).

In the presence of NaNCO, soluble TF protected wild-type and V154A FVIIa from carbamylation, whereas it failed to protect V154G FVIIa from inactivation beyond that seen with free enzyme (Figure 1). This finding suggests strongly that V154G maintains a zymogen-like conformation, even in the presence of TF.

FVII interaction with an active-site inhibitor

Wild-type or mutated FVIIa (2 nM and 8 nM respectively) was incubated with the active-site inhibitor Phe-Pro-Arg chloromethylketone; whereas wild-type FVIIa showed normal binding of the inhibitor as compared with Novoseven™ FVIIa, V154G FVIIa was unable to accommodate the inhibitor in the active site (Figure 2).

TF binding to FVII and FVIIa

In order to evaluate equilibrium parameters for the FVIIa–TF complex assembly in the absence of a macromolecular substrate, we exploited the increased efficiency of FVIIa amidolytic activity in the presence of TF. It has been described previously that dissociation constants that were calculated in the presence of either a peptidyl or macromolecular substrate were comparable [15].

We compared the ability of R152Q FVII, V154G FVIIa or D343H FVIIa to displace functional FVIIa from the soluble TF–FVIIa complex with that of S344A FVIIa (Figure 3). R152Q FVII, V154G FVIIa and D343H FVIIa bound soluble TF with approx. 15-fold lower affinity as compared with S344A FVIIa (Table 2). Similar K_d values were calculated using soluble TF

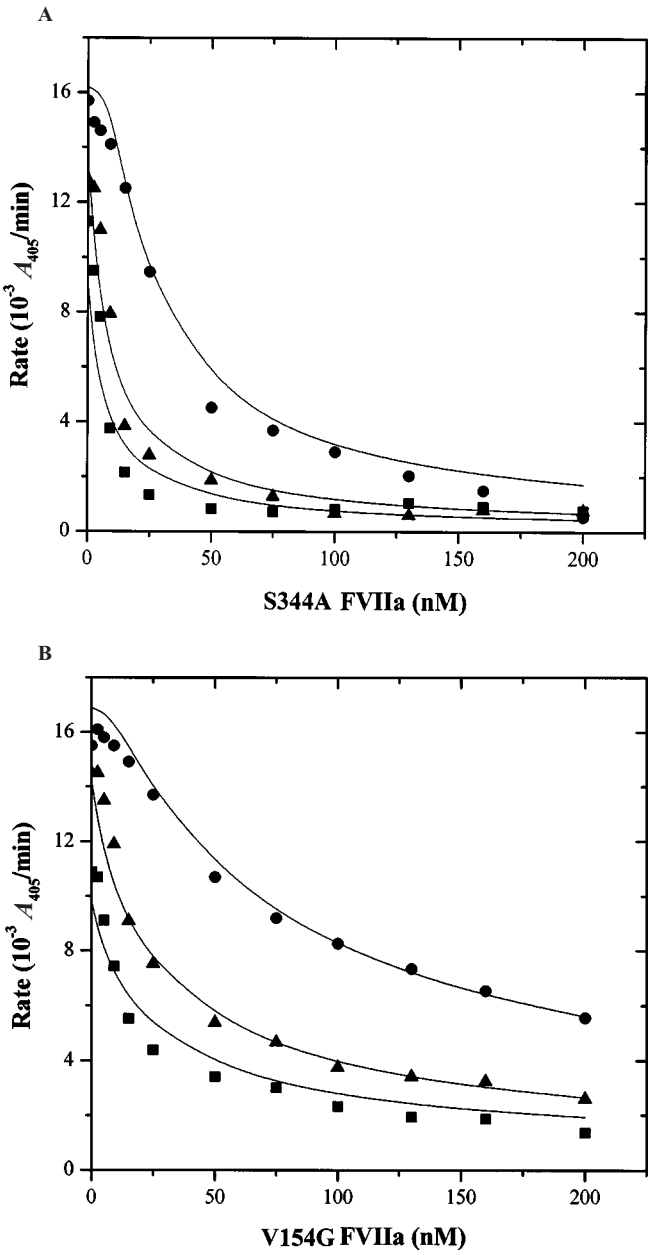


Figure 4 Binding of FVII variants to full-length TF/PC

Binding of FVII variants to full-length TF/PC was performed in reaction buffer by incubating 5 nM Novoseven™ FVIIa with 3 nM TF/12 μM PC (■), 5 nM TF/20 μM PC (▲) or 15 nM TF/60 μM PC (●). S344A FVIIa (**A**) and V154G FVIIa (**B**) were used as competitors at concentrations ranging from 0 to 200 nM. After 30 min of incubation at 37 °C, the activity of the FVIIa–TF complex was assayed using Spectrozyme fVIIa (375 μM).

produced in either *E. coli* or *P. pastoris*. The assay performed in the absence of membranes gave similar results (not shown), supporting the hypothesis that phospholipids do not significantly influence the affinity of soluble TF for FVII/FVIIa [37].

Both V154G FVIIa and R152Q FVII bound full-length TF, produced in *E. coli* and relipidated in PC (Figure 4), with approx. 4-fold decreased affinity as compared with S344A FVIIa (Table 2). Similar results were obtained using full-length TF made in insect cells. Because of sensitivity limitations, the fixed FVIIa concentration (5 nM) used in competition assays for binding to

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reconstituted TF was significantly higher than the inferred dissociation constant between FVIIa and TF. Therefore, calculated K_d values may only provide an upper limit value of these constants, which, however, agree well with previous results [15].

The dissociation constant that we measured for soluble TF and FVII ($K_d = 65.2 \pm 5.4$ nM) was higher than previously reported [38]. This difference could explain the absence of autoactivation of FVII using concentrations of soluble TF and of FVII below the K_d value that we determined [38]. Autoactivation occurs when the soluble TF–FVII concentration exceeds this K_d value, indicating that, as already shown for relipidated TF [39], FVII needs to be bound to soluble TF to be an appropriate substrate of the soluble TF–FVIIa enzymic complex.

In addition, the results shown in Table 2 suggest that the looser binding of all FVII forms to soluble TF, compared with full-length TF, amplifies the range of K_d values, thus making soluble TF useful to estimate differences in TF binding among FVII forms.

DISCUSSION

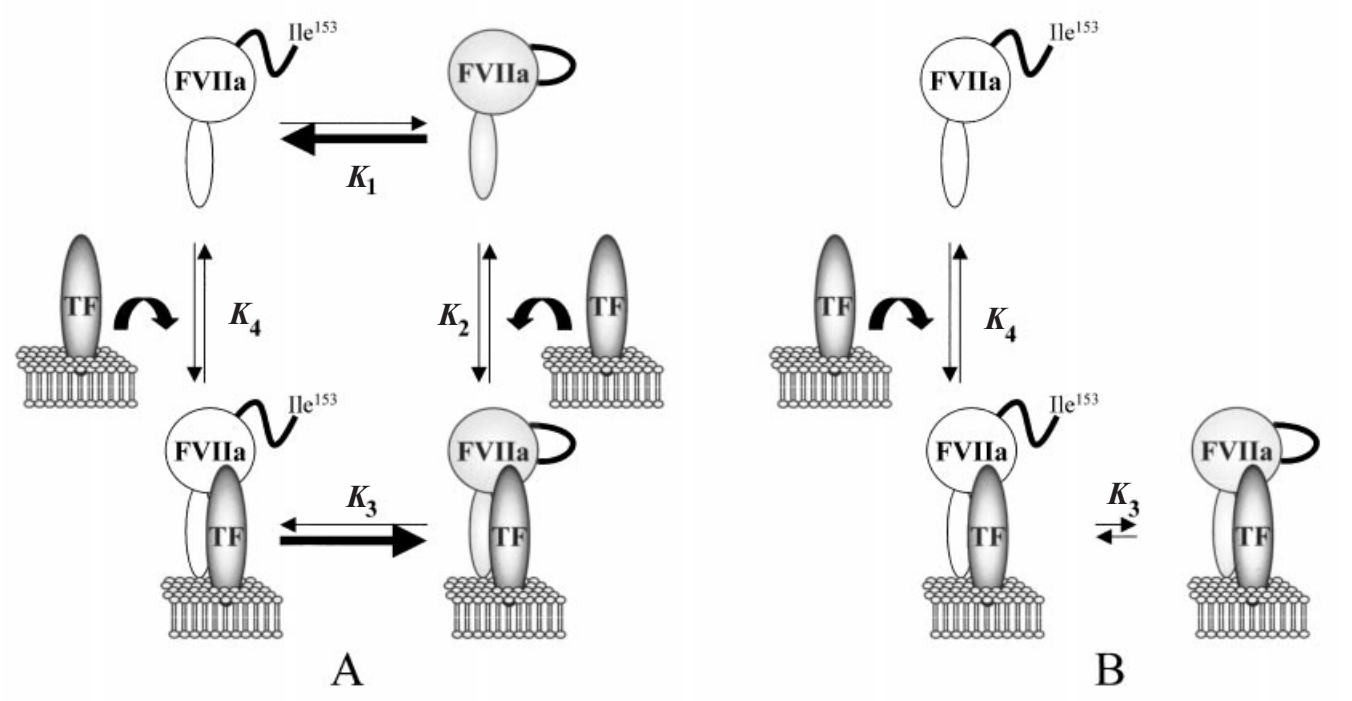
Conversion of the zymogen FVII to fully active FVIIa has undergone extensive scrutiny, in part because of potential benefits that may be gained by a better understanding of the steps required to form enzymically active FVIIa [40–42]. After conversion into the two-chain form, FVIIa remains in a zymogen-like state that renders coagulation initiation dependent on TF exposure. Production of TF-independent FVIIa or the synthesis of inhibitors specific to FVIIa may result in improved strategies for treatment of haemorrhagic or thrombotic disorders [17,18,43,44].

Previous work has shown that the events leading to FVIIa catalytic enhancement include (i) interaction with TF, (ii) salt bridge formation between Ile^{153} and Asp^{343}, and (iii) ordering of the activation domain. However, the mutual and tight linkage among these steps has hampered the precise determination of their chronological sequence. More importantly, the lack of efficient models to represent zymogen-like FVIIa has hindered clarification of the TF mechanism of catalytic enhancement in the FVIIa–TF complex.

In the present paper, we describe the naturally occurring FVII mutant protein V154G, which shows properties consistent with the proposed zymogen-like form of FVIIa, a form that exists between the zymogen FVII and fully active FVIIa and which does not present a salt bridge between Ile^{153} and Asp^{343} [7]. Since salt-bridge formation is expected to partially protect FVIIa from chemical modifications, the FVIIa carbamylation experiments suggest that the majority of V154G FVIIa molecules maintain a zymogen-like form even when associated with TF.

We propose that impairment of salt-bridge formation and/or stability in V154G FVIIa is due to the introduction of a glycine residue at position P2′ producing a series of three glycine residues in a row (I^{153}GGG) at the activation site. This sequence at the N-terminus should be extraordinarily flexible and could easily give rise to a variety of interchangeable structures, thus not favouring the insertion of the newly formed N-terminus into the protein. Consistent with this hypothesis, the V154A mutation did not impair the ability of the heavy chain N-terminus to form a salt bridge. Nevertheless, V154A FVIIa showed reduced catalytic efficiency compared with wild-type FVIIa, suggesting an important role of Val^{154} in the conversion of FVIIa into a fully active enzyme.

The 'immature' V154G FVIIa molecule was exploited to investigate the active-site competence of this salt-bridge-less



Scheme 1 Effect of TF/PC binding to (A) S344A FVIIa and (B) V154G FVIIa with respect to salt-bridge formation

See the text for details.

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form of FVIIa. Its inability to accommodate an active-site inhibitor, even at high FVIIa concentrations, would suggest that the specificity pocket is not properly formed. This result may help to interpret the low catalytic efficiency of FVIIa in the absence of TF as the predominance of an inactive zymogen-like form in the equilibrium after FVII cleavage at Arg^{152}.

The V154G FVIIa variant, modelling a salt-bridge-less form of FVIIa, allowed us to analyse the binding of TF to this intermediate form of FVIIa, therefore representing a more physiological interaction between TF and FVIIa compared with previous studies [7,16]. V154G FVIIa also differs from previously described FVIIa mutants that could be defined as zymogen-like FVIIa [9,41]. These mutations either impaired the contact region between FVIIa and TF [41], or had all the residues in Loop 170s replaced with those of trypsin [9], thus not representing models to study the interaction of a salt-bridge-less FVII form with other macromolecules of interest in blood-clotting initiation.

To investigate TF binding to V154G FVIIa, we also used the FVII mutants R152Q, D343H and S344A to represent zymogen FVII, zymogen-like FVIIa, and inactive FVIIa respectively. V154G FVIIa bound soluble TF with an affinity comparable with that of mutants that lacked the salt bridge (R152Q and D343H). Schemes 1(A) and 1(B) depict binding of TF to mutant S344A and V154G FVIIa respectively, and provide models for interpreting the measured dissociation constants reported in Table 2. In the presence of TF, the equilibrium may be shifted from a zymogen-like FVIIa (white) towards active FVIIa (grey) by TF preferential binding to the species that has already formed the Ile^{153}–Asp^{343} salt bridge ($K_1 + K_2$). Alternatively, TF could bind zymogen-like FVIIa and active FVIIa with comparable affinity, without influencing their equilibrium, leading to an inactive and an active complex (lower left and lower right TF–FVIIa complexes), respectively. Conformational changes due to TF interaction with the zymogen-like form stabilize salt-bridge formation and order the activation domain, giving rise to the active enzymic complex FVIIa–TF $[(K_2) + (K_4 + K_3)]$.

In the case of S344A FVIIa (Scheme 1A), the calculated dissociation constant (0.27 nM) between TF and FVIIa would reflect the combination of the equilibrium constants of all the steps involved in the two different pathways $[K_1 + K_2; (K_2) + (K_4 + K_3)]$. In contrast, binding of TF to V154G FVIIa (Scheme 1B), which retains characteristics of a zymogen-like form, would be insufficient to drive this FVIIa variant into the active conformation. In this case, the quasi-homogeneity, with respect to salt–bridge formation, of zymogen-like V154G FVIIa molecules and the negligible value of K_3 would simplify the scheme to a single pathway. It is possible to assert, with a reasonable approximation, that the calculated dissociation constant (1.15 nM) represents the mere interaction of TF with the zymogen-like V154G FVIIa (K_4).

Based on the comparison of dissociation constants between full-length TF and the different FVII species, and the above mentioned interpretation, the preferential TF binding to the active FVIIa form, as suggested by Bach et al. [24], cannot account for the entire FVIIa catalytic enhancement by TF. Conformational changes driven by the interaction with TF must contribute to FVIIa conversion into the active form.

It is worth noting that the dissociation constants between active FVIIa and TF reconstituted in phospholipid vesicles calculated in this study agree well with previous investigations using bovine and human proteins [7,15], but differ from results obtained in non-equilibrium conditions [24]. The difference in affinity between FVII/FVIIa forms and TF (truncated or reconstituted forms) is consistent with previous reports [7,24] and correlates well with the described tighter binding of TF to active site modified FVIIa [7]. However, these findings do not mirror results obtained by surface plasmon resonance, probably owing to the differences between experimental approaches [16].

In the present study, we characterized a FVII mutant that, after cleavage, still shares characteristics with the FVII zymogen form, thus representing a model of the zymogen-like FVIIa not previously described. Since this form of FVIIa is predominant in the absence of TF, the interaction of V154G FVIIa with an active-site inhibitor and with TF mimics, to date, the most realistic interaction that triggers coagulation initiation.

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(12) **United States Patent**
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(54) **MODIFIED VITAMIN K-DEPENDENT POLYPEPTIDES**

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(57) **ABSTRACT**

The invention provides vitamin K-dependent polypeptides with enhanced membrane binding affinity. These polypeptides can be used to modulate clot formation in mammals. Methods of modulating clot formation in mammals are also described.

56 Claims, 16 Drawing Sheets

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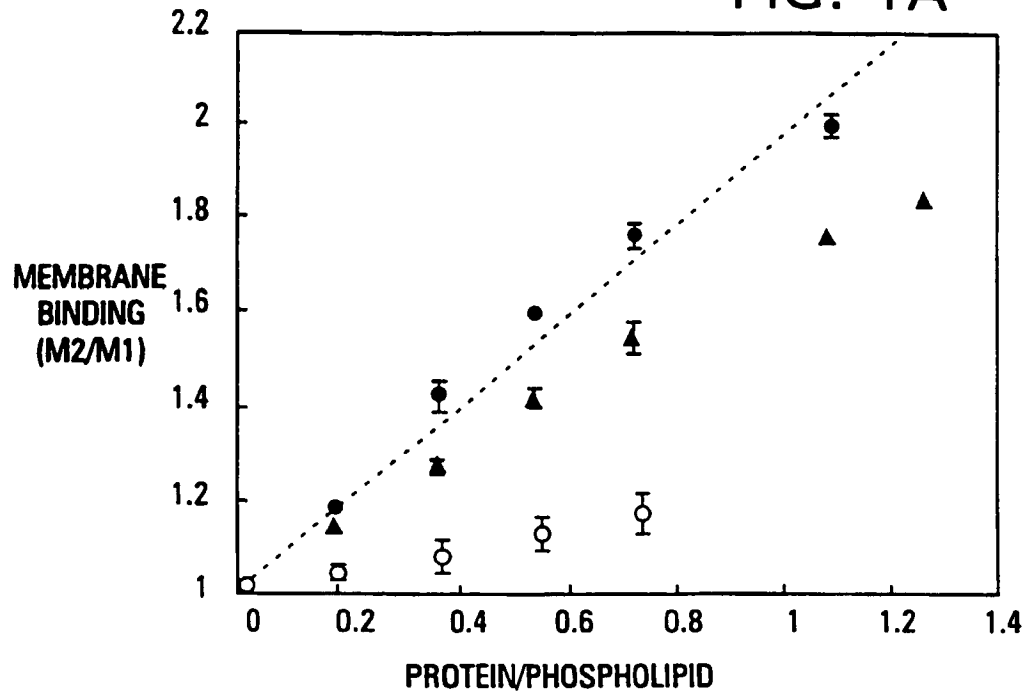
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FIG. 1A


MEMBRANE BINDING (M2/M1)
2.2
2
1.8
1.6
1.4
1.2
1
0
0.2
0.4
0.6
0.8
1
1.2
1.4
PROTEIN/PHOSPHOLIPID

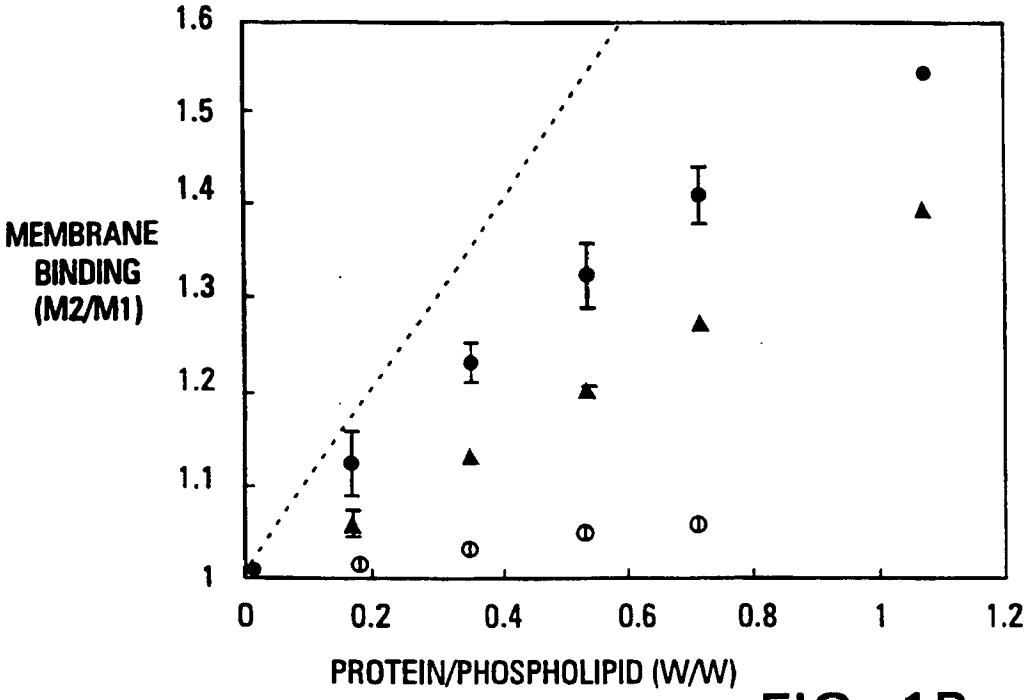

MEMBRANE BINDING (M2/M1)
1.6
1.5
1.4
1.3
1.2
1.1
1
0
0.2
0.4
0.6
0.8
1
1.2
PROTEIN/PHOSPHOLIPID (W/W)

FIG. 1B

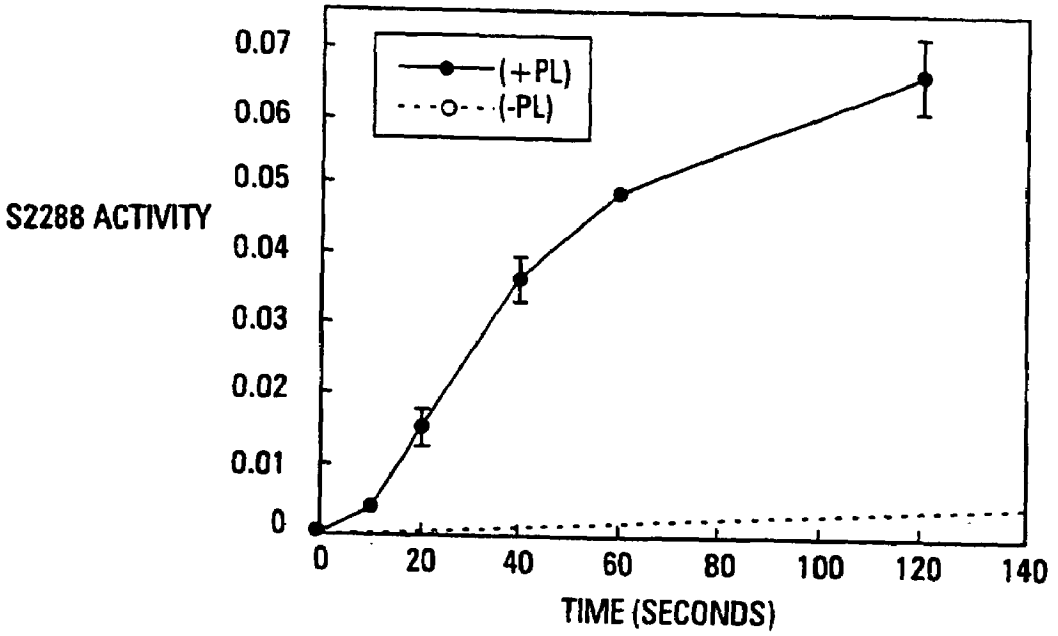

S2288 ACTIVITY
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
(+PL)
(-PL)
0
20
40
60
80
100
120
140
TIME (SECONDS)

FIG. 2

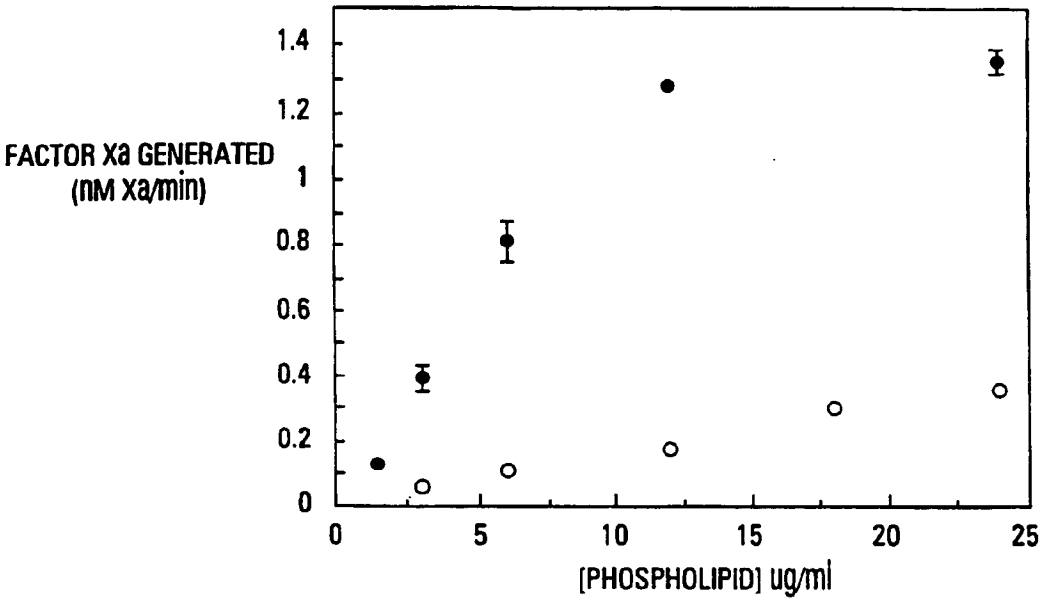
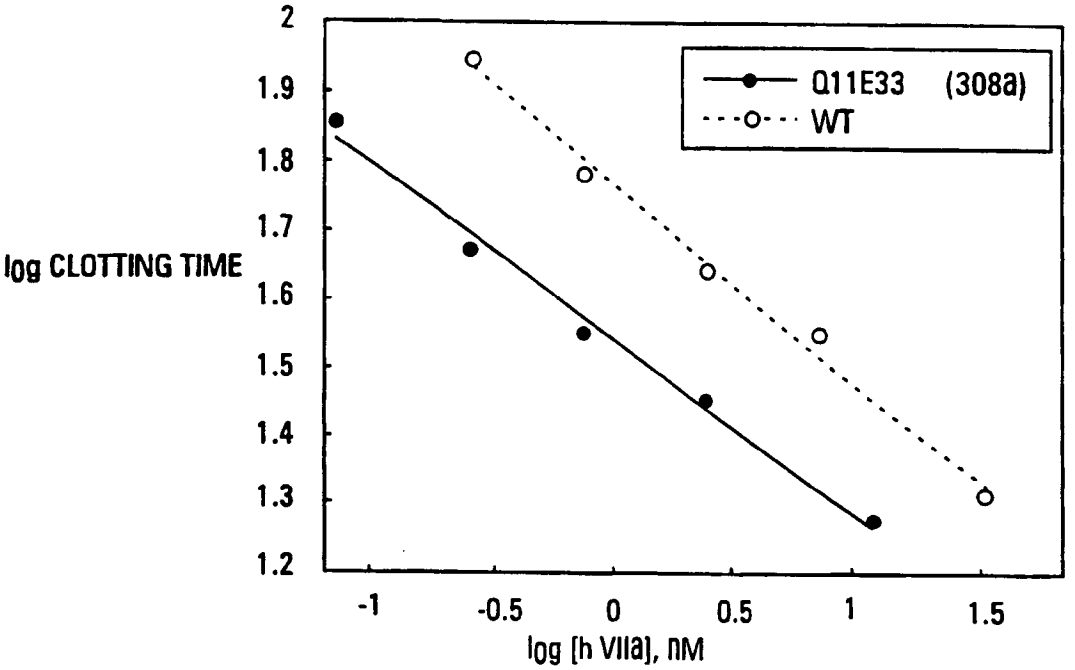

FACTOR Xa GENERATED
(nM Xa/min)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
[PHOSPHOLIPID] ug/ml

FIG. 3

FIG. 4


log CLOTTING TIME
2
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
-1
-0.5
0
0.5
1
1.5
log [h VIIa], nM
Q11E33 (308a)
WT

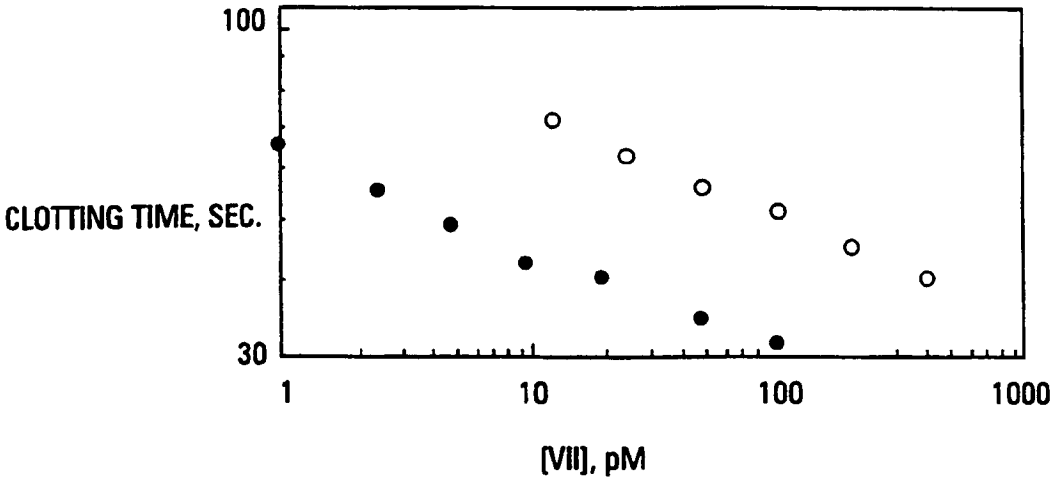


FIG. 5

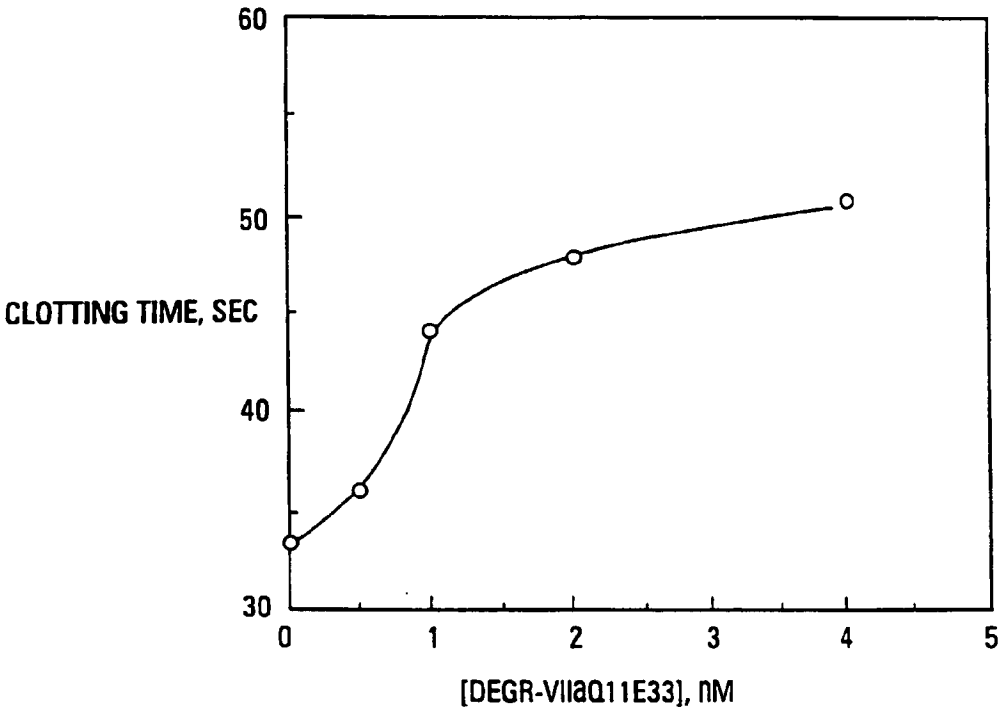


FIG. 6

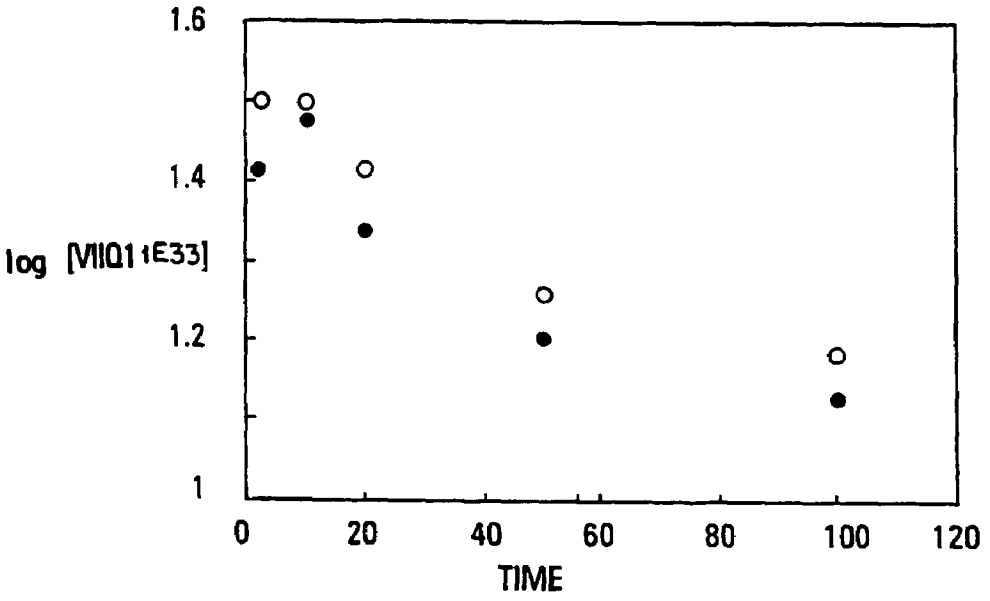
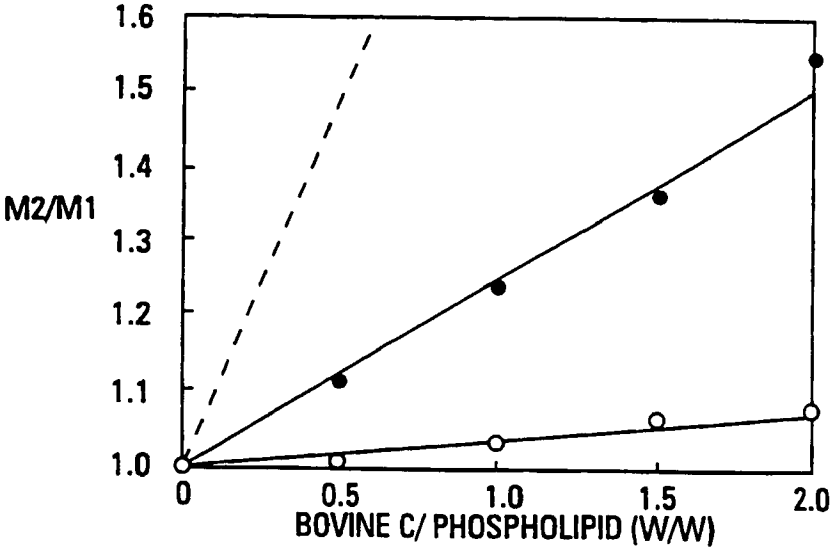


FIG. 7

FIG. 8A


M2/M1
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0
0.5
1.0
1.5
2.0
BOVINE C/ PHOSPHOLIPID (W/W)

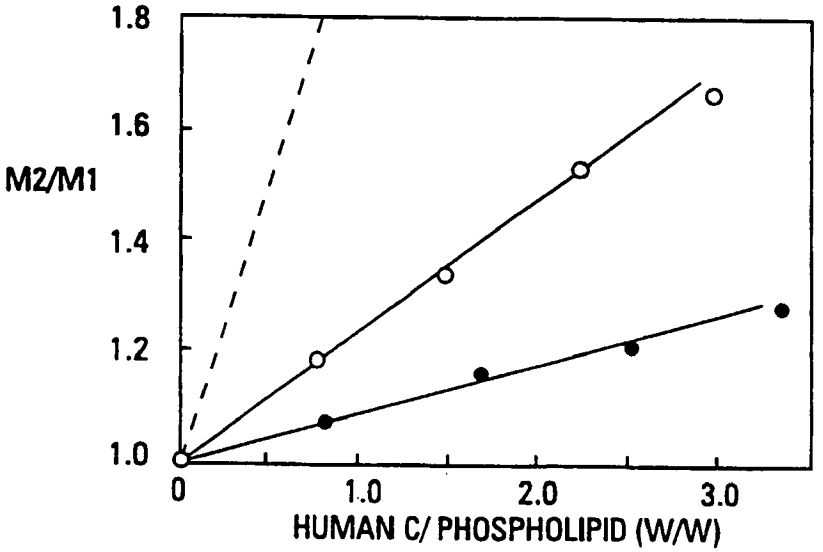

M2/M1
1.8
1.6
1.4
1.2
1.0
0
1.0
2.0
3.0
HUMAN C/ PHOSPHOLIPID (W/W)

FIG. 8B

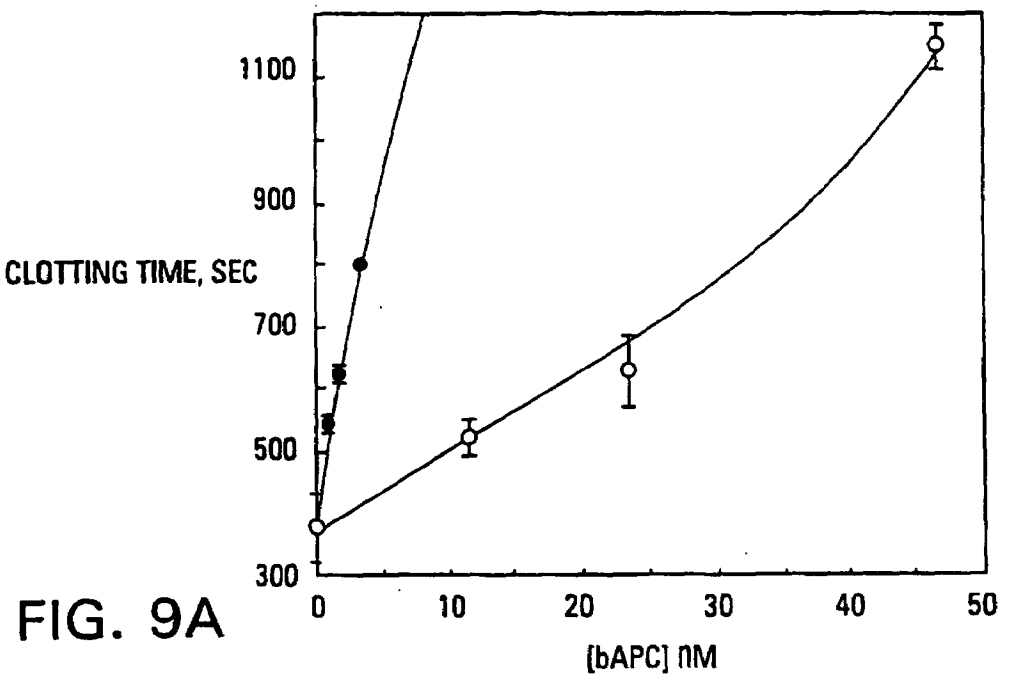

1100
900
CLOTTING TIME, SEC
700
500
300
0
10
20
30
40
50
[bAPC] nM

FIG. 9A

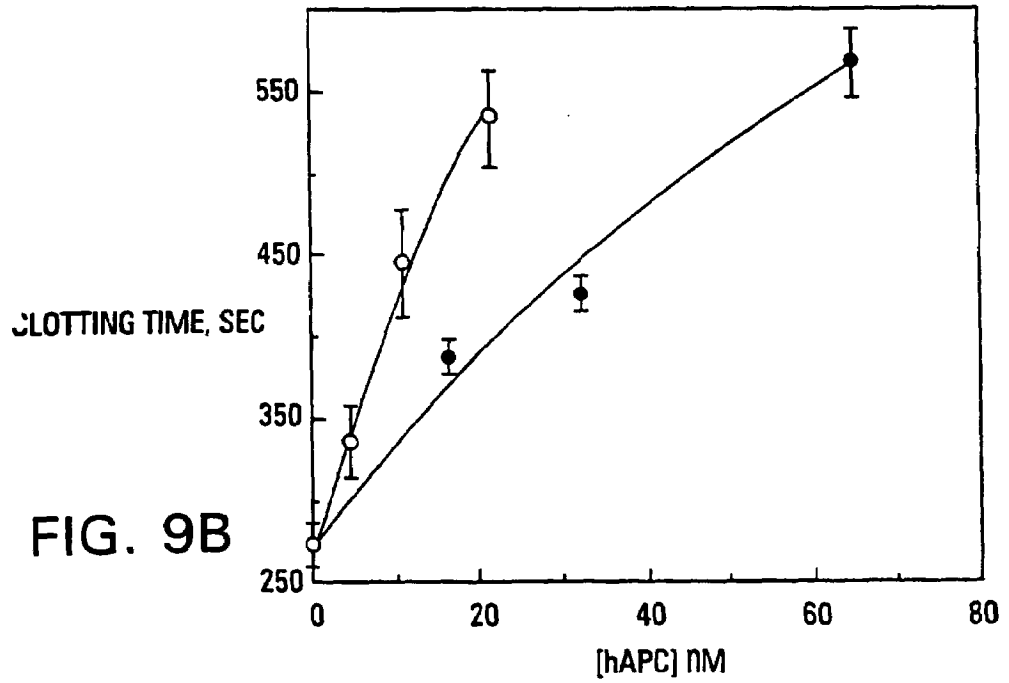
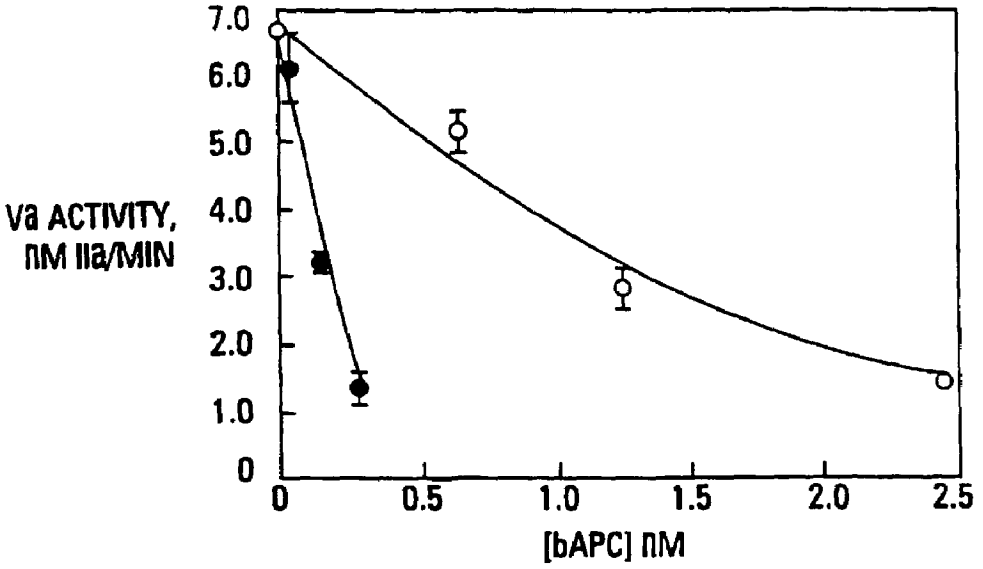

550
450
CLOTTING TIME, SEC
350
250
0
20
40
60
80
[hAPC] nM

FIG. 9B

FIG. 10A



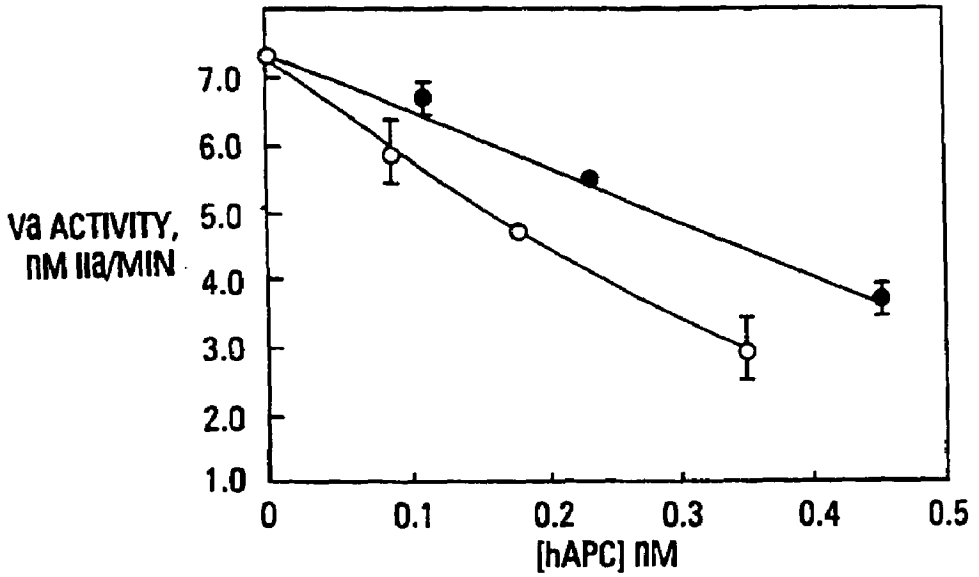
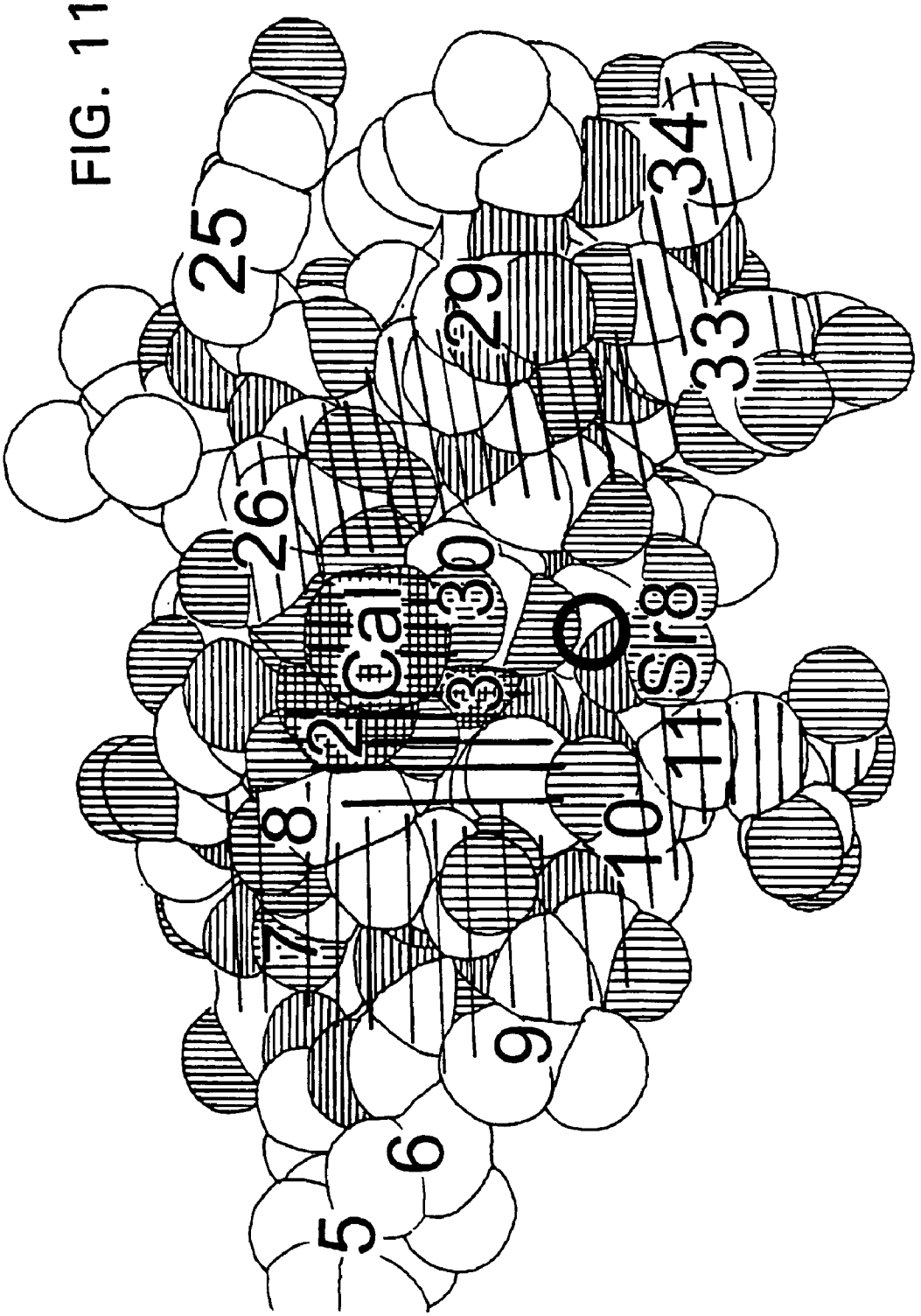


FIG. 10B

FIG. 11



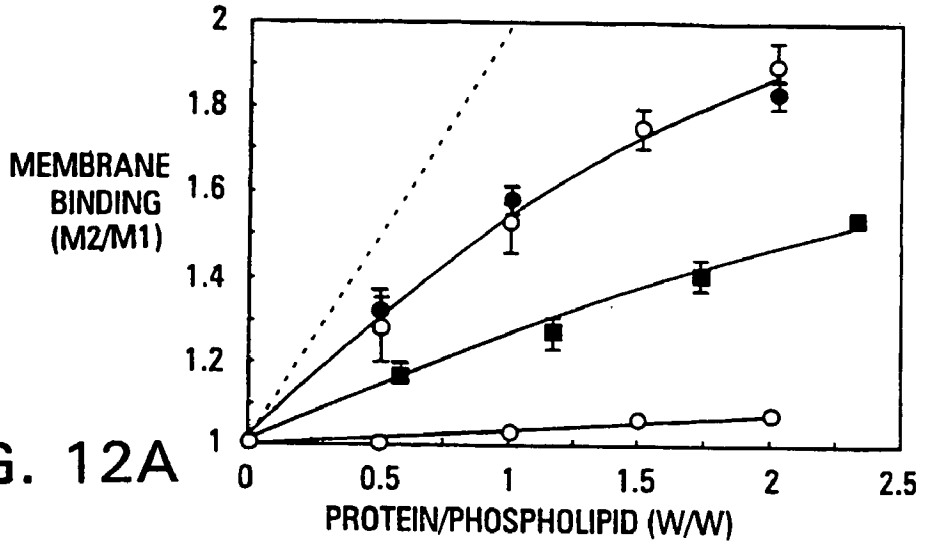


FIG. 12A

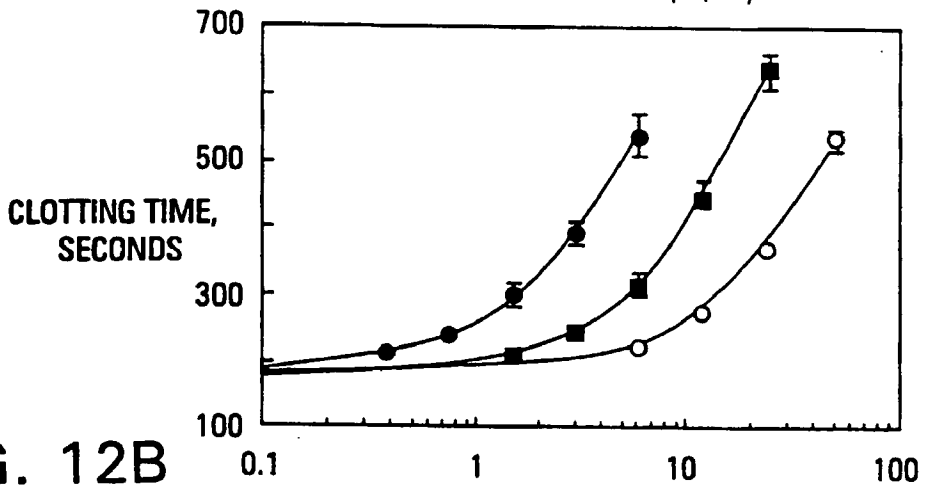


FIG. 12B

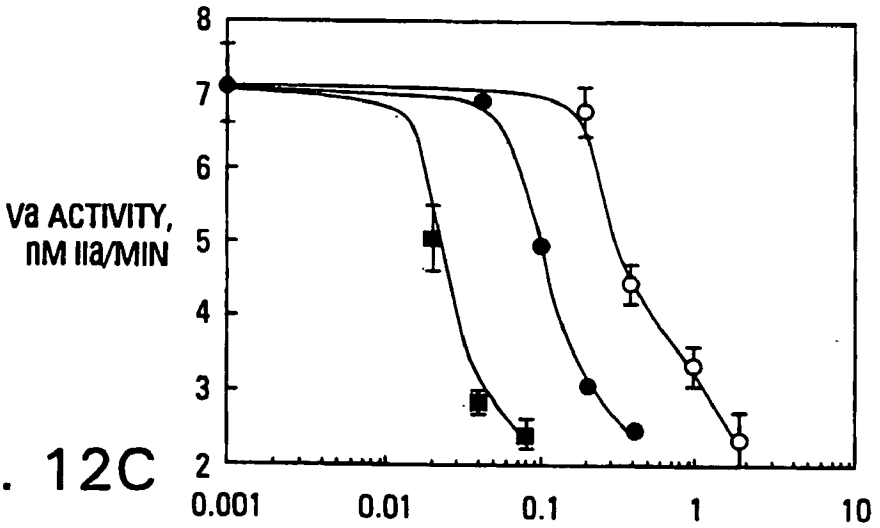
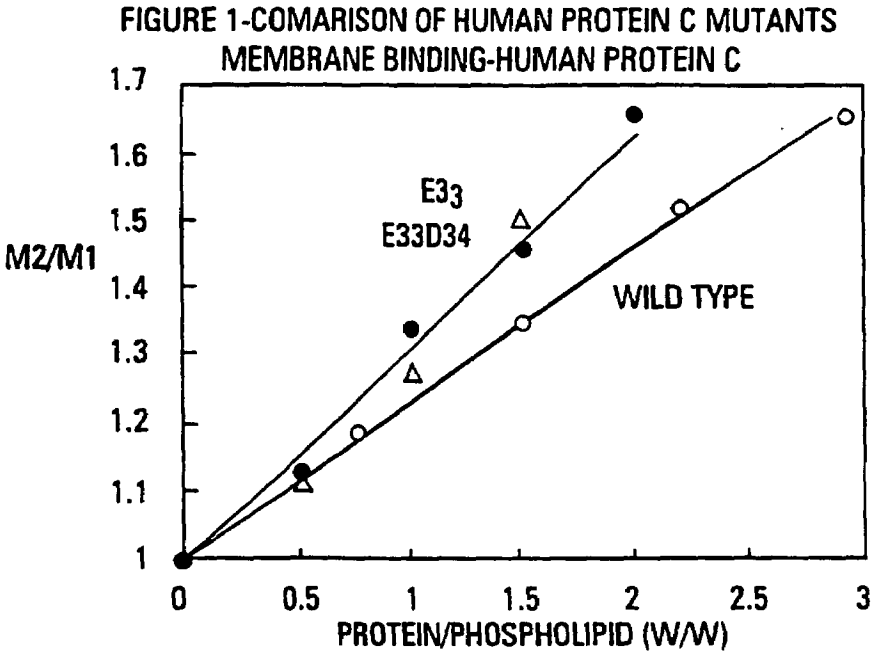


FIG. 12C

FIG. 13A



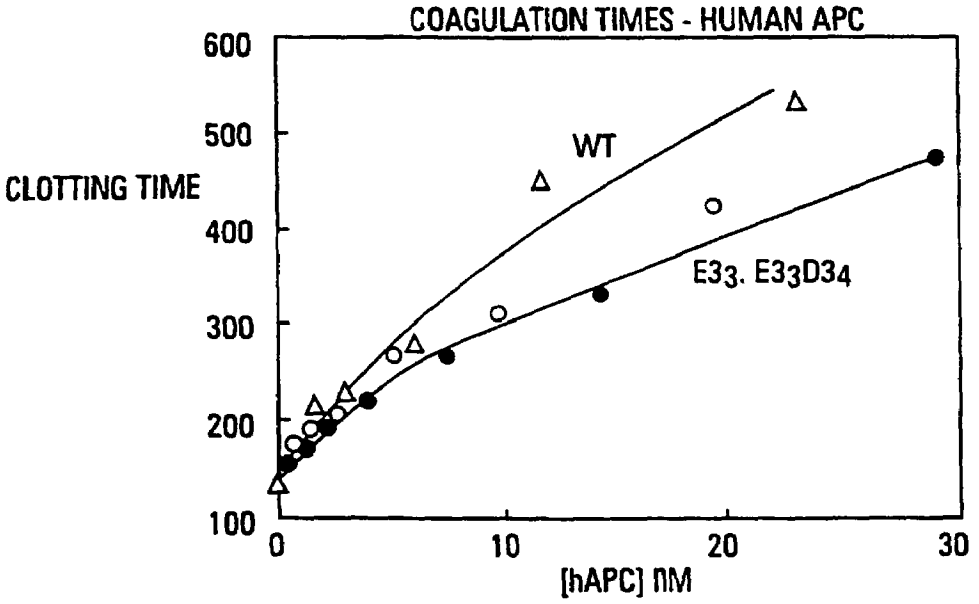


FIG. 13B

FIG. 14A

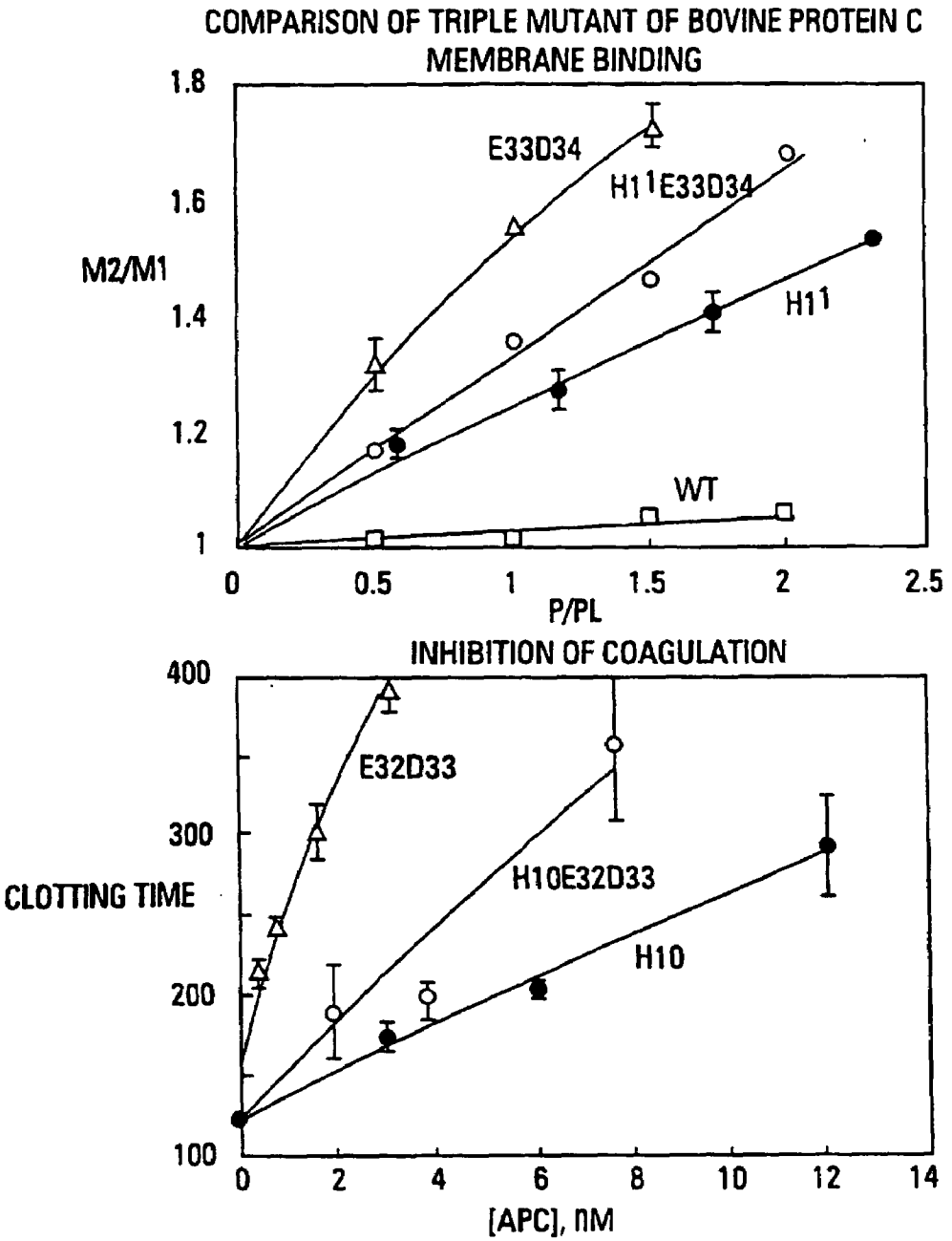


FIG. 14B

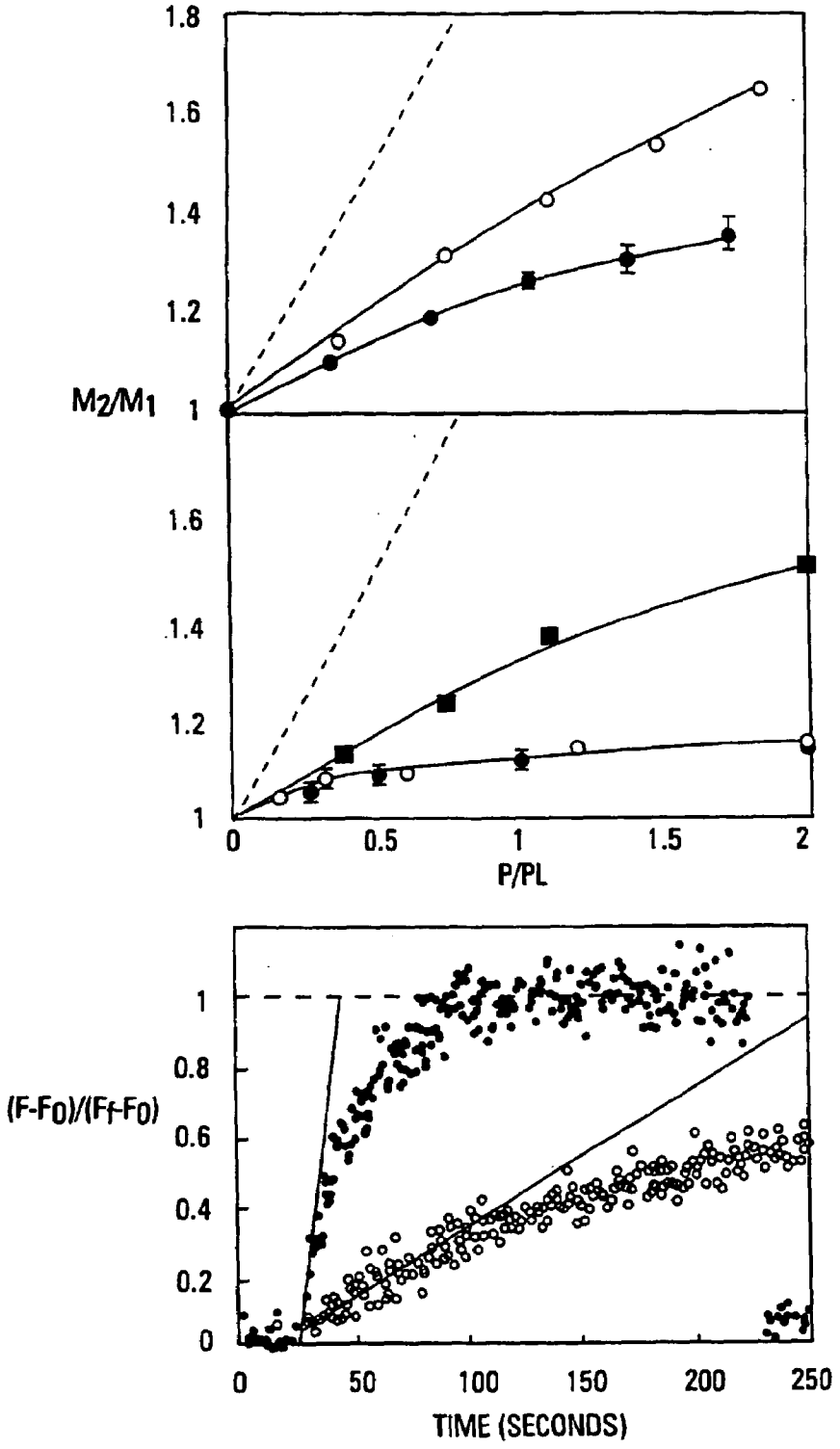

1.8
1.6
1.4
1.2
M2/M1
1
1.6
1.4
1.2
1
0
0.5
1
1.5
2
P/PL
(F-F0)/(Ff-F0)
1
0.8
0.6
0.4
0.2
0
0
50
100
150
200
250
TIME (SECONDS)

FIG. 15

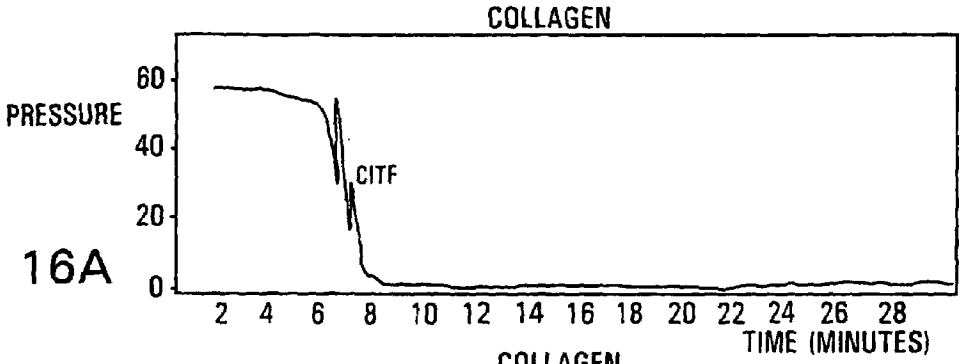


FIG. 16A

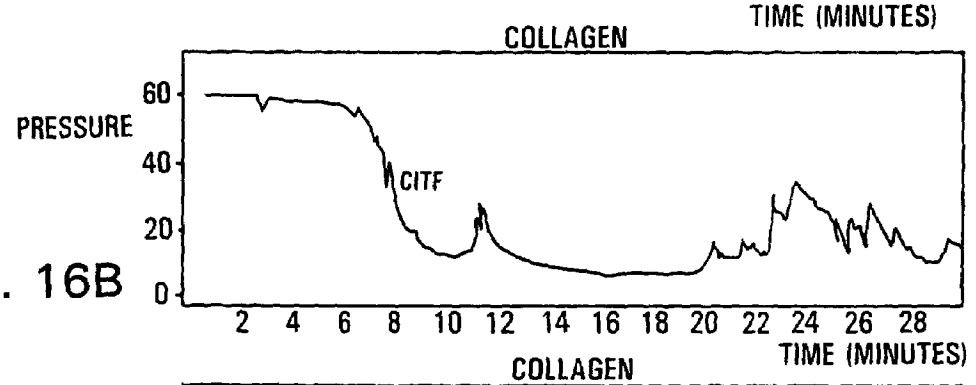


FIG. 16B

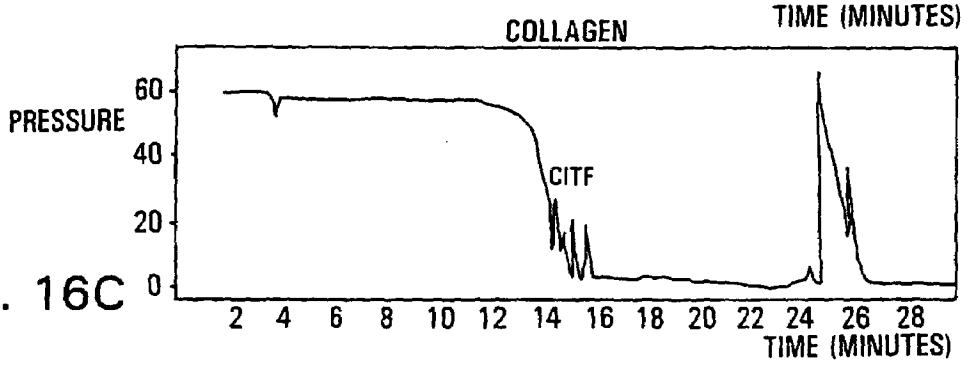


FIG. 16C

MODIFIED VITAMIN K-DEPENDENT POLYPEPTIDES

STATEMENT AS TO FEDERALLY-SPONSORED RESEARCH

Funding for work described herein was provided in part by the National Institutes of Health, grant no. HL15728. The federal government, has certain rights in the invention.

This application is a National Stage application under 35 U.S.C. § 371 and claims benefit under 35 U.S.C. § 119(a) of International Application No. PCT/US00/11416 having an International Filing Date of Apr. 28, 2000.

BACKGROUND OF THE INVENTION

Vitamin K-dependent proteins contain 9 to 13 gamma-carboxyglutamic acid residues (Gla) in their amino terminal 45 residues. The Gla residues are produced by enzymes in the liver that utilize vitamin K to carboxylate the side chains of glutamic acid residues in protein precursors. Vitamin K-dependent proteins are involved in a number of biological processes, of which the most well described is blood coagulation (reviewed in Furie, B. and Furie, B. C., 1988, *Cell,* 53:505–518). Vitamin K-dependent proteins include protein Z, protein S, prothrombin, factor X, factor IX, protein C, factor VII and Gas6. The latter protein functions in cell growth regulation. Matsubara et al., 1996, *Dev. Biol.,* 180: 499–510. The Gla residues are needed for proper calcium binding and membrane interaction by these proteins. The membrane contact site of factor X is thought to reside within amino acid residues 1–37. Evans and Nelsestuen, 1996, *Protein Sci.,* 5:suppl. 1, 163 Abs. Although the Gla-containing regions of the plasma proteins show a high degree of sequence homology, they have at least a 1000-fold range in membrane affinity. McDonald, J. F. et al., 1997, *Biochemistry,* 36:5120–5137.

Factor VII functions in the initial stage of blood clotting and may be a key element in forming blood clots. The inactive precursor, or zymogen, has low enzyme activity that is greatly increased by proteolytic cleavage at the R1521153 bond to form factor VIIa. This activation can be catalyzed by factor Xa as well as by VIIa-tissue factor, an integral membrane protein found in a number of cell types. Fiore, M. M., et al., 1994, *J. Biol. Chem.,* 269:143–149. Activation by VIIa-tissue factor is referred to as autoactivation. It is implicated in both the activation (formation of factor VIIa from factor VII) and the subsequent activity of factor VIIa. The most important pathway for activation in vivo is not known. Factor VIIa can activate blood-clotting factors IX and X.

Tissue factor is expressed at high levels on the surface of some tumor cells. A role for tissue factor, and for factor VIIa, in tumor development and invasion of tissues is possible. Vrana, J. A. et al., *Cancer Res.,* 56:5063–5070. Cell expression and action of tissue factor is also a major factor in toxic response to endotoxic shock. Dackiw, A. A. et al., 1996, *Arch. Sure.,* 131:1273–1278.

Protein C is activated by thrombin in the presence of thrombomodulin, an integral membrane protein of endothelial cells. Esmon, N. L. et al., 1982, *J. Biol. Chem.,* 257: 859–864. Activated protein C (APC) degrades factors Va and VIIa in combination with its cofactor, protein S. Resistance to APC is the most common form of inherited thrombosis disease. Dahlback, B., 1995, *Blood* 85:607–614. Vitamin K inhibitors are commonly administered as a prophylaxis for thrombosis disease.

Vitamin K-dependent proteins are used to treat certain types of hemophilia. Hemophilia A is characterized by the absence of active factor VIII, factor VIIIa, or the presence of inhibitors to factor VIII. Hemophilia B is characterized by the absence of active factor IX, factor IXa. Factor VII deficiency, although rare, responds well to factor VII administration. Bauer, K. A., 1996, *Haemostasis,* 26:155–158, suppl. 1. Factor VIII replacement therapy is limited due to development of high-titer inhibitory factor VIII antibodies in some patients. Alternatively, factor VIIa can be used in the treatment of hemophilia A and B. Factor IXa and factor VIIa activate factor X. Factor VIIa eliminates the need for factors IX and VIII by activating factor X directly, and can overcome the problems of factor IX and VIII deficiencies with few immunological consequences. Hedner et al., 1993, *Transfus. Medi. Rev.,* 7:78–83; Nicolaisen, E. M. et al., 1996, *Thromb. Haemost.,* 76:200–204. Effective levels of factor VIIa administration are often high (45 to 90 μg/kg of body weight) and administration may need to be repeated every few hours. Shulmav, S. et al., 1996, *Thromb. Haemost.,* 75:432–436.

A soluble form of tissue factor (soluble tissue factor or sTF) that does not contain the membrane contact region has been found to be efficacious in treatment of hemophilia when co-administered with factor VIIa. See, for example, U.S. Pat. No. 5,504,064. In dogs, sTF was shown to reduce the amount of factor VIIa needed to treat hemophilia. Membrane association by sTF-VIIa is entirely dependent on the membrane contact site of factor VII. This contrasts to normal tissue-factor VIIa complex, which is bound to the membrane through both tissue factor and VII (a).

SUMMARY OF THE INVENTION

It has been discovered that modifications within the γ-carboxyglutamic acid (GLA) domain of vitamin K-dependent polypeptides enhance their membrane binding affinities. Vitamin K-dependent polypeptides modified in such a manner have enhanced activity and may be used as anticoagulants, pro-coagulants, or for other functions that utilize vitamin K-dependent proteins. For example, an improved factor VII molecule may provide several benefits by lowering the dosage of VIIa needed, reducing the relative frequency of administration and/or by providing qualitative changes that allow more effective treatment of deficiency states.

The invention features vitamin K-dependent polypeptides that include a modified GLA domain that enhances membrane-binding affinity of the polypeptide relative to a corresponding native vitamin K-dependent polypeptide. In some embodiments, activity of the vitamin K-dependent polypeptide also is enhanced. The modified GLA domain is from about amino acid 1 to about amino acid 45 and includes at least one amino acid substitution. For example, the amino acid substitution can be at amino acids 2, 5, 9 11, 12, 29, 33, 34, 35, or 36, and combinations thereof. In particular, the substitution can be at amino acids 11, 12, 29, 33 or 34, amino acids 11, 12, 29, 34, or 35, amino acids 2, 5, or 9, amino acids 5, 9, 35, or 36, amino acids 11 or 12, amino acids 29 or 33, or amino acids 34, 35 or 36, and combinations thereof. The modified GLA domain may include an amino acid sequence, which, in the calcium saturated state, forms a tertiary structure having a cationic core with a halo of

electronegative charge. The vitamin K-dependent polypeptides can be used for treating clotting disorders and can increase or inhibit (decrease) clot formation.

The vitamin K-dependent polypeptide may be, for example, protein C, activated protein C, factor IX, factor IXa or active-site modified factor IXa, factor VII, factor VIIa or active site modified factor VIIa, protein S, protein Z, or factor Xa or active-site modified Xa. The modified GLA domain of protein C or activated protein C may include substitution of a glycine residue at amino acid 12 or a glutamic acid residue at amino acid 33. Further substitutions in the GLA domain of protein C or activated protein C can include one or more of a glutamine or glutamic acid residue at amino acid 11, a phenylalanine residue at amino acid 29, a glutamic acid residue at amino acid 33, a phenylalanine, leucine, isoleucine, or aspartic acid residue at amino acid 34, an aspartic or glutamic acid residue at amino acid 35, or a glutamic acid residue at amino acid 36.

The modified GLA domain of factor VII, factor VIIa, and active site modified factor VIIa may contain a substitution at amino acids 11, 29, 33, 34, or 35, and combinations thereof. For example, a glutamine, glutamic acid, aspartic acid, or an asparagine residue can be substituted at amino acid 11, a glutamic acid or phenylalanine residue can be substituted at amino acid 29, or a glutamic acid residue can be substituted at amino acid 33, and combinations thereof such as substitutions at 11 and 29, 11, 29, and 33, and 11 and 33. Substitution of a glutamine residue at amino acid 11 is particularly useful. In one embodiment, a glutamine residue is substituted at amino acid 11 and a glutamic acid residue is substituted at amino acid 33. The modified GLA domain further can include at least one hydrophobic residue at amino acids 34 or 35. Phenylalanine, leucine, or isoleucine residue may be substituted at amino acid 34, and/or an aspartic acid or glutamic acid residue at amino acid 35.

The modified GLA domain of protein S can include a substitution of an isoleucine, leucine, valine, or phenylalanine residue at amino acid 9. Further substitutions can include a phenylalanine, leucine, isoleucine, aspartic acid, or glutamic acid residue at amino acid 34 or an aspartic acid or glutamic acid residue at amino acids 35. The modified GLA domain of protein S can contain a phenylalanine residue at amino acid 5, and further can include a substitution in the thrombin-sensitive loop, such as at amino acids 49, 60, or 70. The modified GLA domain of active-site modified Factor IXa can include a phenylalanine residue at amino acid 29 or a phenylalanine, leucine, or isoleucine residue at amino acid 5, and combinations thereof. The modified GLA domain further can include a phenylalanine, leucine, isoleucine, an aspartic acid, or glutamic acid residue at amino acid 34 or an aspartic acid or glutamic acid residue at amino acid 35, or substitutions at both amino acids 34 and 35.

The modified GLA domain of active-site modified Factor Xa can include a glutamine at amino acid 11 or an aspartic acid or glutamic acid residue at amino acid 35. The modified GLA domain of protein Z can include an asparagine or glutamine residue at amino acid 2, a phenylalanine, leucine, or isoleucine residue at amino acid 34, or an aspartic acid or glutamic acid residue at amino acid 35.

The modified GLA domain of vitamin K-dependent polypeptides further can include an inactivated cleavage site. For example, factor VII can include an inactivated cleavage site, such as a substitution of an alanine residue at amino acid 152.

In another aspect, the invention features a vitamin K-dependent polypeptide that includes a modified GLA domain that enhances membrane binding affinity and activity of the polypeptide. The modified GLA domain of such a polypeptide includes at least one amino acid insertion at amino acid 4. The polypeptide can be factor VII or VIIa, protein C or activated protein C, factor X or Xa, or protein S. For example, the polypeptide can be factor VII or VIIa, or protein C or activated protein C, and can include the insertion of a tyrosine or glycine residue.

The invention also features a mammalian host cell that includes a vitamin K-dependent polypeptide. The polypeptide includes a modified GLA domain that enhances membrane-binding affinity of the polypeptide relative to a corresponding native vitamin K-dependent polypeptide. The modified GLA domain includes at least one amino acid substitution as described above. The vitamin K-dependent polypeptide may be, for example, factor VII or factor VIIa.

The invention also relates to a pharmaceutical composition that includes a pharmaceutically acceptable carrier and an amount of a vitamin K-dependent polypeptide effective to inhibit clot formation in a mammal. The vitamin K-dependent polypeptide includes a modified GLA domain that enhances membrane-binding affinity of the polypeptide relative to a corresponding native vitamin K-dependent polypeptide. In some embodiments, activity of the polypeptide also is enhanced. The modified GLA domain of the vitamin K-dependent polypeptide includes at least one amino acid substitution as described above. The vitamin K-dependent polypeptide may be, for example, protein C, activated protein C or active site modified factor VIIa, protein S, or active site modified factor IXa. The composition further can include an anticoagulant agent (e.g. aspirin).

The invention also features a pharmaceutical composition that includes a pharmaceutically acceptable carrier and an amount of a vitamin K-dependent polypeptide effective to increase clot formation in a mammal. The vitamin K-dependent polypeptide includes a modified GLA domain that enhances membrane-binding affinity of the polypeptide relative to a corresponding native vitamin K-dependent polypeptide. The modified GLA domain of the vitamin K-dependent polypeptide includes at least one amino acid substitution as described above. The vitamin K-dependent polypeptide may be, for example, factor VII, factor VIIa, factor IX or factor IXa. The pharmaceutical composition may also include soluble tissue factor.

A method of decreasing clot formation in a mammal is also described. The method includes administering an amount of a vitamin K-dependent polypeptide effective to decrease clot formation in the mammal. The vitamin K-dependent polypeptide includes a modified GLA domain that enhances membrane-binding affinity of the polypeptide relative to a corresponding native vitamin K-dependent polypeptide. In some embodiments, activity of the polypeptide also is enhanced. The modified GLA domain includes at least one amino acid substitution as described above. The vitamin K-dependent polypeptide may be, for example, protein C, activated protein C or active site modified factor VIIa or factor IXa, or protein S.

The invention also features a method of increasing clot formation in a mammal. The method includes administering an amount of a vitamin K-dependent polypeptide effective to increase clot formation in the mammal. The vitamin K-dependent polypeptide includes a modified GLA domain that enhances membrane-binding affinity of the polypeptide relative to a corresponding native vitamin K-dependent polypeptide. The modified GLA domain includes at least one amino acid substitution as described above. The vitamin K-dependent polypeptide may be, for example, factor VII, factor VIIa, factor IX or factor IXa.

In another aspect, the invention features a method for identifying a vitamin K-dependent polypeptide having enhanced membrane binding affinity and activity. The method includes modifying the GLA domain of the polypeptide, wherein modifying includes substituting at least one amino acid in the GLA domain; monitoring membrane binding affinity and activity of the polypeptide having the modified GLA domain; and identifying the modified vitamin K-dependent polypeptide as having enhanced membrane binding affinity and activity if membrane binding affinity activity of the modified polypeptide is enhanced relative to a corresponding native vitamin K-dependent polypeptide. Suitable substitutions are described above. The polypeptide can increase clot formation or inhibit clot formation.

The vitamin K-dependent polypeptides can be used in the manufacture of medicaments for the treatment of clotting disorders.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used to practice the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** depicts the binding, with standard deviations, of wild type VIIa (open circles), VIIQ11E33 (filled circles), and bovine factor X (filled triangles) to membranes.

FIG. **2** depicts the autoactivation of VIIQ11E33. The dashed line shows activity in the absence of phospholipid.

FIG. **3** depicts the activation of factor X by factor VIIa. Results for wild type factor VIIa (open circles) and VIIaQ11E33 (filled circles) are given for a concentration of 0.06 nM.

FIG. **4** depicts the coagulation of human plasma by VIIa and VIIaQ11E33 with soluble tissue factor.

FIG. **5** depicts the coagulation of plasma by factor VII zymogens and normal tissue factor.

FIG. **6** depicts the inhibition of clot formation by active site modified factor VIIaQ11E33 (DEGR-VIIaQ11E33).

FIG. **7** depicts the circulatory time of factor VIIaQ11E33 in rats.

FIG. **8** depicts the membrane interaction by normal and modified proteins. Panel A shows the interaction of wild type bovine protein C (open circles) and bovine protein C-H11 (filled circles) with vesicles. Panel B shows the interaction of wild type human protein C (open circles) and human protein C-P11 (filled circles) with membranes. In both cases, the dashed line indicates the result if all of the added protein were bound to the membrane.

FIG. **9** depicts the influence of activated protein C on clotting times. In panel A, the average and standard deviation for three determinations of clotting times for bovine plasma are shown for wild type bovine APC (open circles) and for bAPC-H11 (filled circles). In panel B, the average and standard deviation of three replicates of human plasma coagulation for the wild type human (open circles) and human APC-P11 (filled circles) is shown.

FIG. **10** depicts the inactivation of factor Va by bovine and human APC. Panel A depicts the inactivation of factor Va by wild type bovine APC (open circles) and bovine APC-H11 (filled circles). Panel B depicts the inactivation of human factor Va in protein S-deficient plasma by either wild type human APC (open circles) and human APC-H11 (filled circles).

FIG. **11** depicts the electrostatic distribution of protein Z. Vertical lines denote electropositive regions and horizontal lines denote electronegative regions

FIG. **12** depicts the membrane binding and activity of various protein Cs. Panel A shows membrane binding by wild type protein C (open circles), the P11H mutant of protein C (filled squares), Q33E, N34D mutant (filled circles) and bovine prothrombin (open squares). Panel B shows inhibition of blood coagulation by these mutants. Panel C shows the inactivation of factor Va.

FIG. **13** compares membrane binding and activity of human protein C mutants. Panel A compares the membrane binding of wild-type (open circles), E33 (open triangles) and E33D34 (filled circles). Panel B compares the coagulation times using wild-type (open triangles), E33 (open circles) and E33D34 (filled circles).

FIG. **14** compares membrane binding (Panel A) and coagulation inhibition (Panel B) with wild-type (open squares), H11 (filled circles), E33D34 (open triangles) and the triple H11E33D34 mutant (open circles) of bovine protein C.

FIG. **15** depicts the membrane interaction properties of different vitamin K-dependent proteins. The top panel compares membrane interaction of human (filled circles) and bovine (open circles) Factor X. The middle panel shows membrane interaction by normal bovine prothrombin fragment 1 (open circles), fragment 1 modified with TNBS in the absence of calcium (filled circles) and fragment 1 modified with TNBS in the presence of 25 mM calcium (filled squares). The bottom panel shows the rate of protein Z binding to vesicles at pH 9 (filled circles) and 7.5 (open circles).

FIGS. **16**A–**16**C are clot signature analyzer reports of collagen induced thrombus formation (CITF) for normal blood (A), for blood containing 30 nM wild type APC (B), and blood containing 6 nM Q11G12E33D34 APC(C).

DETAILED DESCRIPTION

In one aspect, the invention features a vitamin K-dependent polypeptide including a modified GLA domain with enhanced membrane binding affinity relative to a corresponding native vitamin K-dependent polypeptide. Activity of the vitamin K-dependent polypeptide also can be enhanced. Vitamin K-dependent polypeptides are a group of proteins that utilize vitamin K in their biosynthetic pathway to carboxylate the side chains of glutamic acid residues in protein precursors. The GLA domain contains 9–13 γ-carboxyglutamic acid residues in the N-terminal region of the polypeptide, typically from amino acid 1 to about amino acid 45. Protein Z, protein S, factor X, factor II (prothrombin), factor IX, protein C, factor VII and Gas6 are examples of vitamin K-dependent polypeptides. Amino acid positions of the polypeptides discussed herein are numbered according to factor IX. Protein S, protein C, factor X, factor VII and human prothrombin all have one less amino acid (position 4) and must be adjusted accordingly. For example, actual position 10 of bovine protein C is a proline, but is numbered herein as amino acid 11 for ease of comparison throughout.

As used herein, the term "polypeptide" is any chain of amino acids, regardless of length or post-translational modification. Amino acids have been designated herein by standard three letter and one-letter abbreviations.

Modifications of the GLA domain include at least one amino acid substitution (e.g, one to 10 substitutions). The substitutions may be conservative or non-conservative. Conservative amino acid substitutions replace an amino acid with an amino acid of the same class, whereas non-conservative amino acid substitutions replace an amino acid with an amino acid of a different class. Non-conservative substitutions may result in a substantial change in the hydrophobicity of the polypeptide or in the bulk of a residue side chain. In addition, non-conservative substitutions may make a substantial change in the charge of the polypeptide, such as reducing electropositive charges or introducing electronegative charges. Examples of non-conservative substitutions include a basic amino acid for a non-polar amino acid, or a polar amino acid for an acidic amino acid. The amino acid substitution may be at amino acid 2, 5, 9, 11, 12, 29, 33, 34, 35, or 36, and combinations thereof. The modified GLA domain may include an amino acid sequence, which, in the calcium-saturated state, contributes to formation of a tertiary structure having a cationic core with a halo of electronegative charge. The highest affinity proteins show an electronegative charge extending to amino acids 35 and 36. Without being bound by a particular theory, enhanced membrane affinity may result from a particular electrostatic pattern consisting of an electropositive core completely surrounded by an electronegative surface.

In addition, modifications of the GLA domain can include an insertion of a residue at position four of a vitamin K-dependent polypeptide. Suitable polypeptides lack an amino acid at this position (Protein S, protein C, factor X, factor VII, and human prothrombin), based on sequence alignments of vitamin K-dependent polypeptides.

Many vitamin K-dependent polypeptides are substrates for membrane-bound enzymes. Since no vitamin K-dependent polypeptides display the maximum potential membrane-binding affinity of a GLA domain, all must contain amino acids whose purpose is to reduce binding affinity. Consequently, many vitamin K-dependent polypeptides contain amino acids that are non-optimal from the standpoint of maximum membrane-binding affinity. These residues effectively disrupt the binding site to provide a more rapid turnover for an enzymatic reaction.

Lowered membrane affinity may serve several purposes. High affinity is accompanied by slow exchange, which may limit reaction rates. For example, when the prothrombinase enzyme is assembled on membranes with high affinity for substrate, protein exchange from the membrane, rather than enzyme catalysis, is the limiting step. Lu, Y. and Nelsestuen, G. L., 1996, *Biochemistry,* 35:8201–8209. Alternatively, adjustment of membrane affinity by substitution with non-optimum amino acids may balance the competing processes of procoagulation (factor X, IX, VII, and prothrombin) and anticoagulation (protein C, S). Although membrane affinities of native proteins may be optimal for normal states, enhancement of membrane affinity can produce proteins that are useful for in vitro study as well as improved therapeutics for regulating blood clotting in pathological conditions in vivo.

Various examples of GLA domain modified vitamin K-dependent polypeptides are described below.

The vitamin K-dependent polypeptide may be protein C or activated protein C (APC). Amino acid sequences of the wild-type human (hC, SEQ ID NO:1) and bovine (bC, SEQ ID NO:2) protein C GLA domains are shown in Table 1. X is a Gla or Glu residue. In general, a protein with neutral (e.g., Q) or anionic residues (e.g., D, E) at positions 11, 33, 34, 35, and 36 will have higher membrane affinity.

TABLE 1

hC:	ANS-FLXXLRH$_{11}$SSLXRXCIXX$_{21}$ICDFXXAKXI$_{31}$FQNVDDTLAF$_{41}$WSKH
bC:	ANS-FLXXLRP$_{11}$GNVXRXCSXX$_{21}$VCXFXXARXI$_{31}$FQNTXDTMAF$_{41}$WSFY

The GLA domain of protein C or APC can contain a substitution at amino acids 11, 12, 29, 33, 34, 35, or 36, and combinations thereof. The modified GLA domain may include, for example, a glutamic acid or aspartic acid residue at position 33. The modified GLA domain may include a substitution at amino acid 12 of a glycine residue for serine, and further may include a glutamic acid residue at amino acid 33 and an aspartic acid or glutamic acid residue at amino acid 34. The glutamic acid at position 33 may be further modified to γ-carboxyglutamic acid in vivo. For optimum activity, the modified GLA domain may include an additional substitution at amino acid 11. For example, a glutamine residue may be substituted at amino acid 11 or, alternatively, a glutamic acid or an aspartic acid residue may be substituted. A histidine residue also may be substituted at amino acid 11 in bovine protein C. Replacement of amino acid 29 by phenylalanine, the amino acid found in prothrombin, is another useful modification. Phenylalanine, leucine, or isoleucine may be substituted at position 34. Glutamic acid or aspartic acid residues also can be substituted at amino acid 35 or a glutamic acid residue at amino acid 36. Thus, modified protein C may contain, for example, substitutions at one or more of 11, 12, 29, 33, 34, 35, or 36, for example, at residues 12 and 33, 12 and 34, 33 and 34, 12 and 35, 33 and 35, 12 and 36, 33 and 36, 11, 12, 33, and 34, 12, 33, and 34, 12, 33, and 35, 12, 33, 34, and 35, or 12, 33, 34, 35, and 36. Modified protein C with enhanced membrane binding affinity may be used in place of, or in combination with, other injectable anticoagulants such as heparin. Heparin is typically used in most types of surgery, but suffers from a low efficacy/toxicity ratio. APC containing a glutamine at 11, a glycine at 12, a glutamic acid at 33, and an aspartic acid at 34 is at least 10-fold more effective than wild type APC for inhibition of clot formation. In addition, modified protein C with enhanced membrane affinity may be used in place of, or in combination with, oral anticoagulants, including aspirin and anticoagulants in the coumarin family, such as warfarin.

These modifications also can be made with active site modified APC. The active site of APC may be inactivated chemically, for example by N-dansyl-glutamyl glycylarginylchloromethylketone (DEGR), phenylalanyl—phenylalanyl arginylchloromethylketone (FFR), or by site-directed mutagenesis of the active site. Sorensen, B. B. et al., 1997, *J. Biol. Chem.,* 272:11863–11868. Active site-modified APC functions as an inhibitor of the prothrombinase complex.

Enhanced membrane affinity of active site modified APC may result in a more therapeutically effective polypeptide.

The vitamin K-dependent polypeptide may be factor VII or the active form of factor VII, factor VIIa. Native or naturally occurring factor VII polypeptide has low affinity for membranes. Amino acid sequences of the wild-type human (hVII, SEQ ID NO: 3) and bovine (bVII, SEQ ID NO: 4) factor VII GLA domains are shown in Table 2.

TABLE 2

hVII:	ANA-FLXXLRP$_{11}$GSLXRXCKXX$_{21}$QCSFXXARXI$_{31}$FKDAXRTKLF$_{41}$WISY
bVII:	ANG-FLXXLRP$_{11}$GSLXRXCRXX$_{21}$LCSFXXAHXI$_{31}$FRNXXRTRQF$_{41}$WVSY

The GLA domain of factor VII or VIIa can contain a substitution, for example at amino acid 11, 29, 33, 34, 35, or 36 and combinations thereof. The modified GLA domain of factor VII or factor VIIa may include, for example, a glutamic acid, a glutamine, an asparagine, or an aspartic acid residue at amino acid 11, a phenylalanine or a glutamic acid residue at amino acid 29, or an aspartic acid or a glutamic acid residue at amino acids 33 or 35. Other neutral residues also may be substituted at these positions. The modified GLA domain can include hydrophobic residues at one or more of positions 34, 35, and 36. The modified GLA domain can include combinations of such substitutions at amino acid residues 11 and 29, 11 and 33, 11 and 35, 11, 33, and 35, 11, 29, and 33, 11, 29, 33, and 35, 29 and 33, 29 and 35, or 29, 33, and 35. For example, the GLA domain of factor VII or factor VIIa may include a glutamine residue at amino acid 11 and a glutamic acid residue at amino acid 33, or a glutamine residue at amino acid 11 and a phenylalanine residue at amino acid 29. The modified GLA domain also may include a substitution at amino acid 34 in combination with one or more substitutions described above at positions 11, 29, 33, and 35. For example, a phenylalanine residue or other hydrophobic residues such as leucine or isoleucine can be substituted at amino acids 34, 35, and/or 36. In addition, the modified GLA domain can include an insertion at position 4 alone (e.g, a tyrosine or glycine residue) or in combination with substitutions described above. Factor VII containing an insertion of a tyrosine residue at position 4 in combination with a glutamine at 11, a glutamic acid at 33, and a phenylalanine at 34 resulted in 160-fold higher activity that wild type factor VIIa.

Factor VII or VIIa modified in these manners has a much higher affinity for membranes than the native or wild type polypeptide. Factor VII or VIIa also have a much higher activity in autoactivation, in factor Xa generation and in several blood clotting assays. Activity is particularly enhanced at marginal coagulation conditions, such as low levels of tissue factor and/or phospholipid. For example, modified factor VII is about 4 times as effective as native VIIa at optimum thromboplastin levels, but is about 20-fold as effective at 1% of optimum thromboplastin levels. Marginal procoagulation signals are probably most predominant in vivo. Presently available clotting assays that use optimum levels of thromboplastin cannot detect clotting time differences between normal plasma and those from hemophilia patients. Clotting differences between such samples are only detected when non-optimal levels of thromboplastin or dilute thromboplastin are used in clotting assays.

Another example of a vitamin K-dependent polypeptide is active site modified Factor VIIa. The active site of factor VIIa may be modified chemically, for example by DEGR, FFR, or by site-directed mutagenesis of the active site. DEGR-modified factor VII is an effective inhibitor of coagulation by several routes of administration. Arnljots, B. et al., 1997, *J. Vasc. Surg.,* 25:341–346. Modifications of the GLA domain may make active site modified Factor VIIa more efficacious. Suitable substitutions or insertions are described above.

The vitamin K-dependent polypeptide may also be Factor IX or the active form of Factor IX, Factor IXa. As with active site-modified factor VIIa, active site modified IXa may be an inhibitor of coagulation. Active site modified factor IXa can bind its cofactor, Factor VIII, but will not form blood clots. Active site modified Factor IXa (wild-type) prevents coagulation without increase of bleeding in an animal model of stroke. See, for example, Choudhri et al., *J. Exp. Med.,* 1999, 190:91–99.

Amino acid sequences of the wild-type human (hIX, SEQ ID NO:5) and bovine (bIX, SEQ ID NO:6) factor IX GLA domains are shown in Table 3. For example, a valine, leucine, phenylalanine, or isoleucine residue may be substituted at amino acid 5, an aspartic acid or glutamic acid residue may be substituted at amino acid 11, a phenylalanine residue at amino acid 29, or an aspartic acid or glutamic acid residue at amino acids 34 or 35, and combinations thereof.

TABLE 3

hIX:	YNSGKLXXFVQ$_{11}$GNLXRXCMXX$_{21}$KCSFXXARXV$_{31}$FXNTXRTTXF$_{41}$WKQY
bIX:	YNSGKLXXFVQ$_{11}$GNLXRXCMXX$_{21}$KCSFXXARXV$_{31}$FXNTXKRTTXF$_{41}$WKQY

A further example of a vitamin K-dependent polypeptide is protein S. The amino acid sequence of human protein S (hPS, SEQ ID NO:19) is shown in Table 4. The modified GLA domain of Protein S can have, for example, a substitution at amino acids 5, 9, 34, or 35, and combinations thereof. For example, a phenylalanine can be substituted at amino acid 5, an isoleucine, leucine, valine, or phenylalanine residue at amino acid 9, or an aspartic acid or glutamic acid residue at amino acids 34 or 35. In addition to the at least one substitution in the GLA domain, protein S further can include a substitution in the thrombin sensitive loop. In particular, residues 49, 60, or 70 of the thrombin sensitive loop, which each are arginine residues, can be replaced with, for example, alanine residues.

TABLE 4

hPS: ANS-LLXXTKQ$_{11}$GNLXRXCIXX$_{21}$LCNKXXARXV$_{31}$FXNDPXTDYF$_{41}$YPKY

The vitamin K-dependent polypeptides of the invention also can include an inactivated cleavage site such that the polypeptides are not converted to an active form. For example, Factor VII containing an inactivated cleavage site would not be converted to Factor VIIa, but would still be able to bind tissue factor. In general, an arginine residue is found at the cleavage site of vitamin K-dependent polypeptides. Any residue can be substituted for the arginine at this position to inactivate the cleavage site. In particular, an alanine residue could be substituted at amino acid 152 of Factor VII. Vitamin K-dependent polypeptides of the invention that further contain an inactivated cleavage site act as inhibitors.

In another aspect, the invention features a mammalian host cell including a vitamin K-dependent polypeptide having a modified GLA domain that enhances membrane-binding affinity of the polypeptide relative to a corresponding native vitamin K-dependent polypeptide. Activity of the vitamin K-dependent polypeptides can be enhanced in some embodiments. Suitable vitamin K-dependent polypeptides and modifications of the GLA domain are discussed above. The mammalian host cell may include, for example, modified factor VII or modified factor VIIa. The GLA domain of modified factor VII or modified factor VIIa may contain an amino acid substitution at amino acid 11 and at amino acid 33. Preferably, the amino acid substitution includes a glutamine residue at amino acid 11 and a glutamic acid residue at amino acid 33 of factor VII or VIIa. Suitable mammalian host cells are able to modify vitamin K-dependent polypeptide glutamate residues to γ-carboxyglutamate. Mammalian cells derived from kidney and liver are especially useful as host cells.

Nucleic Acids Encoding Modified Vitamin K-Dependent Polypeptides

Isolated nucleic acid molecules encoding modified vitamin K-dependent polypeptides of the invention can be produced by standard techniques. As used herein, "isolated" refers to a sequence corresponding to part or all of a gene encoding a modified vitamin K-dependent polypeptide, but free of sequences that normally flank one or both sides of the wild-type gene in a mammalian genome. An isolated polynucleotide can be, for example, a recombinant DNA molecule, provided one of the nucleic acid sequences normally found immediately flanking that recombinant DNA molecule in a naturally-occurring genome is removed or absent. Thus, isolated polynucleotides include, without limitation, a DNA that exists as a separate molecule (e.g., a cDNA or genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences as well as recombinant DNA that is incorporated into a vector, an autonomously replicating plasmid, a virus (e.g., a retrovirus, adenovirus, or herpes virus), or into the genomic DNA of a prokaryote or eukaryote. In addition, an isolated polynucleotide can include a recombinant DNA molecule that is part of a hybrid or fusion polynucleotide.

It will be apparent to those of skill in the art that a polynucleotide existing among hundreds to millions of other polynucleotides within, for example, cDNA or genomic libraries, or gel slices containing a genomic DNA restriction digest, is not to be considered an isolated polynucleotide.

Isolated nucleic acid molecules are at least about 14 nucleotides in length. For example, the nucleic acid molecule can be about 14 to 20, 20–50, 50–100, or greater than 150 nucleotides in length. In some embodiments, the isolated nucleic acid molecules encode a full-length modified vitamin K-dependent polypeptide. Nucleic acid molecules can be DNA or RNA, linear or circular, and in sense or antisense orientation.

Specific point changes can be introduced into the nucleic acid sequence encoding wild-type vitamin K-dependent polypeptides by, for example, oligonucleotide-directed mutagenesis. In this method, a desired change is incorporated into an oligonucleotide, which then is hybridized to the wild-type nucleic acid. The oligonucleotide is extended with a DNA polymerase, creating a heteroduplex that contains a mismatch at the introduced point change, and a single-stranded nick at the 5' end, which is sealed by a DNA ligase. The mismatch is repaired upon transformation of *E. coli* or other appropriate organism, and the gene encoding the modified vitamin K-dependent polypeptide can be re-isolated from *E. coli* or other appropriate organism. Kits for introducing site-directed mutations can be purchased commercially. For example, Muta-Gene7 in-vitro mutagenesis kits can be purchased from Bio-Rad Laboratories, Inc. (Hercules, Calif.).

Polymerase chain reaction (PCR) techniques also can be used to introduce mutations. See, for example, Vallette et al., Nucleic Acids Res., 1989, 17(2):723–733. PCR refers to a procedure or technique in which target nucleic acids are amplified. Sequence information from the ends of the region of interest or beyond typically is employed to design oligonucleotide primers that are identical in sequence to opposite strands of the template to be amplified, whereas for introduction of mutations, oligonucleotides that incorporate the desired change are used to amplify the nucleic acid sequence of interest. PCR can be used to amplify specific sequences from DNA as well as RNA, including sequences from total genomic DNA or total cellular RNA. Primers are typically 14 to 40 nucleotides in length, but can range from 10 nucleotides to hundreds of nucleotides in length. General PCR techniques are described, for example in PCR Primer: A Laboratory Manual, Ed. by Dieffenbach, C. and Dveksler, G., Cold Spring Harbor Laboratory Press, 1995.

Nucleic acids encoding modified vitamin K-dependent polypeptides also can be produced by chemical synthesis, either as a single nucleic acid molecule or as a series of oligonucleotides. For example, one or more pairs of long oligonucleotides (e.g., >100 nucleotides) can be synthesized that contain the desired sequence, with each pair containing a short segment of complementarity (e.g., about 15 nucleotides) such that a duplex is formed when the oligonucleotide pair is annealed. DNA polymerase is used to extend the oligonucleotides, resulting in a double-stranded nucleic acid molecule per oligonucleotide pair, which then can be ligated into a vector.

Production of Modified Vitamin K-Dependent Polypeptides

Modified vitamin K-dependent polypeptides of the invention can be produced by ligating a nucleic acid sequence encoding the polypeptide into a nucleic acid construct such as an expression vector, and transforming a bacterial or

eukaryotic host cell with the expression vector. In general, nucleic acid constructs include a regulatory sequence operably linked to a nucleic acid sequence encoding a vitamin K-dependent polypeptide. Regulatory sequences do not typically encode a gene product, but instead affect the expression of the nucleic acid sequence. As used herein, "operably linked" refers to connection of the regulatory sequences to the nucleic acid sequence in such a way as to permit expression of the nucleic acid sequence. Regulatory elements can include, for example, promoter sequences, enhancer sequences, response elements, or inducible elements.

In bacterial systems, a strain of *E. coli* such as BL-21 can be used. Suitable *E. coli* vectors include without limitation the pGEX series of vectors that produce fusion proteins with glutathione S-transferase (GST). Transformed *E. coli* are typically grown exponentially then stimulated with isopropylthiogalactopyranoside (IPTG) prior to harvesting. In general, such fusion proteins are soluble and can be purified easily from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites such that the cloned target gene product can be released from the GST moiety.

In eukaryotic host cells, a number of viral-based expression systems can be utilized to express modified vitamin K-dependent polypeptides. A nucleic acid encoding vitamin K-dependent polypeptide can be cloned into, for example, a baculoviral vector such as pBlueBac (Invitrogen, San Diego, Calif.) and then used to co-transfect insect cells such as *Spodoptera frugiperda* (Sf9) cells with wild-type DNA from *Autographa californica* multiply enveloped nuclear polyhedrosis virus (AcMNPV). Recombinant viruses producing the modified vitamin K-dependent polypeptides can be identified by standard methodology. Alternatively, a nucleic acid encoding a vitamin K-dependent polypeptide can be introduced into a SV40, retroviral, or vaccinia based viral vector and used to infect suitable host cells.

Mammalian cell lines that stably express modified vitamin K-dependent polypeptides can be produced by using expression vectors with the appropriate control elements and a selectable marker. For example, the eukaryotic expression vector pcDNA.3.1+ (Invitrogen, San Diego, Calif.) is suitable for expression of modified vitamin K-dependent polypeptides in, for example, COS cells, HEK293 cells, or baby hamster kidney cells. Following introduction of the expression vector by electroporation, DEAE dextran-, calcium phosphate-, liposome-mediated transfection, or other suitable method, stable cell lines can be selected. Alternatively, transiently transfected cell lines are used to produce modified vitamin K-dependent polypeptides. Modified vitamin K-dependent polypeptides also can be transcribed and translated in vitro using wheat germ extract or rabbit reticulocyte lysate.

Modified vitamin K-dependent polypeptides can be purified from conditioned cell medium by applying the medium to an immunoaffinity column. For example, an antibody having specific binding affinity for Factor VII can be used to purify modified Factor VII. Alternatively, concanavalin A (Con A) chromatography and anion-exchange chromatography (e.g., DEAE) can be used in conjunction with affinity chromatography to purify factor VII. Calcium dependent or independent monoclonal antibodies that have specific binding affinity for factor VII can be used in the purification of Factor VII.

Modified vitamin K-dependent polypeptides such as modified protein C can be purified by anion-exchange chromatography, followed by immunoaffinity chromatography using an antibody having specific binding affinity for protein C.

Modified vitamin K-dependent polypeptides also can be chemically synthesized using standard techniques. See, Muir, T. W. and Kent, S. B., Curr. Opin. Biotechnol., 1993, 4(4):420–427, for a review of protein synthesis techniques.

Pharmaceutical Compositions

The invention also features a pharmaceutical composition including a pharmaceutically acceptable carrier and an amount of a vitamin K-dependent polypeptide effective to inhibit clot formation in a mammal. The vitamin K-dependent polypeptide includes a modified GLA domain with at least one amino acid substitution or insertion that enhances membrane-binding affinity of the polypeptide relative to a corresponding native vitamin K-dependent polypeptide. In some embodiments, activity of the vitamin K-dependent polypeptide also is enhanced. Useful modified vitamin K-dependent polypeptides of the pharmaceutical compositions can include, without limitation, protein C or APC, active-site modified APC, active-site modified factor VIIa, active-site modified factor IXa, active-site modified factor Xa, or Protein S, as discussed above. Pharmaceutical compositions also can include an anticoagulant agent such as aspirin, warfarin, or heparin.

The concentration of a vitamin K-dependent polypeptide effective to inhibit clot formation in a mammal may vary, depending on a number of factors, including the preferred dosage of the compound to be administered, the chemical characteristics of the compounds employed, the formulation of the compound excipients and the route of administration. The optimal dosage of a pharmaceutical composition to be administered may also depend on such variables as the overall health status of the particular patient and the relative biological efficacy of the compound selected. These pharmaceutical compositions may be used to regulate coagulation in vivo. For example, the compositions may be used generally for the treatment of thrombosis. Altering only a few amino acid residues of the polypeptide as described above, generally does not significantly affect the antigenicity of the mutant polypeptides.

Vitamin K-dependent polypeptides that include modified GLA domains may be formulated into pharmaceutical compositions by admixture with pharmaceutically acceptable non-toxic excipients or carriers. Such compounds and compositions may be prepared for parenteral administration, particularly in the form of liquid solutions or suspensions in aqueous physiological buffer solutions; for oral administration, particularly in the form of tablets or capsules; or for intranasal administration, particularly in the form of powders, nasal drops, or aerosols. Compositions for other routes of administration may be prepared as desired using standard methods.

Formulations for parenteral administration may contain as common excipients sterile water or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, hydrogenated naphthalenes, and the like. In particular, biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxethylene-polyoxypropylene copolymers are examples of excipients for controlling the release of a compound of the invention in vivo. Other suitable parenteral delivery systems include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation administration may contain excipients such as lactose, if desired. Inhalation formulations may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether,

glycocholate and deoxycholate, or they may be oily solutions for administration in the form of nasal drops. If desired, the compounds can be formulated as gels to be applied intranasally. Formulations for parenteral administration may also include glycocholate for buccal administration.

In an alternative embodiment, the invention also features a pharmaceutical composition including a pharmaceutically acceptable carrier and an amount of a vitamin K-dependent polypeptide effective to increase clot formation in a mammal. The vitamin K-dependent polypeptide includes a modified GLA domain with at least one amino acid substitution that enhances membrane binding affinity of the polypeptide relative to a corresponding native vitamin K-dependent polypeptide. These pharmaceutical compositions may be useful for the treatment of clotting disorders such as hemophilia A, hemophilia B and liver disease.

In this embodiment, useful vitamin K-dependent polypeptides of the pharmaceutical compositions can include, without limitations, Factor VII or the active form of Factor VII, Factor VIIa. The modified GLA domain of Factor VII or Factor Vila may include substitutions at amino acid 11 and amino acid 33, for example, a glutamine residue at amino acid 11 and a glutamic acid residue at amino acid 33. The pharmaceutical composition may further comprise soluble tissue factor. Factor VII is especially critical to blood coagulation because of its location at the initiation of the clotting cascade, and its ability to activate two proteins, factors IX and X. Direct activation of factor X by factor VIIa is important for possible treatment of the major forms of hemophilia, types A and B, since the steps involving factors IX and VIII are bypassed entirely. Administration of factor VII to patients has been found to be efficacious for treatment of some forms of hemophilia. Improvement of the membrane affinity of factor VII or VIIa by modification of the GLA domain provides the potential to make the polypeptide more responsive to many coagulation conditions, to lower the dosages of VII/VIIa needed, to extend the intervals at which factor VII/VIIa must be administered, and to provide additional qualitative changes that result in more effective treatment. Overall, improvement of the membrane contact site of factor VII may increase both its activation rate as well as improve the activity of factor VIIa on factor X or IX. These steps may have a multiplicative effect on overall blood clotting rates in vivo, resulting in a very potent factor VIIa for superior treatment of several blood clotting disorders.

Other useful vitamin K-dependent polypeptides for increasing clot formation include Factor IX and Factor IXa, and Factor X and Xa.

In another aspect, methods for decreasing clot formation in a mammal are described. The method includes administering an amount of vitamin K-dependent polypeptide effective to decrease clot formation in the mammal. The vitamin K-dependent polypeptide includes a modified GLA domain that enhances membrane-binding affinity of the polypeptide relative to a corresponding native vitamin K-dependent polypeptide. In some embodiments, activity also is enhanced. The modified GLA domain includes at least one amino acid substitution. Modified protein C or APC, protein S, or active-site modified factors VIIa, IXa, and Xa containing substitutions in the GLA domain may be used for this method. The method further can include administering an anticoagulant agent.

In another aspect, the invention also features methods for increasing clot formation in a mammal that includes administering an amount of vitamin K-dependent polypeptide effective to increase clot formation in the mammal. The vitamin K-dependent polypeptide includes a modified GLA domain that enhances membrane-binding affinity of the polypeptide relative to a corresponding native vitamin K-dependent polypeptide. The modified GLA domain includes at least one amino acid substitution. Modified factor VII or VIIa and modified factor IX or IXa may be used in this method.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

EXAMPLES

Example 1

Factor VII with Enhanced Membrane Affinity and Activity

It has been found that the membrane binding affinity of human blood clotting factor VII can be increased by site-directed mutagenesis. The properties of a P11Q,K33E mutant (referred to herein as Factor VIIQ11E33 or mutant factor VII) have been characterized. Membrane affinity was increased over wild type protein by about 20-fold. Autoactivation by the mutant was increased by at least 100-fold over that of wild type factor VII. The activated form of VIIQ11E33 (referred to as VIIaQ11E33) displayed about 10-fold higher activity toward factor X. The coagulation activity of VIIaQ11E33 with soluble tissue factor in normal plasma was about 10-fold higher than that of wild type VIIa. Coagulation activity of the zymogen, VIIQ11E33, with normal tissue factor (supplied as a 1:100 dilution of thromboplastin-HS), was 20-fold higher than wild type Factor VII. The degree to which activity was enhanced was dependent on conditions, with VIIQ11E33 being especially active under conditions of low coagulation stimuli.

In general, protein concentrations were determined by the Bradford assay using bovine serum albumin as the standard. Bradford, M. M., 1976, *Analyt. Biochem.* 248–254. Molar concentrations were obtained from the molecular weights of 50,000 for factor VII and 55,000 for factor X. Unless indicated, all activity measurements were conducted in standard buffer (0.05 M Tris, pH 7.5, 100 mM NaCl).

Production of Mutant Factor VII: Mutant factor VII was generated from wild type factor VII cDNA (GenBank Accession number M13232, NID g182799). Petersen et al., 1990, *Biochemistry* 29:3451–3457. The P11Q mutation (change of amino acid 11 from a proline residue to a glutamine residue) and the K33E mutation (change of amino acid 33 from a lysine residue to a glutamic acid residue) were introduced into the wild type factor VII cDNA by a polymerase chain reaction strategy essentially as described by Vallette et al., 1989, *Nucleic Acids Res.* 17:723–733. During this process, a mutation-diagnostic XmaIII restriction enzyme site was eliminated. Four PCR primers were designed to prime synthesis of two mutant fragments of M113232, one from MluI to BglII positions 221 to 301, and the other from BglII to SstII, positions 302 to 787. These primers were used under standard PCR cycling conditions (GENEAMP, Perkin Elmer) to prime fragment synthesis using 1 ng of the wild-type factor VII cDNA as template. The resulting fragments were gel purified and digested with MluI and BglII or BglII and SstII. The two purified fragments were then ligated into the factor VIII cDNA in the expression vector Zem219b from which the corresponding wild-type sequence had been removed as a MluI-SstII fragment. Petersen et al., 1990 supra. The mutated fragments

were sequenced in their entirety to confirm the P11Q and K33E substitutions, as well as to eliminate the possibility of other PCR-induced sequence changes.

Transfection, Selection and Purification: Baby hamster kidney (BHK) cells were grown in Dubeccos modified Eagles medium supplemented with 10% fetal calf serum and penicillin-streptomycin. Subconfluent cells were transfected with the factor VII expression plasmid using lipofectAMINE (Gibco BRL) according to the manufacturers recommendations. Two days post-transfection, cells were trypsinized and diluted to selective medium containing 1 μM methotrexate (MTX). Stably-transfected BHK cells were subsequently cultured in serum-free Dubeccos modified Eagles medium supplemented with penicillin-streptomycin, 5 μg/mL vitamin K_1 and 1 μM MTX, and conditioned medium was collected. The conditioned medium was applied twice to an immunoaffinity column composed of a calcium-dependent monoclonal antibody (CaFVII122) coupled to Affi-Gel 10. Nakagaki et al., 1991, *Biochemistry,* 30:10819–10824. The final purified Factor VIIQ11E33 ran as a single band on SDS polyacrylamide gel electrophoresis, with no evidence of factor VIIa in the preparation. The pure VII(P11Q,K33E) mutant showed 1400–2800 factor VII units/mg.

Activation of Factor VII: Activated Factor VIIaQ11E33 was formed by bovine factor Xa cleavage of VIIQ11E33 (1:100 weight ratio, incubation for 1 hr at 37° C.). Alternatively, Factor VIIaQ11E33 was obtained by autoactivation (37° C., 20 min) in a mixture containing 7 μM VIIQ11E33, 0.7 μM sTF and phospholipid (phosphatidylserine/phosphatidylcholine (PS/PC), 25/75, 0.1 g/g protein).

Wild-type factor VIIa was a homogeneous, recombinant protein (NOVO Nordisk). Two preparations consisted of a commercial, lyophilized product and non-lyophilized product. The latter protein was further purified on FPLC mono-Q and showed a specific activity of 80,000 units/mg, calibrated with a George King NPP standard.

Enhanced membrane interaction by Factor VIIQ11E33: Phospholipid preparation, assay and measurement of protein-membrane binding was conducted by the method described by Nelsestuen and Lim, 1977, *Biochemistry* 30:10819–10824. Large unilamellar vesicles (LUVs) and small unilamellar vesicles (SUVs) were prepared by methods described previously. Hope, M. J., et al., *Biochem. Biophys. Acta.,* 812:55–65; Huang, C., 1969, *Biochemistry* 8:344–352. Highly pure phosphatidylserine (bovine brain) and egg phosphatidylcholine (Sigma Chemical Co.) were mixed in chloroform. The solvent was removed by a stream of nitrogen gas. The dried phospholipids were suspended in buffer. SUVs were formed by sonication and gel filtration while LUVs were formed by freeze-thaw and extrusion. Phospholipid concentrations were determined by organic phosphate assay assuming a phosphorous:phospholipid weight ratio of 25.

SUVS of either PS/PC (25/75) or PS/PC (10/90) were prepared. Protein was added to phospholipid at the weight ratios shown in FIG. **1**. Protein-membrane binding was assayed by light scattering at 90° by the method of Nelsestuen and Lim, 1977 supra. Briefly, the light scattering intensity of phospholipid vesicles alone (I_1) and after addition of protein (I_2) were measured and corrected for background from buffer and unbound protein. The molecular weight ratio of the protein-vesicle complex (M_2) to that of the vesicles alone (M1), can be estimated from the relation ship in equation 1, where δn/δc is the refractive index of the respective species.

$$I_2/I_1=(M_2/M_1)^2(\delta n/\delta c_2/\delta n/\delta c_1)^2 \quad \text{(eq. 1)}$$

If phospholipid and protein concentrations are known, the concentration of bound [P*PL] and free protein [P] can be estimated. These values, together with the maximum protein binding capacity [P^*PL_{max}] of the vesicles (assumed to be 1.0 g/g for all proteins) can be used to obtain the equilibrium constant for protein-membrane interaction by the relationship in equation 2, where all concentrations are expressed as molar protein or protein binding sites.

$$K_D=[P][P^*PL_{max}-P^*PL]/[P^*PL] \quad \text{(eq. 2)}$$

Binding was assessed at 5 mM calcium and is expressed as the ratio, M2/M1.

FIG. **1** shows the binding of wild type VIIa (open circles) and factor VIIQ11E33 (filled circles) to membranes of either PS/PC=25/75, 25 μg/ml (FIG. **1**A) or PS/PC=10/90, 25 μg/ml (FIG. **1**B). VIIQ11E33 had much higher affinity than wild type protein. Binding to PS/PC (25/75) was at the quantitative level so that [$Protein_{free}$] was essentially zero. Consequently, Kd values could not be estimated from this data. Membrane binding of bovine factor X (filled triangles) is shown in FIG. **1** as a reference. Bovine factor X is one of the highest affinity proteins in this family, giving a Kd for PS/PC (20/80) at 2 mM calcium of 40 nM. McDonald et al., 1997, *Biochemistry,* 36:5120–5127. The Kd for bovine factor X, obtained from the result at a protein/phospholipid ratio of 0.55 (FIG. **1**), was 0.025 μM.

Binding of wild-type and mutant Factor VII to membranes of PS/PC (10/90) was also determined (FIG. **1**B). The VIIQ11E33 bound at less than the quantitative level, which allowed a binding constant to be estimated from the relationship in equation 3.

$$Kd=[Protein_{free}][Binding\ sites_{free}]/[Protein_{bound}] \quad \text{(eq. 3)}$$

[$Binding\ sites_{free}$] were estimated from equation 4, assuming a maximum M2/M1 of 1.0 (i.e., [$Binding\ sites_{total}$]=[$Phospholipid_{weight\ conc.}$/$Protein_{MW}$]). This is a common value observed for several proteins of this family. See McDonald et al., 1997, supra.

$$[Binding\ sites_{free}]=[Binding\ sites_{total}]-[Protein_{bound}] \quad \text{(eq. 4)}$$

Using these assumptions and the data at a protein to phospholipid ratio of 0.37, Kd values were 0.7 μM for bovine factor X, 5.5 μM for wild type factor VII and 0.23 μM for VIIQ11E33. Thus, it was clear that factor VIIQ11E33 was greatly improved in membrane binding affinity over wild type factor VII and had one of the highest membrane-binding affinities among the vitamin K-dependent proteins.

It also has been observed that the difference between wild-type VIIa and VIIa-Q11E33 varied somewhat with the composition of the phospholipid vesicles that were used. For example, membranes containing PS/PE/PC (20/40/40) produced a 33-fold higher activity for VIIQ11E33, while certain preparations of PS/PC (20/80 to 25/75) showed a 10 to 19 fold higher activity for VIIQ11E33.

Enhanced activation of factor VIIQ11E33: The first step in coagulation involves the activation of factor VII. Autoactivation of VII was conducted in a solution containing 100 nM sTF (highly purified recombinant product from Dr. Walter Kisiel, Fiore et al., 1994, *J. Biol. Chem.,* 269: 143–149), 36 nM VIIQ11E33 and PS/PC (25/75, 22 μg/mL). Activity of VIIaQ11E33 was estimated at various time intervals by addition of 0.15 mm substrate S-2288 (Kabi)

and assessing the rate of p-nitrophenylphosphate product release by absorbance change at 405 nm. Initial activity of the VIIQ11E33 preparation was less than 4% that of fully active VIIaQ11E33.

VIIQ11E33 was found to be a much better substrate for activation than wild-type factor VII. FIG. **2** shows autoactivation of factor VIIQ11E33. The data were analyzed by the relationship in equation 5 (equation 7 of Fiore et al., 1994, supra).

$$1n[VIIa]_t = 1n[VIIa]_0 + kcat*y*t \quad \text{(eq. 5)}$$

$1n[VIIa]_t$ is the factor VIIa concentration at time t, kcat is the catalytic rate constant for factor VIIa acting on VII and y is the fractional saturation of VIIa sites. For wild-type factor VIIa, this relationship and 1 μM sTF gave a kcat of 0.0045/s and a kcat/Km ratio of $7*10^3$ $M^{-1}s^{-1}$. See, Fiore et al., 1994, supra. For the VIIQ11E33 enzyme, autoactivation was rapid (FIG. **2**) and it was only possible to estimate a lower limit for kcat. This was obtained from the VIIa doubling time of about 25 seconds ($kcat=(1n2)/t_{1/2}$). The resulting value ($kcat_{min}$=0.03/s), along with the substrate concentration of this reaction ($3.6*10^{-8}$ M) and the assumption that y=1.0, gave a value for kcat/[S]=$8*10^5$ $M^{-1}s^{-1}$. This should be far below the true kcat/Km for VIIaQ11E33, but was about 100-times greater than the value of kcat/Km for wild type factor VIIa/sTF estimated by Fiore et al., 1994, supra. Thus, the combination of VIIaQ11E33 enzyme and Factor VIIQ11E33 substrate was superior to wild type proteins in the activation step of coagulation. This suggested that VIIQ11E33 was superior to wild type enzyme when coagulation conditions were minimal.

Enhanced activity of VIIaQ11E33: Once generated, factor VIIa activates either factor X or factor IX. Activation of bovine factor X (0.1 μM) by factor VIIa was carried out in 50 mM Tris HCl buffer, pH 7.5 containing 100 mM NaCl, 5 mM calcium, various amounts of phospholipid (PS/PC, 25/75) and 1 mg/mL bovine serum albumin at 22.5° C. Factor VIIa (0.06 nM of VIIaQ11E33 or 0.6 nM wild type VIIa) was added at zero time and Xa activity at 1, 3 and 5 minute time points was determined. Aliquots of the reaction mixture (0.2 mL) were mixed with buffer (0.2 mL) containing 10 mM EDTA and 0.4 mM S-2222 (Kabi), a chromogenic substrate for factor Xa. Absorbance change at 405 nm was determined in a Beckman DU8 spectrophotometer. The amount of factor Xa generated was calculated from the extinction coefficient ($1*10^4$ M^{-1} cm^{-1}) of the p-nitrophenylphosphate reaction product and a velocity of 33/sec for substrate hydrolysis by purified bovine Xa under the conditions of this assay.

FIG. **3** compares the ability of wild type factor VIIa (open circles) and VIIaQ11E33 (closed circles) to activate factor X in a purified system. Again, VIIaQ11E33 was far superior to wild type factor VIIa in this reaction. The difference was greatest at low phospholipid concentrations and diminished to 2-fold at 200 μg phospholipid per mL. This was expected from the fact that high membrane concentrations cause a greater portion of wild type VIIa to bind to the membrane. Once again, the increased function of VIIaQ11E33 was greatest under conditions of low phospholipid exposure.

The ability of factor VIIa to activate factor X on activated platelet surfaces also was determined. Platelets were isolated by standard techniques consisting of centrifugation onto a bovine serum albumin cushion and gel filtration to obtain monomeric platelets. The platelets were activated with calcium ionophore (A23187) and diluted to a concentration of 2×10^8/mL. The buffer was 0.05 M Tris, pH 7.5–0.1 M NaCl and contained 5 mM $CaCl_2$. Factor VIIa or Q11E33-VIIa (50 nM) were added along with 200 mM factor X. At various time intervals, the reaction was quenched by adding excess EDTA. The amount of factor Xa generated was determined by its activity toward S-2222, a specific factor Xa substrate. The amount of factor Xa was calculated by comparison to known factor Xa concentrations. At 20 minutes the reaction containing wild type VIIa had generated 0.34 nM Xa while the one containing Q11E33-VIIa had generated 7.7 nM Xa. This assay is independent of tissue factor and utilizes a biological membrane.

Another assay with biological membranes was conducted with J82 cells. This cell line expresses high levels of tissue factor on its surface and is often used to study factor VII binding to tissue factor (e.g. Sakai, T. et al., 1989, *J. Biol. Chem.* 264, 9980–9988). The cells were grown as monolayers by standard methods and were released from the surface by mild trypsin treatment. The rate of factor Xa generation by cells in suspension was determined in the presence of 2 nM active site-modified factor VIIa (DEGR-VIIa). The DEGR-VIIa is an inhibitor and must be displaced from the tissue factor by active VIIa added to the reaction. This is a competitive reaction. The rate of factor Xa generation was used as a measure of VIIa displacement of active site-modified VIIa from the tissue factor. The Q11E33-VIIa displaced DEGR-VIIa at ½8th the concentration required by wild type VIIa. This superiority was similar to that observed in many other competitive assays.

Superior coagulation of VIIaQ11E33: Blood clotting assays were conducted at 37° C. using the hand tilt method to detect clot formation. Human plasma (0.1 mL) was allowed to equilibrate at 37° C. for 1 minute. Various reagents were added in a volume of 0.1 mL of standard buffer. Soluble tissue factor (50 nM) and phospholipid (PS/PC, 10/90, 75 μg/mL) were added to the plasma, along with the factor VIIa concentration shown in FIG. **4** (0.1–32 nM). Finally, 0.1 mL of 25 mM $CaCl_2$ was added to start the reaction. Time to form a clot was measured. In most cases, the average and standard deviations of replicate samples was reported.

FIG. **4** shows the coagulation times of wild type VIIa versus VIIaQ11E33 in normal human plasma. Coagulation was supported by sTF and added phospholipid vesicles. Endogenous wild type factor VII is approximately 10 nM in concentration, and had virtually no impact on coagulation times. The background coagulation was 120 seconds, with or without sTF. Factor VIIaQ11E33 showed approximately 8-fold higher activity than the wild type VIIa under these assay conditions. Similar results were obtained with factor VIII-deficient plasma, suggesting that the major pathway for blood clotting in this system involved direct activation of factor X by factor VIIa. Overall, factor VIIaQ11E33 was superior to wild type VIIa in procoagulant activity supported by membrane vesicles and soluble tissue factor. Wild type zymogen had virtually no activity under these conditions, as indicated by similar background clotting times of 2 minutes, whether or not sTF was added.

Procoagulant activity with normal tissue factor: Coagulation supported by normal tissue factor was assayed with standard rabbit brain thromboplastin-HS (HS=high sensitivity) containing calcium (Sigma Chemical Co.). This mixture contains both phospholipids and membrane-bound tissue factor. Rabbit brain thromboplastin-HS was diluted 1:100 in buffer and used in the assay of VII (added in the form of normal human plasma, which contains 10 nM factor VII) and VIIQ11E33 (added as the pure protein). The thromboplastin (0.2 mL) was added to plasma (0.1 mL) to start the reaction and the time required to form a blood clot was

measured. Assays were also conducted with full strength thromboplastin, as described by the manufacturer.

At optimum levels of human thromboplastin, wild type VII showed a normal level of activity, about 1500 units per mg. This is approximately 25-fold less than the activity of wild type factor VIIa (80,000 units per mg). The VIIQ11E33 gave approximately 1500–3000 units per mg under the standard assay conditions, only 2-fold greater than wild type VII.

The difference between wild type VII and VIIQ11E33 was much greater when the coagulation conditions were sub-optimal. FIG. **5** shows the clotting times and zymogen concentrations in assays that contained 0.01-times the normal thromboplastin level. Under these conditions, VIIQ11E33 was approximately 20-fold more active than wild type factor VII. Thus, greater efficacy of the VIIQ11E33 mutant was especially evident when coagulation conditions were limited, which is relevant to many situations in vivo.

Anticoagulant Activities of DEGR-VIIaQ11E33: Standard coagulation assays were performed with normal human serum and human thromboplastin that was diluted 1:10 with buffer. The active site of factor VIIaQ11E33 was modified by DEGR as described by Sorenson, B. B. et al., 1997, supra. FIG. **6** shows the clotting time of DEGR-VIIaQ11E33 (0–4 nM) incubated with thromboplastin, in calcium buffer, for 15 seconds before addition of the plasma. The time to form a clot was determined with the hand tilt method. Clotting time was approximately 45 seconds with about 1 nM of DEGR-VIIaQ11E33. Active-site modified Factor VIIaQ11E33 had enhanced anticoagulation activity.

Example 2

Purification of Factor VII

Factor VII (wild-type or mutant) was purified by Concanavalin A (Con A), DEAE, and affinity chromatography. Crude media of transfected 293 cells were incubated with Con A resin (Pharmacia) for four hours at 4° C. The resin then was washed with a solution containing 50 mM Tris, pH 7.5, 10 mM benzamidine, 1 mM $CaCl_2$, and 1 mM $MgCl_2$, and factor VII was eluted with 0.2 M D-methyl mannoside, 0.5 M NaCl in 50 mM Tris buffer, pH 7.5. Factor VII was dialyzed against 50 mM Tris, pH 8.0, 50 mM NaCl, 10 mM benzamidine, and 25 mM D-methyl mannoside overnight.

Dialyzed factor VII then was incubated with DEAE resin (Pharmacia) for one hour and the mixture was packed into a column. The DEAE column was washed with 50 mM Tris, pH 8.0, 10 mM Benzamidine, and 50 mM NaCl, and factor VII was eluted with a gradient from 50 mM to 500 mM NaCl in 50 mM Tris buffer, pH 8.0 at a flow rate of 2 mL/min. Fractions containing factor VII activity were pooled and dialyzed against 50 mM Tris, pH 8.0, 50 mM NaCl, and 5 mM $CaCl_2$ overnight. The Con A and DEAE partially purified factor VII was activated by bovine factor Xa (weight ratio 1:10, Enzyme Research Laboratory) at 37EC for one hour.

Activated factor VII was purified further by affinity chromatography. A calcium-independent monoclonal antibody for factor VII (Sigma) was coupled to affigel-10 (Bio-Rad Laboratory) as described by Broze et al., *J. Clin. Invest.*, 1985, 76:937–946, and was incubated with the affinity column overnight at 4EC. The column was washed with 50 mM Tris, pH 7.5, 0.1 M NaCl, and factor VIIa was eluted with 50 mM Tris, pH 7.5, 3 M NaSCN at a flow rate of 0.2 mL/min. The eluted fractions were immediately diluted five fold into 50 mM Tris, pH 7.5, 0.1 M NaCl. Fractions containing factor VIIa activity were pooled, concentrated, and dialyzed against 50 mM Tris, pH 7.5, 0.1 M NaCl overnight.

The protein concentration of factor VIIa was determined with a Bio-Rad protein assay kit, using BSA as the standard. The purity of factor VIIa was assayed by Coomassie gel and Western Blotting under reduced and denatured conditions. Proteolytic activity of factor VIIa was measured using a synthetic peptide substrate spectrozyme-FVIIa (American Diagnostica) in the presence of thromboplastin (Sigma). Purified factor VIIa was stored in 0.1 mg/ml BSA, 0.1 M NaCl, 50 mM Tris, pH 7.5 at −80EC.

Procoagulant effectiveness of factor VIIa mutants was assessed using standard in vitro clotting assays (and modifications thereof); specifically, the prothrombin time (PT) assay and the activated partial thromboplastin (aPTT) assay. Various concentrations of Factor VIIa mutants were evaluated in pooled normal human donor plasma and in coagulation factor-deficient (Factor VIII, Factor IX, Factor VII) human plasmas (Sigma). Clotting times were determined at 37EC using both a FibroSystem fibrometer (BBL) with a 0.3 mL probe and a Sysmex CA-6000 Automated Coagulation Analyzer (Dade Behring).

Platelet poor plasma (PPP) was prepared from pooled normal human donor blood. Blood (4.5 mLs) was drawn from each healthy donor into citrated (0.5 mLs of 3.2% buffered sodium citrate) Vacutainer tubes. Plasma was obtained after centrifugation at 2,000 g for ten minutes and was kept on ice prior to use. Purchased factor-deficient plasmas were reconstituted according to the manufacturer's instructions. Serial dilutions from each stock of Factor VIIa mutant were all prepared in plasma. All plasmas (with or without Factor Vila mutants) were kept on ice prior to use. In all cases, the time to form a clot was measured. The average and standard deviation of replicate samples was reported.

The prothrombin time (PT) assay was performed in plasmas (with or without serial dilutions of added FVIIa mutants) using either of the following PT reagents: Thromboplastin C-Plus (Dade), Innovin (Dade), Thromboplastin With Calcium (Sigma), or Thromboplastin HS With Calcium (Sigma). Assays were conducted according to manufacturer's instructions. In addition to using PT reagents at the manufactured full-strength concentration, the PT assay also was performed using various dilutions of PT reagent. In all cases, the time to form a clot was measured. The average and standard deviation of replicate samples was reported.

The activated partial thromboplastin (aPTT) assay was performed in plasmas (with or without serial dilutions of added Factor VIIa mutants) using either Actin FS (Dade) or APTT reagent (Sigma). Clotting was initiated using 0.025M $CaCl_2$ (Dade) for Actin FS (Dade) or 0.02M $CaCl_2$ (Sigma) for APTT reagent (Sigma). Assays were conducted according to manufacturer's instructions. In addition to using aPTT reagents at the manufactured full-strength concentration, the aPTT assay also was performed using various dilutions of aPTT reagent. In all cases, the time to form a clot was measured. The average and standard deviation of replicate samples was reported.

Clotting assays also were conducted at 37° C. in which different concentrations of phospholipid vesicles [at varying

ratios of PS/PC or PS/PC/PE (PE=phosphatidylethanolamine)] were added to plasmas (with or without serial dilutions of added FVIIa mutants). Clotting was initiated by the addition of 20 mM $CaCl_2$. Various reagents were added in standard buffer. In all cases, the time to form a clot was measured. The average and standard deviation of replicate samples was reported.

Specific Factor VIIa clotting activity was assessed in plasmas (with or without added Factor VIIa mutants) using the STACLOT VIIa-rTF kit (Diagnostica Stago), as per the manufacturer's instructions. This kit is based on the quantitative clotting assay for activated FVII (Morrissey et al., 1993, *Blood,* 81(3):734–744). Clotting times were determined using both a FibroSystem fibrometer (BBL) with a 0.3 mL probe and a Sysmex CA-6000 Automated Coagulation Analyzer (Dade Behring).

Example 3

Circulatory Time of Factor VIIQ11E33 in the Rat

Two anesthetized (sodium nembutol) Sprague Dawley rats (325–350 g) were injected with 36 μg of factor VIIQ11E33 at time zero. Injection was through the juggler vein, into which a cannula had been placed. At the times shown in FIG. **7**, blood was withdrawn from the carotid artery, into which a cannula had been inserted by surgery. The amount of factor VIIQ11E33 in the circulation was estimated from the clotting time of human factor VII-deficient plasma, to which 1 μL of a 1:10 dilution of the rat plasma was added. A 1:100 dilution of rabbit brain thromboplastin-HS (Sigma Chemical Co.) was used. Coagulation was assessed by the manual tube tilt method as described in Example 1. The amount of factor VII activity in the plasma before injection of VIIQ11E33 was determined and was subtracted as a blank. The concentration of factor VIIQ11E33 in the circulation is given as log nM. A sham experiment in which a third animal received the operation and cannulation but no factor VIIQ11E33 was conducted. The amount of factor VII activity in that animal did not change over the time of the experiment (100 minutes). At the end of the experiment, the animals were euthanized by excess sodium nembutol.

The rats appeared normal throughout the experiment with no evidence of coagulation. Therefore, the factor VIIQ11E33 did not cause indiscriminate coagulation, even in the post-operative rat. The circulation life-time of the VIIQ11E33 was normal (FIG. **7**), with approximately 40% of the protein being cleared in about 60 minutes and an even slower disappearance of the remaining protein. This was similar to the rate of clearance of bovine prothrombin from the rat. Nelsestuen and Suttie, 1971, *Biochem. Biophys. Res. Commun.* 45:198–203. This is superior to wild-type recombinant factor VIIa that gave a circulation half-time for functional assays of 2045 minutes. Thomsen, M. K., et al., 1993, *Thromb. Haemost.,* 70:458–464. This indicated that factor VIIQ11E33 was not recognized as an abnormal protein and that it was not rapidly destroyed by coagulation activity. It appeared as a normal protein and should have a standard circulation lifetime in the animal.

Example 4

Enhancement of the Membrane Site and Activity of Protein C

Bovine and human protein C show a high degree of homology in the amino acids of their GLA domains (amino terminal 44 residues), despite about 10-fold higher membrane affinity of the human protein. Bovine protein C contains a proline at position 11 versus a histidine at position 11 of human protein C. The impact of replacing proline-11 in bovine protein C with histidine, and the reverse change in human protein C, was examined. In both cases, the protein containing proline-11 showed lower membrane affinity, about 10-fold for bovine protein C and 5-fold for human protein C. Activated human protein C (hAPC) containing proline at position 11 showed 2.4 to 3.5-fold lower activity than wild type hAPC, depending on the assay used. Bovine APC containing histidine-11 displayed up to 15-fold higher activity than wild type bAPC. This demonstrated the ability to improve both membrane contact and activity by mutation.

Mutagenesis of Protein C: A full-length human protein C cDNA clone was provided by Dr. Johan Stenflo (Dept. of Clinical Chemistry, University Hospital, Malmö, Sweden). The bovine protein C cDNA clone was provided by Dr. Donald Foster (ZymoGenetics, Inc., USA). The GenBank accession number for the nucleotide sequence of bovine protein C is KO2435, NID g163486 and is K02059, NID g190322 for the nucleotide sequence of human protein C.

Site-directed mutagenesis was performed by a PCR method. For human protein C mutagenesis of histidine-11 to proline, the following oligonucleotides were synthesized: A, 5'-AAA TTA ATA CGA CTC ACT ATA GGG AGA CCC AAG CTT-3' (corresponding to nucleotides 860–895 in the vector pRc/CMV, SEQ ID NO:7) to create a Hind III site between pRc/CMV and protein C. B, 5'-GCA CTC CCG CTC CAG GCT GCT GGG ACG GAG CTC CTC CAG GAA-3' (corresponding to the amino acid residues 4–17 in human protein C, the 8th residue in this sequence was mutated from that for human protein C to that of bovine protein C, as indicated by the underline, SEQ ID NO:8).

For bovine protein C mutagenesis of proline-11 to histidine, the following oligonucleotides were synthesized: A, (as described above); C, 5'-ACG CTC CAC GTT GCC GTG CCG CAG CTC CTC TAG GAA-3' (corresponding to amino acid residues 4–15 in bovine protein C, the 6th amino acid was mutated from that for bovine protein C to that of human protein C as marked with underline SEQ ID NO:9); D, 5'-TTC CTA GAG GAG CTG CGG CAC GGC AAC GTG GAG CGT-3' (corresponding to amino acid residues 4–15 in bovine protein C, the 7th amino acid was mutated from that for bovine protein C to that of human protein C; mutated nucleotides are underlined, SEQ ID NO:10); E, 5'-GCA TTT AGG TGA CAC TAT AGA ATA GGG CCC TCT AGA-3' (corresponding to nucleotides 984–1019 in the vector pRc/CMV), creating a Xba I site between pRc/CMV and protein C, SEQ ID NO:11).

Both human and bovine protein C cDNAs were cloned into the Hind III and Xba I sites of the expression vector pRc/CMV. Human protein C cDNA containing the 5' terminus to amino acid-17 was PCR amplified with intact human protein C cDNA and primers A and B. The volume for the PCR reaction was 100 μl and contained 0.25 μg of template DNA, 200 μM each of the four deoxyribonucleoside triphosphates, 0.5 mM of each primer and 2.5 U of Pwo-DNA polymerase (Boehringer Mannheim) in Tris-HCl buffer (10 mM Tris, 25 mM KCl, 5 mM $(NH_4)_2SO_4$, and 2 mM $MgSO_4$, pH 8.85). Samples were subjected to 30 cycles of PCR consisting of a 2 minute, 94° C. denaturation period, a 2 minute, 55° C. annealing period, and a 2 minute, 72° C. elongation period. After amplification, the DNA was electrophoresed through an 0.8% agarose gel in 40 mM Tris-acetate buffer containing 1 mM EDTA. PCR products were purified with JET Plasmid Miniprep-Kit (Saveen Biotech

AB, Sweden). Human protein C cDNA containing respective mutations was cleaved by Hind III and Bsr BI, and then cloned into pRc/CMV vector that was cleaved by Hind III/Xba I and that contained human protein C fragment from Bsr BI to the 3' terminus to produce a human protein C full length cDNA with the mutation.

Bovine protein C cDNA, containing the 5' terminus through amino acid-11, was PCR amplified with intact human protein C cDNA and primers A and C. Bovine protein C cDNA from amino acid 11 to the 3' terminus was amplified with intact human protein C cDNA and primers D and E. These two cDNA fragments were used as templates to amplify full length bovine protein C cDNA containing mutated amino acids with primers A and E. PCR reaction conditions were identical to those used for hAPC. The bovine protein C cDNA containing the respective mutations was cleaved by Hind III and Bsu 36I, and the Hind III/Bsu36I fragment was cloned into pRc/CMV vector containing intact bovine protein C fragments from the Bsu 36I to the 3' terminus to produce full-length bovine protein C cDNA containing the mutation. All mutations were confirmed by DNA sequencing prior to transfection.

Cell Culture and Expression: The adenovirus-transfected human kidney cell line 293 was grown in DMEM medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml of penicillin, 100 U/ml streptomycin and 10 μg/ml vitamin K_1. Transfection was performed using the lipofectin method. Felgner, P. L. et al., 1987, *Proc. Natl. Acad. Sci. USA,* 84:7413–7417. Two μg of DNA was diluted to 0.1 mL with DMEM containing 2 mM of L-glutamine medium. Ten μL of Lipofectin (1 mg/ml) was added to 100 μL of DMEM containing 2 mM L-glutamine medium. DNA and lipofectin were mixed and left at room temperature for 10–15 min. Cell monolayers (25–50% confluence in 5-cm petri-dishes) were washed twice in DMEM with 2 mM L-glutamine medium. The DNA/lipid mixture was diluted to 1.8 mL in DMEM containing 2 mM L-glutamine medium, added to the cells and incubated for 16 hours. The cells were fed with 2 mL of complete medium containing 10% calf serum, left to recover for another 48–72 hours and then trypsinized and seeded into 10-cm dishes with selection medium (DMEM containing 10% serum and 400 μg/mL of Geneticin) at 1:5. Yan, S. C. B. et al., 1990, *Bio/Technology* 655–661. Geneticin-resistant colonies were obtained after 3–5 weeks of selection. Twenty four colonies from each DNA transfection were picked, grown to confluence and the media screened for protein C expression with a dot-blot assay using monoclonal antibody HPC4 (for human protein C) and monoclonal antibody BPC5 (for bovine protein C). Clones producing high amounts of protein were isolated and grown until confluence in the presence of 10 μg/mL of vitamin K_1.

The purification of bovine recombinant protein C and its mutant were based on the method described previously with some modifications. Rezair, A. R., and Esmon, C. T., 1992, *J. Biol. Chem.,* 267:26104–26109. Conditioned serum-free medium from stably transfected cells was centrifuged at 5000 rpm at 4° C. for 10 minutes. The supernatant was filtered through 0.45 μm of cellulose nitrate membranes (Micro Filtration Systems, Japan). EDTA (5 mM, final concentration) and PPACK (0.2 μM, final concentration) were added to the conditioned medium from 293 cells, then passed through a Pharmacia FFQ anion-exchange column at room temperature using Millipore Con Sep LC100 (Millipore, USA). The protein was eluted with a $CaCl_2$ gradient (starting solution, 20 mM Tris-HCl/150 mM NaCl, pH 7.4; limiting solution, 20 mM Tris-HCl/150 mM NaCl/30 mM $CaCl_2$, pH 7.4). After removal of the $CaCl_2$ by dialysis and Chelex 100 treatment, the protein was reabsorbed to a second FFQ column, then eluted with an NaCl gradient (starting solution 20 mM Tris-HCl/150 mM NaCl, pH 7.4; limiting solution, 20 mM Tris-HCl/500 mM NaCl, pH 7.4). At this point in the purification, wild-type and the mutant recombinant bovine protein C were homogeneous as determined by SDS-PAGE.

The first column used for purification of wild-type and mutant recombinant human protein C was the same as that described for bovine protein C. The chromatographic method described by Rezair and Esmon was employed with some modifications described for the method of protein S purification. Rezair, A. R., and Esmon, C. T., 1992, supra; He, Z. et al., 1995, *Eur. J. Biochem.* 227:433–440. Fractions containing protein C from anion-exchange chromatography were identified by dot-blot. Positive fractions were pooled and applied to an affinity column containing the Ca^{2+}-dependent antibody HPC-4. The column was equilibrated with 20 mM Tris-HCl, 150 mM NaCl, pH 7.4, containing 5 mM Benzamidine-HCl and 2 mM $CaCl_2$. After application, the column was washed with the same buffer containing 1 M NaCl. Protein C was then eluted with 20 mM Tris-HCl, 150 mM NaCl and 5 mM EDTA, pH 7.4, containing 5 mM Benzamidine-HCl. After purification, the purity of all human and bovine recombinant protein C preparations was estimated by SDS-PAGE followed by silver staining. Proteins were concentrated using YM 10 filters (Amicon), then dialyzed against buffer (50 mM Tris-HCl and 150 mM NaCl, pH 7.4) for 12 hours and stored at −70° C. The concentrations of proteins were measured by absorbance at 280 nm.

Association of normal and mutant protein C molecules with membranes: LUVs and SUVs were prepared by methods described in Example 1. Light scattering at 90° to the incident light was used to quantitate protein-membrane binding as described above for Factor VII (25 μg/mL of PS/PC, (25/75) at 5 mM calcium (0.05 M Tris buffer-0.1 M NaCl, pH 7.5).

Bovine protein C containing histidine at position 11 interacted with membranes with about 10-fold higher affinity than wild type protein. When fit to equation 2, the data gave K_D values of 93080 nM for protein C-H11 and 9200950 nM for wild type protein C (FIG. **8**A). The difference in affinity corresponded to about 1.4 kcal/mol at 25° C. In fact, membrane affinity of bovine protein C-H11 was almost identical to that of native human protein C (660 nM, FIG. **8**B). This suggested that proline 11 formed a major basis for differences between the membrane binding site of human and bovine proteins.

The reverse substitution, replacement of His-11 of human protein C by proline, decreased membrane affinity (FIG. **8**B). When fit to equation 2, these data gave K_D values of 66090 nM for wild type human protein C and 335Q 110 nM for human protein C-P11. The impact of proline introduction was only slightly less than that of proline in the bovine proteins.

Impact of proline-11 on activity of activated protein C: Activated protein C was generated by thrombin cleavage, using identical conditions for both the wild type and mutant proteins. Approximately 150 μg of the various protein C preparations (1 mg/mL) were mixed with bovine thrombin (3 μg) and incubated at 37° C. for 5 hours. The reaction product was diluted to 0.025 M Tris buffer-0.05 M NaCl and applied to a one mL column of SP-Sephadex C-50. The column was washed with one mL of the same buffer and the flow-through was pooled as activated protein C. Approximately 65–80% of the protein applied to the column was

recovered. APC activity was determined by proteolysis of S2366 (0.1 mM) at 25° C. The preparations were compared to standard preparations obtained on larger scale. Standard human APC was provided by Dr. Walter Kisiel. For bovine proteins, the standard was a large-scale preparation of thrombin-activated APC. The activity of bovine APC was consistent for all preparations of normal and mutant proteins (5%). Two preparations of bovine APC were used for comparisons. Human APC generated from thrombin was 55 to 60% as active as the standard. The concentrations reported in this study were based on activity toward S2366, relative to that of the standard.

Standard APTT test used bovine or human plasma and standard APTT reagent (Sigma Chemical Co.) according to manufacturers instructions. Alternatively, phospholipid was provided in the form of vesicles formed from highly purified phospholipids. In this assay, bovine plasma (0.1 mL) was incubated with either kaolin (0.1 mL of 5 mg/mL in 0.05 M Tris buffer, 0.1 M NaCl, pH 7.5) or ellagic acid (0.1 mM in buffer) for 5 minutes at 35□ C. Coagulation was started by adding 0.1 mL of buffer containing phospholipid and the amounts of APC shown, followed by 0.1 mL of 25 mM calcium chloride. All reagents were in standard buffer containing 0.05 M Tris buffer, 0.1 M NaCl, pH 7.5. An average of a 14-fold higher concentration of wild type bAPC was needed to duplicate the impact of the H11 mutant. Coagulation time at 10 nM bAPC-H11 was greater than 120 minutes. Standard APTT reagent (Sigma Chemical Co.) gave a clotting time of about 61 seconds at 35° C. with this plasma. Time required to form a clot was recorded by manual technique. The amount of phospholipid was designed to be the limiting component in the assay and to give the clotting times shown. The phospholipids used were SUVs (45 μg/0.4 mL in the final assay, PS/PC, 10/90) or LUVs (120 μg/0.4 mL in the final assay, PS/PC, 25/75).

The anticoagulant activity of activated protein C was tested in several assays. FIG. **9** shows the impact on the APTT assay, conducted with limiting phospholipid. Under the conditions of this assay, coagulation times decreased in a nearly linear, inverse relationship with phospholipid concentration. Approximately 14-times as much wild type bovine APC was needed to equal the effect of bovine APC-H11.

Parts of the study in FIG. **9** were repeated for membranes of PS/PC (25/75, LUV). Again, activity was limited by phospholipid, and its concentration was adjusted to give a control clotting time of 360 seconds (120 μg of 25% PS in the 0.4 mL assay). Approximately 15-fold more wild type enzyme was needed to equal the impact of the H11 mutant. Finally, standard APTT reagent (Sigma Chemical Co., standard clotting time 502 seconds) was used. Approximately 10.00.7 nM of wild type enzyme was needed to double the coagulation time to 1025 seconds. The same impact was produced by 2.20.1 nM bovine APC-H11. Phospholipid was not rate limiting in the standard assay so a smaller impact on membrane affinity may be expected.

Results for human proteins are shown in FIG. **8**B. About 2.5 times as much human APC containing proline-11 was required to prolong coagulation to the extent of wild type APC. A lower impact of proline-11 introduction may reflect the smaller differences in membrane affinity of the human proteins (FIG. **9**B).

Inactivation of factor Va: Factor Va inactivation was assayed by the method of Nicolaes et al., 1996, *Thrombosis and Haemostasis,* 76:404–410. Briefly, for bovine proteins, bovine plasma was diluted 1000-fold by 0.05 M Tris, 0.1 M NaCl, 1 mg/mL bovine serum albumin and 5 mM calcium at pH 7.5. Phospholipid vesicles (5 μg/0.24 mL assay) and 5 μL of 190 nM thrombin were added to activate factor V. After a 10-minute incubation at 37° C., APC was added and the incubation was continued for 6 minutes. Bovine prothrombin (to 10 μM final concentration) and factor Xa (0.3 nM final concentration) were added and the reaction was incubated for one minute at 37° C. A 20 μL sample of this activation reaction was added to 0.38 mL of buffer (0.05 M Tris, 0.1 M NaCl, 5 mM EDTA, pH 7.5) containing S2288 substrate (60□M). The amount of thrombin was determined by the change in absorbance at 405 nM (ϵ=1.0*10^4 M^{-1}s^{-1}, k_{cat} for thrombin=100/s). For human proteins, human protein S-deficient plasma (Biopool Canada, Inc.) was diluted 100-fold, factor Va was activated by human thrombin and the factor Va produced was assayed with the reagents used for the bovine proteins.

Bovine APC-H11 was 9.2-fold more active than wild type (FIG. **10**A) in inactivating factor Va. As for membrane binding (above), the impact of proline-11 was less with the human proteins, with an average of 2.4-fold difference between the curves drawn for wild type and P-11 mutant (FIG. **10**B). Similar results were obtained with normal human plasma.

Example 5

Identification of an Archetype Membrane Affinity for the Membrane Contact Site of Vitamin K-Dependent Proteins

Comparison of various human and bovine protein C mutants and other vitamin K-dependent polypeptides led to a proposed membrane contact site archetype. The electrostatic archetype consists of an electropositive core on one surface of the protein, created by bound calcium ions, surrounded by a halo of electronegative charge from amino acids of the protein. The closer a member of this protein family approaches this electrostatic pattern, the higher its affinity for membranes.

Phospholipid vesicles, wild type bovine protein C, protein-membrane interaction studies, activation and quantitation of protein C, and activity analysis were as described in Example 4.

Recombinant, mutant protein C was generated by the following procedures. Site-directed mutagenesis was performed by a PCR method. The following oligonucleotides were synthesized: A, as described in Example 4; F, 5'-GCA TTT AGG TGA CAC TAT AGA ATA GGG CCC TCT AGA-3' (corresponding to nucleotides 984–1019 in the vector pRc/CMV, SEQ ID NO:11), creating a Xba I site between pRc/CMV and protein C; G, 5'-GAA GGC CAT TGT GTC TTC CGT GTC TTC GAA AAT CTC CCG AGC-3' (corresponding to amino acid residues 40–27 in bovine protein C, the 8th and 9th amino acids were mutated from QN to ED as marked with underline, SEQ ID NO:12); H, 5'-CAG TGT GTC ATC CAC ATC TTC GAA AAT TTC CTT GGC-3' (corresponding to amino acid residues 38–27 in human protein C, the 6th and 7th amino acids in this sequence were mutated from QN to ED as indicated with the underline, SEQ ID NO:13); I, 5'-GCC AAG GAA ATT TTC GAA GAT GTG GAT GAC ACA CTG-3' (corresponding to amino acid residues 27–38 in human protein C, the 6th and 7th amino acids in this sequence were mutated from QN to ED as indicated with underline, SEQ ID NO:14); J, 5'-CAG TGT GTC ATC CAC ATT TTC GAA AAT TTC CTT GGC-3 (corresponding to amino acid residues 38–27 in human protein C, the 7th amino acids in this sequence were

mutated from Q to E as indicated with underline, SEQ ID NO:15); K, 5'-GCC AAG GAA ATT TTC GAA AAT GTG GAT GAC ACA CTG-3' (corresponding to amino acid residues 27–38 in human protein C, the 6th amino acid in this sequence was mutated from Q to E as indicated with underline, SEQ ID NO:16);

Both bovine and human protein C full length cDNAs were cloned into the Hind III and Xba I site of the vector pRc/CMV. To obtain bovine protein C mutant E33D34, PCR amplification of the target DNA was performed as follows. Bovine protein C cDNA containing the 5' terminus to the amino acid at position 40, was amplified with intact bovine protein C cDNA and primers A and C. The PCR reaction conditions were as described in Example 3. The sample was subjected to 30 cycles of PCR consisting of a 2 min denaturation period at 94° C., a 2 min annealing period at 55° C. and a 2 min elongation period at 72° C. After amplification, the DNA was electrophoresed through an 0.8% agarose gel in 40 mM Tris-acetate buffer containing 1 mM EDTA. The PCR products were purified with The Geneclean III kit (BIO 101, Inc. USA), and the PCR fragment of bovine protein C cDNA containing the respective mutations was cleaved by Hind III and Bbs I. The Hind III/Bbs I fragment and the human protein C fragment (Bbs I-3' terminus) were cloned into the Hind II and Xba I sites of pRc/CMV vector to produce a full-length bovine protein C cDNA with the mutations. Bovine protein C mutant H11 E33 D34 was created in the same way, but used bovine protein C mutant H11 as a template in the PCR reaction.

Human protein C cDNA containing the 5' terminus to amino acid-38 was PCR amplified with intact human protein C cDNA and primers A and D. Human protein C cDNA from amino acid 27 to the 3' terminus was amplified with intact human protein C cDNA and primers B and E. These two cDNA fragments were used as templates to amplify full length bovine protein C cDNA containing mutated amino acids (E33 D34) with primers A and B. Human protein C mutant E33 was obtained by the following steps: human protein C cDNA containing the 5' terminus to amino acid 38 was amplified with intact human protein C cDNA and primers A and F. Human protein C cDNA from amino acid 27 to the 3' terminus was amplified with intact human protein C cDNA and primers B and G. These two cDNA fragments were used as templates to amplify full length bovine protein C cDNA containing mutated amino acids (E33) with primers A and B. The PCR mixture and program were described above. The human protein C PCR products containing respective mutations were cleaved by Hind III and Sal I, and then the fragment (Hind III-Sal I) together with intact human protein C fragment (Sal I-3' terminus) were cloned into the Hind III and Xba I sites of pRc/CMV vector to produce the full length human protein C cDNA with the respective mutations. Human protein C containing a glycine residue at position 12, in conjunction with the E33D34 mutations also was made. All mutations were confirmed by DNA sequencing prior to transfection.

The adenovirus-transfected human kidney cell line 293 was cultured and transfected as described in Example 4. Bovine and human recombinant protein C and mutants were purified as described in Example 4.

The vitamin K-dependent proteins were classified into four groups on the basis of their affinities for a standard membrane (Table 5). Sequences of the amino terminal residues of some relevant proteins including human protein C (hC, SEQ ID NO:1), bovine protein C (bC, SEQ ID NO:2), bovine prothrombin (bPT, SEQ ID NO:17), bovine factor X (bX, SEQ ID NO:18), human factor VII (hVII, SEQ ID NO:3), human protein Z (hZ, SEQ ID NO:20), and bovine protein Z (bZ, SEQ ID NO:21) are given for reference, where X is Gla (γ-carboxyglutamic acid) or Glu.

bPT: ANKGFLXXVRK$_{11}$GNLXRXCLXX$_{21}$PCSRXXAFXA$_{31}$LXSLSATDAF$_{41}$WAKY

bX: ANSFLXXVKQ$_{11}$GNLXRXCLXX$_{21}$ACSLXXARXV$_{31}$FXDAXQTDXF$_{41}$WSKY

hC: ANSFLXXLRH$_{11}$SSLXRXCIXX$_{21}$ICDFXXAKXI$_{31}$FQNVDDTLAF$_{41}$WSKH

bC: ANSFLXXLRP$_{11}$GNVXRXCSXX$_{21}$VCXFXXARXI$_{31}$FQNTXDTMAF$_{41}$WSFY

hVII: ANAFLXXLRP$_{11}$GSLXRXCKXX$_{21}$QCSFXXARXI$_{31}$FKDAXRTKLF$_{41}$WISY

hZ: AGSYLLXXLFX$_{11}$GNLXKXCYXX$_{21}$ICVYXXARXV$_{31}$FXNXVVTDXF$_{41}$WRRY

bZ: AGSYLLXXLFX$_{11}$GHLXKKCWXX$_{21}$ICVYXXARXV$_{31}$FXDDXTTDXF$_{41}$WRTY

TABLE 5

	Charges and Affinity						
	Residue						
	11 + 29 + 33 + 34 = Sum					Total	K_D (nM)
Class I							
bZ	−2	+	−2	−1	−4	−6	0.2[a]–32
hZ	−2	+	−2		−3	−5	2.0[a]–170
Class II							
bPT-TNBS			−2		−2	−1	<10
hVII-Q11E33		+	−2	−1	−2	−2	10
hS		+	−2	−1	−2	−2	40
bX		+	−2	−1	−2	−3	40
bC-E33D34	P	+	−2	−1	−2	−4	125
hX	+	+	−2	−1	−1	−2	160
bPT	+		−2		−1	0	100
hPT	+		−2		−1	−1	—
bS	+	+	−2		0	0	120
Class III							
bIX		+	−2		−1	−1	1000
hIX		+	−2		−1	−1	1000
hC		+			+1	−2	660
bC-H11		+			+1	−1	930
Class IV							
hC	P	+			+1	−2	3300
hVII	P	+	+	−1	+1	+1	4000
bC	P	+			+1	−1	9200
bVII	P	+	+		+1	0	15000

[a]Higher affinity value equals $k_{dissociation}/1 * 10^7 M^{-1}S^{-1}$; the denominator is a typical $k_{association}$ for other proteins.

In Table 5, vitamin K-dependent polypeptide mutants are in bold. The total charge (residues 1–34) includes 7 calcium ions (+14) and the amino terminus (+1).

Protein Z was assigned to class I on the basis of its dissociation rate constant, which was 100 to 1000 times slower than that of other proteins. If protein Z displayed a normal association rate constant (about 10^7 $M^{-1}s^{-1}$) the K_D would be about 10^{-10} M. Wei, G. J. et al., 1982, *Biochemistry* 21:1949–1959. The latter affinity may be the maximum

possible for the vitamin K proteins. Protein Z is a candidate for anticoagulation therapy as a cofactor for inhibition of factor Xa by the protein Z-dependent protease inhibitor (ZPI). Incubation of protein Z and ZPI with factor Xa reduces the procoagulant activity of factor Xa. See, for example, Han et al., *Proc. Natl. Acad. Sci. USA,* 1998, 95:9250–9255. Enhancement of association kinetics would speed inhibition rates and increase binding affinity at equilibrium. Protein Z contains gly-2, which is thought to abolish interaction of asn-2 with bound calcium in other proteins. The presence of a glycine residue at position 2 may destabilize protein folding and lower association kinetics. In addition, the lower affinity of protein Z relative to that of bovine protein Z may arise from fewer anionic charges in positions 34–36 (Table 5).

Class IV proteins differed from class III in the presence of proline-11, which may alter affinity by non-electrostatic means.

While a relatively weak correlation existed between membrane affinity and net negative charge on residues 1–34, an excellent correlation was found when only residues 5, 11, 29, 33 and 34 were considered (Table 5). The latter amino acids are located on the surface of the protein. A number of proteins were modeled by amino acid substitution into the prothrombin structure and their electrostatic potentials were estimated by the program DelPhi. A sketch patterned after the electrostatic potential of bovine protein Z is shown in FIG. **11**. Electronegative sites at 7, 8, 26, 30, 33, 34 and 11 produce a halo of electronegative charge surrounding a cationic core produced by the calcium-lined pore (FIG. **11**). The closer a protein structure approaches this structure, the higher its affinity for the membrane. The highest affinity proteins show an electronegative charge extending to amino acids 35 and 36. This correlation is apparent from the wild-type proteins, the mutants and chemically modified proteins.

The pattern for other structures can be extrapolated from examination of the charge groups that are absent in other proteins. For example, Lys-11 and Arg-10 of bovine prothrombin generate high electropositive regions in their vicinity; the lack of Gla-33 in protein C and Factor VII create less electronegativity in those protein regions. In all cases, highest affinity corresponded to a structure with an electropositive core that was completely surrounded by electronegative protein surface, as shown for protein Z. The exceptions to this pattern are the proteins with Pro-11, which may lower affinity by a structural impact and ser-12 (human protein C), which is a unique uncharged residue.

To further test the hypothesis of an archetype for electrostatic distribution, site-directed mutagenesis was used to replace Gln33Asn34 of bovine and human protein C with Glu33Asp34. Glu33 should be further modified to Gla during protein processing. These changes altered the electrostatic potential of bovine protein C to that of bovine factor X. The membrane affinity of the mutant protein was expected to be lower than that of factor X due to the presence of proline-11. Indeed, the bovine protein C mutant gave a membrane affinity similar to that of bovine prothrombin (FIG. **12**A), and slightly less than that of bovine factor X (Table 5).

More interesting was that clot inhibition by APC was greater for the mutant than for the wild type enzyme (FIG. **12**B, C). Inclusion of results for the P11H mutant of bovine protein C from Example 3 showed that a family of proteins could be produced, each with different membrane affinity and activity, by varying the amino acid substitutions at positions 11, 33 and 34.

Human protein C mutants containing E33 and E33D34 resulted in a small increase in membrane binding affinity (FIG. **13***a*). Activity of these mutants was slightly less than the wild-type enzyme (FIG. **13***b*). Results with mutants of bovine protein C suggest that failure of the E33D34 mutation in the human protein may arise from H11 and/or other unique amino acids in the protein. FIG. **14**A shows that the H11 mutant of bovine protein C bound to the membrane with about 10-fold higher affinity than wild type protein, the E33D34 mutant bound with about 70-times the affinity, but that the triple mutant, H11E33D34, was only slightly better than the H11 mutant. This relationship was mirrored in the activity of APC formed from these mutants (FIG. **14**B). This result suggested that the presence of H11 reduced the impact of E33D34 on membrane binding affinity.

These results indicated that introduction of E33D34 alone may not be optimal for all proteins. Consequently, other mutations may be desirable to create human protein C that will use E33D34 and have maximum increased membrane affinity. The result with the bovine protein suggested that histidine 11 may be the primary cause of this phenomenon. Consequently, H11 may be altered to glutamine or to another amino acid in human protein C, along with the E33D34 mutation. Another amino acid that may impact the affinity is the serine at position 12, an amino acid that is entirely unique to human protein C. These additional changes should produce proteins with enhanced membrane affinity. Substitution of a glycine residue for serine at position 12 of human activated protein C, in conjunction with E33D34 resulted in 9-fold higher activity than wild-type activated human protein C. Activity of the G12E33D34 mutant was assessed with a dilute thromboplastin assay using a control clotting time of 30 seconds and normal human plasma. The electrostatic archetype also was tested by comparison of human and bovine factor X. The presence of lysine-11 in human factor X suggests that it should have lower affinity than bovine factor X. This prediction was borne out, by the result shown in FIG. **15**.

Earlier studies had shown that trinitrobenzenesulfonic acid (TNBS) modification of bovine and human prothrombin fragment 1 had relatively little impact (0 to 5-fold) on membrane affinity. Weber, D. J. et al., 1992, *J. Biol. Chem.,* 267:4564–4569; Welsch, D. J. et al., 1988, *Biochemistry,* 27:4933–4938. Conditions used for the reaction resulted in derivatization of the amino terminus, a change that is linked to lowered membrane affinity. Welsch, D. J. and Nelsestuen, G. L., 1988, *Biochemistry,* 27:4939–4945. Protein modification in the presence of calcium, which protects the amino terminus, resulted in TNBS-modified protein with much higher affinity for the membrane than native fragment 1.

The suggestion that protein Z constitutes the archetype was based on its dissociation rate constant and that a normal association rate would generate a $K_D=10^{-10}$ M. Whether this value can be reached is uncertain. It is possible that the slow association rate of protein Z is caused by improper protein folding, resulting in a low concentration of the membrane-binding conformation. If conditions can be altered to improve protein folding, association rates of protein Z should improve. Indeed, the association rate constant for protein Z was improved by alteration of pH. The basis for this observation may be related to an unusual feature of the prothrombin structure, which is the close placement of the amino terminus (+1 at pH 7.5) to calcium ions 2 and 3. The +1 charge on the amino terminus is responsible for the slight electropositive region just above Ca-1 in FIG. **11**. Charge repulsion between Ca and the amino terminal may destabi-

lize protein folding and could be a serious problem for a protein that had low folding stability.

Table 6 provides additional support for the archetype model. It shows the relationship between distance of ionic groups from strontium ions 1 and 8 (corresponding to calcium 1 and an extra divalent metal ion found in the Sr x-ray crystal structure of prothrombin). The pattern suggests that the closer an ionic group is to these metal ions, the higher its impact on membrane affinity. The exception is Arg-16, which contributes to the charge of the electropositive core. Higher affinity is correlated with electronegative charge at all other sites. This correlation also applies to the GLA residues.

TABLE 6

Distance to Sr-1, 8 and Ion Importance.

Position	Atom[a]	Distance (Å) to: Sr-1	Sr-8	Impact/ion on $K_D(K_M)$
(A.A-Protein)				
3(K-PT)	ϵ-N	22.1	21.7	Low or Unknown
5(K-IX)	para-C(F)	20.1	20.8	"
19(K/R-VII)	C5(L)	20.2	17.8	"
22(K-IX)	C4(P)	17.0	18.5	"
10(R)	C6(R)	16.8	12.9	"
25(R-PT)	C6(R)	11.2	13.8	"
24(X/D-PC)	O(S)	8.1	12.0	"
11(K-PT, hX, bS; Gla-PZ)	ϵ-C(K)	14.7	7.4	3–10-fold[b]
33(Gla)	γ-C(E)	11.6	7.5	"
34(D)	O(S)	15.3	12.1	"
29(R)	para-C(F)	7.5	8.4	"
16(R)	C6(R)	14.2	10.6	3–10-fold[c]
Gla residue[d]				
Low importance:				
7		12.8	13.3	+2(<2)
15		20	16	<2(<2)
20		19.4	17.8	<2(<2)
21		17.2	15	4(3)
33		11.6	7.5	?[e](<2)
High importance:				
8		8.7	10.9	?[e](20)
26		3.6	9.5	?[e](50)
17		11.1	9.1	>200(100)
27		8.4	10.6	>200(85)
30		3.4	4.2	>200(25)

[a]Distances are from this atom of bovine prothrombin (residue of prothrombin used in measurement is given in parentheses) to strontium 1 and 8 of the Sr-Prothrombin fragment 1 structure. Seshadri et al. 1994, Biochemistry 33:1087–1092.
[b]For all but 16-R, cations lower affinity and anions increase affinity.
[c]Thariath et al. 1997 Biochem. J. 322:309–315.
[d]Impact of Glu to Asp mutations, distances are averages for the gamma-carboxyl-carbons. $K_D(K_M)$ data are from Ratcliffe et al. 1993 J. Biol. Chem. 268:24339–45.
[e]Binding was of lower capacity or caused aggregation, making comparisons less certain.

The results in the bottom panel of FIG. **15** show that the association rate for protein Z was substantially improved at pH 9, where an amino terminal should be uncharged. The rate constant obtained from these data was about 12-fold higher at pH 9 than at pH 7.5.

Example 6

Competition Assay for Assessing Affinity of Modified Factor VII

Clotting activity of wild type and VIIaQ11E33 was assessed using reconstituted tissue factor (Innovin, Dade) and membrane. Saturating amounts of factor VIIa were used (approximately 0.7 μl of Innovin/0.15 ml assay). Factor VIIa and Innovin were added to the plasma-free buffer (6.7 mM CaCl2, 50 mM Tris, pH 7.5, 100 mM NaCl) in 112.5 μl. After 15 minutes, 37.5 μl of factor VII-deficient plasma was added and clotting time was recorded. Tissue factor-dependent activity of VIIaQ11E33 was similar to that of wild-type protein when assayed in this manner. As use of Factor VII-deficient plasma is not representative of in vivo conditions, the relative affinity of modified factor VII for membrane-bound tissue factor was evaluated in the presence of a competing protein. In particular, active site-modified factor VIIa was used as the competing protein and was present in abundance (2 nM). The modified factor VII to be assessed was used above the concentration of tissue factor. Under such conditions, free protein concentrations of all proteins were approximately equal to total proteins added. Subtraction of bound protein from total to obtain free protein concentrations represents a small correction. Based on the competition assay, factor VIIaQ11E33 was 41-times more effective than wild type VIIa.

Example 7

Enhanced Membrane Binding Affinity and Activity of FACTOR VII, Protein S, and Other Vitamin K-Dependent Polypeptides

Protein S (GenBank Accession number M57853 J02917) is a high affinity membrane-binding protein and a cofactor for action of APC. Deficiency in protein S is a strong indicator of thrombosis disease and may be used in patients who have low levels of this protein or who have increased danger of thrombosis. See, for example, Dahlback, *Blood* 1995, 85:607–614 and U.S. Pat. No. 5,258,288.

Substitutions at amino acids 5 or 9 can enhance membrane binding affinity and activity of Protein S. Residue 9 of Protein S is a threonine residue, while most vitamin K-dependent proteins contain a hydrophobic residue at this position. See, for example, McDonald et al., *Biochemistry* 1997, 36:5120–5127. Replacement of the analogous residue in human Protein C (a leucine residue) with a glutamine, a hydrophobic to hydrophilic replacement, resulted in a severe loss of activity. See, Christiansen et al., 1995, *Biochemistry,* 34:10376–10382. Thus, there has been confusion about the importance of position 8 in membrane association by vitamin K-dependent proteins.

Human factor VII containing a threonine substitution at amino acid 9 in conjunction with the Q11E33 mutations described above, were prepared by ATG Laboratories, Inc. and provided in an appropriate vector for transfection into human kidney cell line 293, as described above in Example 4.

The vector containing the nucleic acid sequence encoding the mutant factor VII was transfected into human kidney cell line 293, using commercially available kits. The cells were grown and colonies providing high levels of factor VII antigen were selected by dot blot assay, as described for protein C, using commercially available polyclonal antibodies. The amount of factor VII in the conditioned medium also was determined by a coagulation assay, using the competition assay described in Example 6. In general, Factor VII was converted to VIIa by incubation with tissue factor before initiation of coagulation. Identical amounts of VIIa-Q11E33 and VIIa-T9Q11E33 were used in the assays. The factor VII polypeptides were mixed with an appropriate amount of membrane-associated tissue factor (Innovin,

Dade). Active site modified VIIa (FFR-VIIa) was added (0 to 3.5 nM) and the reactions were allowed to equilibrate for 60 minutes in 112.5 μL of buffer containing 6.7 mM $CaCl_2$, 50 mM Tris, pH 7.5, and 100 m NaCl. Finally, factor VII-deficient plasma was added and time required to form a clot was measured. Efficacy was determined by the clotting time as a function of added inhibitor. Human factor VIIa-T9Q11E33 exhibited a severe loss of competitive binding affinity. Thus, a threonine residue was not optimal at this position. Introduction of a leucine residue into protein 9 of protein S should enhance the membrane affinity and activity of protein S under many conditions.

The basis for high membrane affinity of protein S, despite the presence of substantial sub-optimum residues, may come from other parts of its structure. That is, protein S contains a sequence region known as the 'second disulfide loop' or thrombin sensitive region (residues 46 to 75 of the mature polypeptide). Prothrombin also contains a second disulfide loop in a shorter version. Proteolytic cleavage of the loop in prothrombin or protein S results in loss of membrane affinity. See, Schwalbe et al., *J. Biol. Chem.,* 1989, 264: 20288–20296. The cleavage may be involved in regulation of protein S activity by providing a negative control to protein S action. The second disulfide loop may serve to produce an optimum membrane binding site such that residues 46–75 fold back onto residues 1–45 to create an optimum binding site. Isolated residues 1–45 of protein S do not associate with membranes in a calcium-dependent manner, which is unlike residues 1–41 or 1–38 of prothrombin, residues 1–44 of factor X, residues 1–41 of protein C, or residues 1–45 of protein Z that do bind membranes in a calcium-dependent manner. Thus, despite intact protein S having high affinity, results with the isolated 1–45 GLA domain of protein S suggest that the intact protein suffers a substantial loss of affinity in the GLA domain.

Proteolysis prevents proper function of the second disulfide loop in this role, and the resultant protein S shows loss of activity as the membrane affinity declines to that expected by the amino acids in residues 1–45. Thus, enhancement of membrane affinity by introduction of Leu9 and other changes (see below), would create a protein S which is no longer down-regulated by proteolysis and which will be a more effective anticoagulant.

A conserved residue in most vitamin K-dependent proteins is position 5. Leucine is found at this position in both protein S and protein Z. Protein C, factors X and VII, and prothrombin contain phenylalanine, another hydrophobic residue, at the corresponding position. Factor IX contains a lysine at residue 5, a major deviation from other proteins. There appears to be no apparent connection to this unconserved residue and the membrane affinity of factor IX, making the role of position 5 ambiguous.

Substitution of a glutamine residue at position 5 of human protein C resulted in a protein displaying similar affinity for membranes and similar calcium titrations, with reduced clotting activity. Substitution of a leucine for a phenylalanine residue at position 5 of factor VII (which contained the Q11E33 mutations) was carried out by the methods described above. The product, L5Q11E33, had only 33% of the activity of the Q11E33 mutant when assayed by the competition method with active-site modified VIIa (0–3.2 nM) described above. Thus, activity of protein S can be improved by substitution, for example, of a phenylalanine residue at position 5. Substitution of F5K in factor VII also resulted in a protein with 33% of the activity of wild type factor VII when assayed by the competitive assay described in Example 6. Membrane binding affinity of Factor IX can be enhanced by a K5E mutation. Substitution of P11Q in Factor VII had a positive impact, while a further change of Q11E (to make this position like that of protein Z) was without further impact, i.e., P11Q and P11E both were better than wild-type, but were not better than each other. Consequently, the impact of substitutions of individual residues varies with the protein. Appropriate combined substitutions, however, elucidate the universal importance of these residues.

A factor VIIa molecule was produced by procedures outlined above. This mutant contained the Q11E33 mutations and also contained R29F and D34F mutations (Q11F29E33F34). This mutant had 2.5-fold higher activity than the Q11E33 mutant alone when assayed in the competition assay described in Example 6. Thus, the correct combination of amino acid residues at the important sites described are needed to maximize the function of the important carboxyl groups in the protein. The optimum combination at these sites may include anionic as well as neutral and hydrophobic residues.

The importance of position 35 is indicated by modification of the Factor VII mutant Q11E33F34 to further include A351. The activity of the resulting mutant Q11E33F34I35 was 60% that of the Q11E33F34 mutant when evaluated in the competitive assay described in Example 6. Another mutant, Q11E33I34F35, was produced and displayed similar activity as the Q11E33F34I35 mutant. These results indicate that the presence of a large hydrophobic group at position 35 is not desirable.

Stability of the Q11E33F34 mutant in cell culture medium was reduced by 75% relative to that of the Q11E33 mutant. Reduced stability resulted in decrease of yield from the culture medium. Loss of stability appeared to arise from proteolysis. Modification of position 35 to Gla may be required to increase the stability of the protein with highest activity, as Gla residues prevent proteolysis. Introduction of A35E mutation may increase the stability of the Q11E33F34 mutant.

The importance of position 36 was indicated by addition of a E36D mutation to the Q11E33 factor VII mutant. The resulting mutant, Q11E33D36, had 70% the activity of the Q11E33 mutant in the competitive assay described in Example 6.

Modifications of positions 34–36 would appear to have the highest impact on human protein C, in which the wild-type protein contains N34V35D36. Introduction of F34X35E36, where X is an amino acid resistant to proteolytic digestion, may enhance the membrane binding affinity of human protein C.

Example 8

Insertion of Residue at Amino Acid 4

Factor VII containing a tyrosine residue was prepared by ATG Laboratories, Inc. and assayed by the competition assay described in Example 6. Factor VII was activated by incubation with Innovin (20 ml) in 5 mM calcium. Samples containing sufficient amounts of Innovin to give a minimum clotting time of 28 seconds (about 0.7 μl) were transferred to buffer. Factor VII deficient plasma was added and clotting times were recorded. Samples were assayed at various times until maximum activity was reached (usually ≦30 min.). The amount of Factor VII in the conditioned media needed to generate a clotting time of 30 seconds was determined. This ratio of media/Innovin represents a nearly 1:1 ratio of factor VIIa/Tissue Factor. After activation, samples of the

media/Innovin sufficient to give a 19 second clotting time (about 4 μl of Innovin) were diluted to 112.51 μl with buffer containing calcium and BSA (1 g/L). Various amounts of active-site modified factor VIIa (DEGR-VIIa) were added and allowed to incubate until equilibrium was reached; typically 60 minutes at 37° C. Human factor VII deficient plasma was added (37.5 μl) and clotting times were measured. Results were compared to similar experiments conducted with media containing wild-type VIIa. Based on results from the competitive displacement assay, human factor VII containing an insertion of a tyrosine residue at position 4 had two-fold higher activity than a similar factor VII molecule lacking this residue.

Combination of the beneficial mutations was performed to generate a factor VII mutant, Q11E33F34 with a Tyrosine inserted at position 4. This mutant showed 5-fold higher activity than the factor VII Q11E33 mutant in the competitive assay described above. Overall, this mutant has 160-fold higher activity than wild type factor VIIa in the competition assay described in example 6. This result showed that the benefits of individual residues are additive when presented in combination.

Example 9

Anticoagulation of Human Blood by Activated Protein C (APC)

The Clot Signature Analyzer (CSA) apparatus (Xylum Company, Scarsdale, N.Y.) was used to test relative efficacy of wild type APC versus the Q11G12E33D34 mutant. In one assay, this apparatus pumps freshly drawn (<2 min), non-anticoagulated blood through a tube containing a fiber that has a collagen surface. The pressure of blood flow (mm Hg) is similar to that of the circulation system. Platelets in the blood bind to the collagen, become activated and support coagulation. The instrument detects pressure at the outlet of the tube, as shown in FIG. **16**. Upon clot formation, pressure at the outlet of the tube declines and the half-reaction point is described as the "Collagen-Induced Thrombus Formation" (CITF) time. Without additives, the CITF time for the human subject was 5.0 minutes (FIG. **16**A). Background time is subtracted from the value in FIG. **16** to obtain CITF. With 30 nM wild type APC added to the blood, the average CITF time was 6.5 min (5 determinations) for the same subject (FIG. **16**B). The average CITF time was 15.5 min with 6 nM mutant APC (Q11G12E33D34), based on 5 determinations (FIG. **16**C), and was 9.5 min with 3 nM of the same mutant APC, based on 5 determinations. Thus, the mutant APC was at least 10-fold more effective than wild type APC for inhibition of clot formation in whole human blood under flow pressure, using activated human platelet membranes as the phospholipid source. In one additional experiment, in which CITF was assessed in a subject that had ingested two aspirins, an even larger difference was observed between mutant and wild type APC. Lower membrane activity may correlate with a larger impact of the mutant.

This result differed from the outcome of an in vitro coagulation test using the hand tilt assay procedure, described earlier in this document. Using the standard conditions and full strength reagents for the APTT test (materials obtained from, and procedures described by the manufacturer, the Sigma Chemical Co., St. Louis, Mo., 1998), the mutant protein showed only 1.5-fold higher activity than the wild type protein. The APTT assay, however, contains high levels of phospholipid,, a condition that minimizes the difference between mutant and wild type proteins. Once again, these results indicate that assay conditions are important in characterizing the benefit of the mutant proteins produced by this invention and that low phospholipid concentration is characteristic of the biological membrane provided by platelets.

The impact of a procoagulant protein on blood from two hemophilia patients was tested in the CSA. The CITF for the first hemophilia patient was 13.2 minutes and the clot was unstable with continual breaks (with increases in pressure) similar to those shown for anticoagulated blood in FIG. **16**. In a second sample, 60 nM wild type factor VIIa was added to the blood before analysis. This dose correlates with therapeutic levels. The CITF time (9.7 min) was shortened and the clot was more stable. A third sample used 60 nM factor VIIa Q11E33. The CITF time (3.7 min) was below the range for normal individuals and the clot was stable. The second hemophilia patient gave similar responses.

The CSA represents an important assay since it utilizes biological membranes to support blood coagulation. Another assay that uses biological membranes also was tested. The assay provided by the Hemochron Jr. Signature Whole Blood Microcoaguation System is a common tool for testing coagulation in clinical situations. Whole blood is drawn into the cassette and time to reach coagulation is determined. Using this system, the Q11G12E33D34 mutant of activated protein C was 5 to 10-fold more active than wild type human activated protein C. This activity reflects the advantage of the mutant in many other assays.

Example 10

Carboxylation State of Modified Polypeptides

Carboxylation of the GLA domain of vitamin K-dependent polypeptides was assessed by MALDI-TOF Mass spectrometry after release of the GLA domain from intact protein by mild chymotrypsin digestion (1:500, protease:substrate protein, pH 7.5 at 37' for 3 hr). Chymotrypsin cleaves preferentially around residues 40–45 of vitamin K-dependent proteins, releasing Gla domains that can be isolated for study. The peptides were desalted by adsorption onto a C18 reverse phase gel and elution with 75% acetonitrile in 0.1% trifluoroacetic acid. 2-Hydroxy-5-methoxybenzoic acid was used as the matrix. This analysis showed that wild type recombinant factor VIIa (NOVO Nordisk) had one less Gla residue than expected. The principal product formed from chymotrypsin digestion was the 1–40 peptide, which gave a M+1 ion of 5145.6 mass units, approximately one carboxyl group (44 mass units) lower than the theoretical value for fully carboxylated 1–40 peptide (theoretical=5190.4 mass units). Plasma-derived factor VIIa was purchased from Enzyme Research Laboratories. The M+1 ion for the 1–40 peptide was 5189.8 mass units, equal to the fully carboxylated Gla domain. A second ion observed for the NOVO-recombinant VIIa product corresponding to residues 1–32. The M+1 ion for this peptide was 4184.6 mass units, equal to the theoretical value for fully carboxylated 1–32 peptide (theoretical=4185.3 mass units). Thus, recombinant wild type factor VIIa is severely undercarboxylated, with almost all of this expressed at position 36. In contrast to this result, the Q11E33 mutant of factor VII gave M+1 ions of 5312.8 and 5269.0 in approximately equal abundance. The M+1 for this fully carboxylated peptide is 5311.4. The second peak

corresponds to a peptide with one less carboxyl group (−44 mass units). This result shows that incorporation of the Q11E33 mutations resulted in fully carboxylated Gla domain after tissue culture production. An advantage of the Q11E33 mutant may arise from more complete carboxylation of position 36. Overall, protein production with an optimum Gla domain entails selection of residues that facilitate full carboxylation in the tissue culture. Proper selection of residues in positions 33–36 appear important in this regard.

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

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Xaa Asp Asp Xaa Thr Thr Asp Xaa Phe Trp Arg Thr Tyr
         35                  40                  45
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What is claimed is:

1. A Factor VII or Factor VIIa polypeptide comprising a modified GLA domain that enhances membrane binding affinity of said polypeptide relative to a corresponding native Factor VII or Factor VIIa polypeptide, said modified GLA domain comprising an amino acid substitution selected from a hydrophobic amino acid residue or a glutamic acid residue at position 34, wherein amino acid positions of the Factor VII or Factor VIIa polypeptide are numbered according to SEQ ID NO:3.

2. The polypeptide of claim **1**, wherein a phenylalanine, leucine or isoleucine residue is substituted at position 34.

3. The polypeptide of claim **1**, wherein a glutamic acid residue is substituted at position 34.

4. The polypeptide of claim **1**, further comprising an amino acid substitution at position 10.

5. The polypeptide of claim **4**, wherein a glutamine, asparagine, glutamic acid, or aspartic acid residue is substituted at position 10.

6. The polypeptide of claim **5**, wherein a glutamine residue is substituted at position 10.

7. The polypeptide of claim **1**, further comprising an amino acid substitution at position 32.

8. The polypeptide of claim **7**, wherein a glutamic acid residue is substituted at position 32.

9. The polypeptide of claim **1**, further comprising an amino acid substitution at position 28.

10. The polypeptide of claim 9, wherein a phenylalanine or a glutamic acid residue is substituted at position 28.

11. The polypeptide of claim 10, wherein a phenylalanine residue is substituted at position 28.

12. The polypeptide of claim 1, further comprising an insertion at position 4.

13. The polypeptide of claim 12, wherein a tyrosine or glycine residue is inserted at position 4.

14. The polypeptide of claim 13, wherein a tyrosine residue is inserted at position 4.

15. An isolated nucleic acid molecule comprising a nucleic acid sequence encoding the polypeptide of claim 1.

16. The isolated nucleic acid of claim 15, wherein a glutamic acid residue is substituted at position 34.

17. The isolated nucleic acid of claim 15, further comprising an amino acid substitution at position 10.

18. The isolated nucleic acid of claim 17, wherein a glutamine, asparagine, glutamic acid, or aspartic acid residue is substituted at position 10.

19. The isolated nucleic acid of claim 18, wherein a glutamine residue is substituted at position 10.

20. The isolated nucleic acid of claim 15, further comprising an amino acid substitution at position 32.

21. The isolated nucleic acid of claim 20, wherein a glutamic acid residue is substituted at position 32.

22. The isolated nucleic acid of claim 15, wherein the Factor VII or Factor VIIa polypeptide further comprises a glutamine residue substituted at position 10 and a glutamic acid residue substituted at position 32.

23. The polypeptide of claim 1, wherein the Factor VII or Factor VIIa polypeptide further comprises a glutamine residue substituted at position 10 and a glutamic acid residue substituted at position 32.

24. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and an amount of Factor VII or Factor VIIa polypeptide effective to increase clot formation, wherein said Factor VII or Factor VIIa polypeptide comprises a modified GLA domain that enhances membrane binding affinity of said polypeptide relative to a corresponding native Factor VII or Factor VIIa polypeptide, said modified GLA domain comprising an amino acid substitution selected from a hydrophobic amino acid residue at position 34, wherein amino acid positions of the Factor VII or Factor VIIa polypeptide are numbered according to SEQ ID NO:3.

25. The pharmaceutical composition of claim 24, wherein the Factor VII or Factor VIIa polypeptide further comprises a glutamine residue substituted at position 10 and a glutamic acid residue substituted at position 32.

26. A method for treating a bleeding disorder in a patient, said method comprising administering the pharmaceutical composition of claim 24 to said patient.

27. The pharmaceutical composition of claim 24, wherein a glutamic acid residue is substituted at position 34.

28. The pharmaceutical composition of claim 24, further comprising an amino acid substitution at position 10.

29. The pharmaceutical composition of claim 28, wherein a glutamine, asparagine, glutamic acid, or aspartic acid residue is substituted at position 10.

30. The pharmaceutical composition of claim 29, wherein a glutamine residue is substituted at position 10.

31. The pharmaceutical composition of claim 24, further comprising an amino acid substitution at position 32.

32. The pharmaceutical composition of claim 31, wherein a glutamic acid residue is substituted at position 32.

33. An isolated mammalian host cell that expresses a Factor VII or Factor VIIa polypeptide, said Factor VII or Factor VIIa polypeptide comprising a modified GLA domain that enhances membrane binding affinity of said polypeptide relative to a corresponding native Factor VII or Factor VIIa polypeptide, said modified GLA domain comprising an amino acid substitution selected from a hydrophobic amino acid residue or a glutamic acid residue at position 34, wherein amino acid positions of the Factor VII or Factor VIIa polypeptide are numbered according to SEQ ID NO:3.

34. The host cell of claim 33, wherein a glutamic acid residue is substituted at position 34.

35. The host cell of claim 33, further comprising an amino acid substitution at position 10.

36. The host cell of claim 35, wherein a glutamine, asparagine, glutamic acid, or aspartic acid residue is substituted at position 10.

37. The host cell of claim 36, wherein a glutamine residue is substituted at position 10.

38. The host cell of claim 33, further comprising an amino acid substitution at position 32.

39. The host cell of claim 38, wherein a glutamic acid residue is substituted at position 32.

40. The host cell of claim 33, wherein the Factor VII or Factor VIIa polypeptide further comprises a glutamine residue substituted at position 10 and a glutamic acid residue substituted at position 32.

41. A method of increasing clot formation in a mammal comprising administering an amount of a Factor VII or Factor VIIa polypeptide effective to increase clot formation in said mammal, wherein said Factor VII or Factor VIIa polypeptide comprises a modified GLA domain that enhances membrane binding affinity of said polypeptide relative to a corresponding native Factor VII or Factor VIIa polypeptide, said modified GLA domain comprising at least one amino acid substitution selected from the group consisting of a hydrophobic amino acid residue or a glutamic acid residue at position 34, wherein amino acid positions of the Factor VII or Factor VIIa polypeptide are numbered according to SEQ ID NO:3.

42. The method of claim 41, wherein a glutamic acid residue is substituted at position 34.

43. The method of claim 41, further comprising an amino acid substitution at position 10.

44. The method of claim 43, wherein a glutamine, asparagine, glutamic acid, or aspartic acid residue is substituted at position 10.

45. The method of claim 44, wherein a glutamine residue is substituted at position 10.

46. The method of claim 41, further comprising an amino acid substitution at position 32.

47. The method of claim 46, wherein a glutamic acid residue is substituted at position 32.

48. The method of claim 41, wherein the Factor VII or Factor VIIa polypeptide further comprises a glutamine residue substituted at position 10 and a glutamic acid residue substituted at position 32.

49. A method for producing a Factor VII or Factor VIIa polypeptide having a modified GLA domain comprising at least one amino acid substitution selected from the group consisting of a) substitution of a hydrophobic amino acid residue at position 33, and b) substitution of a hydrophobic amino acid residue or a glutamic acid residue at position 34, wherein amino acid positions of the Factor VII or Factor VIIa polypeptide are numbered according to SEQ ID NO:3, the method comprising (a) providing a culture of the mammalian host cell of claim 18 under conditions which permit expression of the polypeptide, and (b) recovering the polypeptide.

50. The method of claim 49, wherein a glutamic acid residue is substituted at position 34.

51. The method of claim 49, further comprising an amino acid substitution at position 10.

52. The method of claim 51, wherein a glutamine, asparagine, glutamic acid, or aspartic acid residue is substituted at position 10.

53. The method of claim 52, wherein a glutamine residue is substituted at position 10.

54. The method of claim 49, further comprising an amino acid substitution at position 32.

55. The method of claim 54, wherein a glutamic acid residue is substituted at position 32.

56. The method of claim 49, wherein the Factor VII or Factor VIIa polypeptide further comprises a glutamine residue substituted at position 10 and a glutamic acid residue substituted at position 32.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 7,220,837 B1
APPLICATION NO. : 10/031005
DATED : May 22, 2007
INVENTOR(S) : Gary L. Nelsestuen

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title Page (Page 2), References Cited, Other Publications, Dahlback reference, please delete "Thromboemolism" and insert --Thromboembolism--therefor;

Title Page (Page 2), References Cited, Other Publications, Evans and Nelsestuan reference, please delete "Nelsestuan" and insert --Nelsestuen--therefor;

Title Page (Page 2), References Cited, Other Publications, Hope et al. reference, please delete "Protential" and insert --Potential--therefor;

Title Page (Page 2), References Cited, Other Publications, first Lu and Nelsestuen reference, please delete "Inferation" and insert --Interaction--therefor;

Title Page (Page 2), References Cited, Other Publications, first Shen et al. reference, please delete "Mutagenetis" and insert --Mutagenesis--therefore;

Title Page (Page 3), References Cited, Other Publications, Jurlander et al. reference, please delete "(rFVVIa)" and insert --(rFVIIa)--therefor;

Title Page (Page 3), References Cited, Other Publications, Bharadwaj et al. reference, after "binding" please insert --,--;

Title Page (Page 3), References Cited, Other Publications, Bharadwaj et al. reference, please delete "27130685" and insert --271:30685-30691--therefor;

Title Page (Page 3), References Cited, Other Publications, first Chang et al. reference, please delete "recombiant" and insert --recombinant--therefor;

Title Page (Page 3), References Cited, Other Publications, first Chang et al. reference, after "Biochemistry" please insert --,--;

Title Page (Page 3), References Cited, Other Publications, second Chang et al. reference, please delete "199" and insert --100--therefor;

Title Page (Page 3), References Cited, Other Publications, Higashi et al. reference, please delete "27" and insert --271--therefor;

Title Page (Page 3), References Cited, Other Publications, Iakhiaev et al. reference, please delete "Thromobsis" and insert --Thrombosis--therefor;

Title Page (Page 3), References Cited, Other Publications, second Jin et al. reference, please delete "mediated" and insert --mediate--therefor;

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 7,220,837 B1
APPLICATION NO. : 10/031005
DATED : May 22, 2007
INVENTOR(S) : Gary L. Nelsestuen

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title Page (Page 3), References Cited, Other Publications, Petrovan et al. reference, please delete "seerine" and insert --serine--therefor;

Column 50, lines 33-34, please delete "at least one" and insert --an--therefor;

Column 50, lines 34-35, please delete "the group consisting of";

Column 50, lines 57-58, please delete "at least one" and insert --an--therefor;

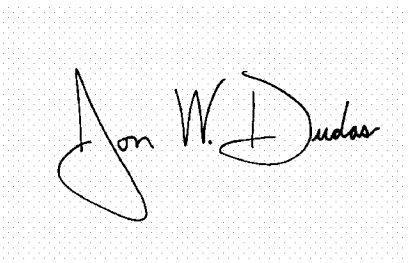
Column 50, lines 58-59, please delete "the group consisting of a substitution of";

Column 50, lines 60-61, please delete "at position 33, and b) substitution of a hydrophobic amino acid residue";

Column 50, line 65, please delete "18" and insert --33--therefor.

Signed and Sealed this

Fourth Day of September, 2007



JON W. DUDAS
Director of the United States Patent and Trademark Office

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 7,220,837 B1
APPLICATION NO. : 10/031005
DATED : May 22, 2007
INVENTOR(S) : Gary L. Nelsestuen

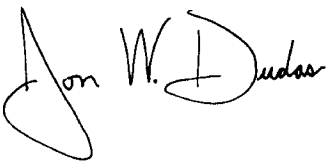
It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 49, line 42 (Claim 24), after "residue" please insert --or a glutamic acid residue--

Column 50, line 59 (Claim 49), please delete "a)" and insert --a-- therefor.

Signed and Sealed this

Fifteenth Day of July, 2008



JON W. DUDAS
Director of the United States Patent and Trademark Office

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Haaning et al. (43) Pub. Date: May 25, 2006

(54) FACTOR VII OR VIIA POLYPEPTIDE VARIANTS

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(52) **U.S. Cl.** **514/12**; 530/383

(57) **ABSTRACT**

The present invention relates to novel polypeptide variants of factor VII (FVII) or factor VIIa (FVIIa) polypeptides, where said variants comprise an amino acid substitution in position 10 and 32 and where said variants further comprise a sugar moiety covalently attached to an introduced in vivo N-glycosylation site located outside the Gla domain. Such polypeptide variants are useful in therapy, in particular for the treatment of a variety of coagulation-related disorders, such as trauma.

FACTOR VII OR VIIA POLYPEPTIDE VARIANTS

FIELD OF THE INVENTION

[0001] The present invention relates to novel polypeptide variants of factor VII (FVII) or factor VIIa (FVIIa) polypeptides, where said variants comprise an amino acid substitution in position 10 and 32 and where said variants further comprise a sugar moiety covalently attached to an introduced in vivo N-glycosylation site.

[0002] The present invention also relates to use of such polypeptide variants in therapy, in particular for the treatment of a variety of coagulation-related disorders.

BACKGROUND OF THE INVENTION

[0003] Blood coagulation is a process consisting of a complex interaction of various blood components (or factors) that eventually results in a fibrin clot. Generally, the blood components participating in what has been referred to as the "coagulation cascade" are proenzymes or zymogens, i.e. enzymatically inactive proteins that are converted into an active form by the action of an activator. One of these coagulation factors is FVII.

[0004] FVII is a vitamin K-dependent plasma protein synthesized in the liver and secreted into the blood as a single-chain glycoprotein with a molecular weight of 53 kDa (Broze & Majerus, J. Biol. Chem 1980; 255:1242-1247). The FVII zymogen is converted into an activated form (FVIIa) by proteolytic cleavage at a single site, R152-I153, resulting in two chains linked by a single disulfide bridge. FVIIa in complex with tissue factor (FVIIa complex) is able to convert both factor IX and factor X into their activated forms, followed by reactions leading to rapid thrombin production and fibrin formation (Osterud & Rapaport, Proc Natl Acad Sci USA 1977; 74:5260-5264).

[0005] FVII undergoes post-translational modifications, including vitamin K-dependent carboxylation resulting in ten γ-carboxyglutamic acid residues in the N-terminal region of the molecule. Thus, residue number 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35 shown in SEQ ID NO:2 are γ-carboxyglutamic acids-residues in the Gla domain important for FVII activity. Other post-translational modifications include sugar moiety attachment at two naturally occurring N-glycosylation sites at position 145 and 322, respectively, and at two naturally occurring O-glycosylation sites at position 52 and 60, respectively.

[0006] The gene coding for human FVII (hFVII) has been mapped to chromosome 13 at q34-qter 9 (de Grouchy et al., Hum Genet 1984; 66:230-233). It contains nine exons and spans 12.8 Kb (O'Hara et al., Proc Natl Acad Sci USA 1987; 84:5158-5162). The gene organisation and protein structure of FVII are similar to those of other vitamin K-dependent procoagulant proteins, with exons Ia and Ib encoding for signal sequence; exon 2 the propeptide and Gla domain; exon 3 a short hydrophobic region; exons 4 and 5 the epidermal growth factor-like domains; and exon 6 through 8 the serine protease catalytic domain (Yoshitake et al., Biochemistry 1985; 24: 3736-3750).

[0007] Reports exist on experimental three-dimensional structures of hFVIIa (Pike et al., PNAS. U.S.A., 1999; 96:8925-30 and Kemball-Cook et al., J. Struct. Biol, 1999; 127:213-223); of hFVIIa in complex with soluble tissue factor using X-ray crystallographic methods (Banner et al., Nature, 1996; 380:41 and Zhang et al., J. Mol. Biol, 1999; 285: 2089); and of smaller fragments of hFVII (Muranyi et al., Biochemistry, 1998; 37:10605 and Kao et al., Biochemistry, 1999; 38:7097).

[0008] Some protein-engineered variants of FVII have been reported. See, e.g., Dickinson & Ruf, J Bio Chem, 1997;272:19875-19879, Kemball-Cook et al., J Biol Chem, 1998; 273:8516-8521, Bharadwaj et al., J Biol Chem, 1996; 271:30685-30691, Ruf et al., Biochemistry, 1999; 38:1957-1966; WO 99/20767; WO 00/11416; WO 02/22776; WO 02/38162; WO 01/83725; WO 01/58935; U.S. Pat. No. 5,580,560.

[0009] Reports exist on expression of FVII in BHK or other mammalian cells (WO 92/15686, WO 91/11514 and WO 88/10295) and co-expression of FVII and kex2 endoprotease in eukaryotic cells (WO 00/28065).

[0010] Commercial preparations of human recombinant FVIIa are sold as NovoSeven®. NovoSeven® is indicated for the treatment of bleeding episodes in hemophilia A or B patients. NovoSeven® is the only rhFVIIa for effective and reliable treatment of bleeding episodes available on the market.

[0011] An inactive form of FVII in which arginine 152 and/or isoleucine 153 is/are modified has been reported in WO91/1154. These amino acids are located at the activation site. WO 96/12800 describes inactivation of FVIIa by a serine proteinase inhibitor; inactivation by carbamylation of FVIIa at the α-amino acid group I153 has been described by Petersen et al., Eur J Biochem, 1999;261:124-129. The inactivated form is capable of competing with wild-type FVII or FVIIa for binding to tissue factor and inhibiting clotting activity. The inactivated form of FVIIa is suggested to be used for treatment of patients being in hypercoagulable states, such as patients with sepsis, in risk of myocardial infarction or of thrombotic stroke.

[0012] A circulating rhFVIIa half-life of 2.3 hours was reported in "Summary Basis for Approval for NovoSeven®", FDA reference number 96-0597. Relatively high doses and frequent administration are necessary to reach and sustain the desired therapeutic or prophylactic effect. As a consequence adequate dose regulation is difficult to obtain and the need of frequent intravenous administrations imposes restrictions on the patient's way of living.

[0013] In connection with treatment of uncontrolled bleedings, such as trauma, it is believed that factor VIIa is capable of activating factor X to factor Xa without binding to tissue factor, and this activation reaction is believed to occur primarily on activated blood platelets (Hedner et al. Blood Coagulation & Fibrinolysis, 2000;1 1;107-111). However, hFVIIa or rhFVIIa has a low activity towards factor X in the absence of tissue factor and, consequently, treatment of uncontrolled bleeding, for example in trauma patients, requires relatively high and multiple doses of hFVIIa or rhFVIIa Therefore, in order to treat uncontrolled bleedings more efficiently (to minimize blood loss) there is need for improved FVIIa molecules, which possess a high activity toward factor X in the absence of tissue factor. Such improved FVIIa molecules will exhibit a lowered clotting time (or faster action) as compared to rhFVIIa when administered in connection with uncontrolled bleedings.

[0014] A molecule with a longer circulation half-life would decrease the number of necessary administrations. Given the association of current the rhFVIIa product with frequent injections, and the potential for obtaining more optimal therapeutic FVIIa levels with concomitant enhanced therapeutic effect, there is a clear need for improved FVII- or FVIIa-like molecules.

[0015] One way to increase the circulation half-life of a protein is to ensure that renal clearance of the protein is reduced. This may be achieved by conjugating the protein to a chemical moiety, which is capable of conferring reduced renal clearance to the protein.

[0016] Furthermore, attachment of a chemical moiety to the protein or substitution of amino acids exposed to proteolysis may effectively block a proteolytic enzyme from contact leading to proteolytic degradation of the protein. Polyethylene glycol (PEG) is one such chemical moiety that has been used in the preparation of therapeutic protein products. WO 98/32466 suggests that FVII, among many other proteins, may be PEGylated but does not contain any further information in this respect WO 01/58935 discloses a new strategy for developing FVII or FVIIa molecules having inter alia an increased half-life.

[0017] As indicated above, another problem in current rhFVIIa treatment is the relative instability of the molecule with respect to proteolytic degradation. Proteolytic degradation is a major obstacle for obtaining a preparation in solution as opposed to a lyophilized product. The advantage of obtaining a stable soluble preparation lies in easier handling for the patient, and, in the case of emergencies, quicker action, which potentially can become life saving. Attempts to revent proteolytic degradation by site directed mutagenesis at major proteolytic sites have been disclosed in WO 88/10295.

[0018] One object of the present invention is to provide improved FVII or FVIIa molecules FVII or FVIIa variants) with a longer circulation half-life (thereby decreasing the number of necessary administrations) and which are capable of activating factor X to factor Xa (without binding to tissue factor) more efficiently than hFVIIa or rhFVIIa (thereby being able to treat uncontrolled bleadings, such as a trauma, more efficiently).

[0019] Another object of the present invention is to provide improved FVII or FVII molecules (FVII or FVIIa variants) with an increased bioavailability (such as an increased Area Under the Curve as compared to rhFVIIa when administered intravenously) and which are capable of activating factor X to factor Xa (without binding to tissue factor) more efficiently than hFVIIa or rhFVIIa (thereby being able to treat uncontrolled bleadings, such as a trauma, more efficiently).

[0020] These objects are met by the FVII or FVIIa variants provided herein.

BRIEF DISCLOSURE OF THE INVENTION

[0021] In its broadest aspect the present invention relates to a FVII or FVIIa polypeptide variant having an amino acid sequence comprising 3-15 amino acid modifications relative to hFVII or hFVIIa having the amino acid sequence shown in SEQ ID NO:2, wherein said amino acid sequence of the variant comprises an amino acid substitution in position 10 and 32 and wherein a sugar moiety is covalently attached to an introduced in vivo N-glycosylation site located outside the Gla domain.

[0022] Another aspect of the invention relates to a nucleotide sequence encoding the polypeptide variant of the invention.

[0023] In a further aspect the invention relates to an expression vector comprising the nucleotide sequence of the invention.

[0024] In a still further aspect the invention relates to a host cell comprising the nucleotide sequence of the invention or the expression vector of the invention.

[0025] In an even further aspect the invention relates to a pharmaceutical composition comprising the polypeptide variant of the invention, and a pharmaceutical acceptable carrier or excipient.

[0026] Still another aspect of the invention relates to the polypeptide variant of the invention, or the pharmaceutical composition of the invention, for use as a medicament.

[0027] Further aspects of the present invention will be apparent from the below description as well as from the appended claims.

DETAILED DISCLOSURE OF THE INVENTION

DEFINITIONS

[0028] In the context of the present application and invention the following definitions apply:

[0029] The term "conjugate" (or interchangeably "conjugated polypeptide") is intended to indicate a heterogeneous (in the sense of composite or chimeric) molecule formed by the covalent attachment of one or more polypeptide(s) to one or more non-polypeptide moieties such as polymer molecules, lipophilic compounds, sugar moieties or organic derivatizing agents. Preferably, the conjugate is soluble at relevant concentrations and conditions, i.e. soluble in physiological fluids such as blood. Examples of conjugated polypeptides of the invention include glycosylated and/or PEGylated polypeptides.

[0030] The term "covalent attachment" or "covalently attached" means that the polypeptide variant and the non-polypeptide moiety are either directly covalently joined to one another, or else are indirectly covalently joined to one another through an intervening moiety or moieties, such as a bridge, spacer, or linkage moiety or moieties.

[0031] The term "non-polypeptide moiety" is intended to mean a molecule, different from a peptide polymer composed of amino acid monomers and linked together by peptide bonds, which molecule is capable of conjugating to an attachment group of the polypeptide variant of the invention. Preferred examples of such molecules include polymer molecules, sugar moieties, lipophilic compounds or organic derivatizing agents. When used in the context of a conjugated variant of the invention it will be understood that the non-polypeptide moiety is linked to the polypeptide part of the conjugated variant through an attachment group of the polypeptide. As explained above, the non-polypeptide moiety can be directly covalently joined to the attachment group or it can be indirectly covalently joined to the attachment

group through an intervening moiety or moieties, such as a bridge spacer or linker moiety or moieties.

[0032] A "polymer molecule" is a molecule formed by covalent linkage of two or more monomers, wherein none of the monomers is an amino acid residue, except where the polymer is human albumin or another abundant plasma protein. The term "polymer" may be used interchangeably with the term "polymer molecule". The term is also intended to cover carbohydrate molecules attached by in vitro glycosylation, i.e. a synthetic glycosylation performed in vitro normally involving covalently linking a carbohydrate molecule to an attachment group of the polypeptide variant, optionally using a cross-linking agent. In vitro glycosylation is discussed in detail further below.

[0033] The term "sugar moiety" is intended to indicate a carbohydrate-containing molecule comprising one or more monosaccharide residues, capable of being attached to the polypeptide variant (to produce a polypeptide variant conjugate in the form of a glycosylated polypeptide variant) by way of in vivo glycosylation. The term "in vivo glycosylation" is intended to mean any attachment of a sugar moiety occurring in vivo, i.e. during posttranslational processing in a glycosylating cell used for expression of the polypeptide variant, e.g. by way of N-linked and O-linked glycosylation. The exact oligosaccharide structure depends, to a large extent, on the glycosylating organism in question.

[0034] An "N-glycosylation site" has the sequence N-X-S/T/C, wherein X is any amino acid residue except proline, N is asparagine and S/TIC is either serine, threonine or cysteine, preferably serine or threonine, and most preferably threonine. Preferably, the amino acid residue in position +3 relative to the asparagines residue is not a proline residue.

[0035] An "O-glycosylation site" is the OH-group of a serine or threonine residue.

[0036] The term "attachment group" is intended to indicate a functional group of the polypeptide variant, in particular of an amino acid residue thereof or a carbohydrate moiety, capable of attaching a non-polypeptide moiety such as a polymer molecule, a lipophilic molecule, a sugar moiety or an organic derivatizing agent. Useful attachment groups and their matching non-polypeptide moieties are apparent from the table below.

Attachment group	Amino acid	Examples of non-polypeptide moiety	Conjugation method/-Activated PEG	Reference
—NH_2	N-terminal, Lys	Polymer, e.g. PEG, with amide or imine group	mPEG-SPA Tresylated mPEG	Shearwater Inc. Delgado et al, critical reviews in Therapeutic Drug Carrier Systems 9(3, 4): 249–304 (1992)
—COOH	C-terminal, Asp, Glu	Polymer, e.g. PEG, with ester or amide group	mPEG-Hz	Shearwater Inc.
		Carbohydrate moiety	In vitro coupling	
—SH	Cys	Polymer, e.g. PEG, with disulfide, maleimide or vinyl sulfone group	PEG-vinylsulphone PEG-maleimide	Shearwater Inc. Delgado et al, critical reviews in Therapeutic Drug
		Carbohydrate moiety	In vitro coupling	Carrier Systems 9(3, 4): 249–304 (1992)
—OH	Ser, Thr, Lys, OH—	Sugar moiety PEG with ester, ether, carbamate, carbonate	In vivo O-linked glycosylation	
—$CONH_2$	Asn as part of an N-glycosylation site	Sugar moiety Polymer, e.g. PEG	In vivo N-glycosylation	
Aromatic residue	Phe, Tyr, Trp	Carbohydrate moiety	In vitro coupling	
—$CONH_2$	Gln	Carbohydrate moiety	In vitro coupling	Yan and Wold, Biochemistry, 1984, Jul 31; 23(16): 3759–65
Aldehyde Ketone	Oxidized oligo-saccharide	Polymer, e.g. PEG, PEG-hydrazide	PEGylation	Andresz et al., 1978, Makromol. Chem. 179: 301, WO 92/16555, WO 00/23114
Guanidino	Arg	Carbohydrate moiety	In vitro coupling	Lundblad and Noyes, Chimical Reagents for Protein Modification, CRC Press Inc., Florida, USA
Imidazole ring	His	Carbohydrate moiety	In vitro coupling	As for guanidine

[0037] For in vivo N-glycosylation, the term "attachment group" is used in an unconventional way to indicate the amino acid residues constituting a N-glycosylation site (with the sequence N-X-S/T/C, wherein X is any amino acid residue except proline, N is asparagine and S/T/C is either serine, threonine or cysteine, preferably serine or threonine, and most preferably threonine). Although the asparagine residue of the N-glycosylation site is the one to which the sugar moiety is attached during glycosylation, such attachment cannot be achieved unless the other amino acid residues of the N-glycosylation site is present.

[0038] Accordingly, when the non-polypeptide moiety is a sugar moiety and the conjugation is to be achieved by in vivo N-glycosylation, the term "amino acid residue comprising an attachment group for a non-polypeptide moiety" as used in connection with alterations of the amino acid sequence of the polypeptide is to be understood as meaning that one or more amino acid residues constituting an in vivo N-glycosylation site are to be altered in such a manner that a functional in vivo N-glycosylation site is introduced into the amino acid sequence.

[0039] In the present application, amino acid names and atom names (e.g. CA, CB, CD, CG, SG, NZ, N, O, C, etc) are used as defined by the Protein DataBank (PDB) (www-.pdb.org) based on the IUPAC nomenclature (IUPAC Nomenclature and Symbolism for Amino Acids and Peptides (residue names, atom names, etc.), *Eur. J. Biochem.,* 138, 9-37 (1984) together with their corrections in *Eur. J. Biochem.,* 152, 1 (1985)).

[0040] The term "amino acid residue" is intended to indicate an amino acid residue contained in the group consisting of alanine (Ala or A), cysteine (Cys or C), aspartic acid (Asp or D), glutamic acid (Glu or E), phenylalanine (Phe or F), glycine (Gly or G), histidine (His or H), isoleucine (Ile or I), lysine (Lys or K), leucine (Leu or L), methionine (Met or M), asparagine (Asn or N), proline (Pro or P), glutamine (Gln or Q), arginine (Arg or R), serine (Ser or S), threonine (Thr or T), valine (Val or V), tryptophan (Trp or W), and tyrosine (Tyr or Y) residues.

[0041] The terminology used for identifying amino acid positions is illustrated as follows: I205 indicates that position 205 is occupied by an isoleucine residue in the amino acid sequence shown in SEQ ID NO:2. I205T indicates that the isoleucine residue of position 205 has been substituted with a threonine residue. Alternative substitutions are indicated with a "/", e.g. I205S/T means an amino acid sequence in which isoleucine in position 205 is substituted with either serine or threonine. Multiple substitutions are indicated with a "+", e.g. K143N+N145T means a substitution of the lysine residue in position 143 with an asparagine residue and a substitution of the asparagine residue in position 145 with a threonine residue. Insertion of an additional amino acid residue is indicated in the following way: Insertion of a tyrosine residue after A3 (i.e. in position 4) is indicated by A3AY (leading to insertion of a tyrosine residue in position 4). A deletion of an amino acid residue is indicated by an asterix. For example, deletion of a valine residue in position 172 is indicated by V172*. Simultaneous insertion and substitution are indicated in the following way: Substitution of an alanine residue in position 175 with a threonine residue followed by insertion of a leucine residue after position 175 is indicated A175TL.

[0042] Unless otherwise indicated, the numbering of amino acid residues made herein is made relative to the amino acid sequence of the hFVII/VIIa polypeptide (SEQ ID NO:2).

[0043] The term "differs from" as used in connection with specific mutations is intended to allow for additional differences being present apart from the specified amino acid difference. For instance, in addition to the introduction of in vivo N-glycosylation sites (located outside the Gla domain), the polypeptide may comprise other modifications that are not related to introduction of such amino acid residues. In a similar way, in addition to the modifications performed in the Gla domain aiming at increasing the phospholipid membrane binding affinity, the polypeptide may contain other modifications that are not necessarily related to this effect.

[0044] Thus, in addition to the amino acid modifications disclosed herein, it will be understood that the amino acid sequence of the polypeptide variant of the invention may, if desired, contain other alterations, i.e. other substitutions, insertions or deletions. These may, for example, include truncation of the N- and/or C-terminus by one or more amino acid residues (e.g. by 1-10 amino acid residues), or addition of one or more extra residues at the N- and/or C-terminus, e.g. addition of a methionine residue at the N-terminus or introduction of a cysteine residue near or at the C-terminus, as well as "conservative amino acid substitutions", i.e. substitutions performed within groups of amino acids with similar characteristics, e.g. small amino acids, acidic amino acids, polar amino acids, basic amino acids, hydrophobic amino acids and aromatic amino acids.

[0045] Examples of such conservative substitutions are shown in the below table.

1	Alanine (A)	Glycine (G)	Serine (S)	Threonine (T)
2	Aspartic acid (D)	Glutamic acid (E)		
3	Asparagine (N)	Glutamine (Q)		
4	Arginine (R)	Histidine (H)	Lysine (K)	
5	Isoleucine (I)	Leucine (L)	Methionine (M)	Valine (V)
6	Phenylalanine (F)	Tyrosine (Y)	Tryptophan (W)	

[0046] Still other examples of additional modifications are disclosed in the section entitled "*Other modifications outside the Gla domain*" below.

[0047] The term "nucleotide sequence" is intended to indicate a consecutive stretch of two or more nucleotide molecules. The nucleotide sequence may be of genomic, cDNA, RNA, semisynthetic, synthetic origin, or any combinations thereof.

[0048] The term "polymerase chain reaction" or "PCR" generally refers to a method for amplification of a desired nucleotide sequence in vitro, as described, for example, in U.S. Pat. No. 4,683,195. In general, the PCR method involves repeated cycles of primer extension synthesis, using oligonucleotide primers capable of hybridising preferentially to a template nucleic acid.

[0049] The term "vector" refers to a plasmid or other nucleotide sequences that are capable of replicating within a host cell or being integrated into the host cell genome, and as such, are useful for performing different functions in

conjunction with compatible host cells (a vector-host system): to facilitate the cloning of the nucleotide sequence, i.e. to produce usable quantities of the sequence, to direct the expression of the gene product encoded by the sequence and to integrate the nucleotide sequence into the genome of the host cell. The vector will contain different components depending upon the function it is to perform.

[0050] "Cell", "host cell", "cell line" and "cell culture" are used interchangeably herein and all such terms should be understood to include progeny resulting from growth or culturing of a cell.

[0051] "Transformation" and "transfection" are used interchangeably to refer to the process of introducing DNA into a cell.

[0052] "Operably linked" refers to the covalent joining of two or more nucleotide sequences, by means of enzymatic ligation or otherwise, in a configuration relative to one another such that the normal function of the sequences can be performed. For example, the nucleotide sequence encoding a presequence or secretory leader is operably linked to a nucleotide sequence coding for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide: a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the nucleotide sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, then synthetic oligonucleotide adaptors or linkers are used, in conjunction with standard recombinant DNA methods.

[0053] In the context of the present invention the terms "modification" or "amino acid modification" is intended to cover replacement of an amino acid side chain, substitution of an amino acid residue, deletion of an amino acid residue and/or insertion of an amino acid residue.

[0054] The terms "mutation" and "substitution" are used interchangeably herein.

[0055] The term "introduce" refers to introduction of an amino acid residue by substitution of an existing amino acid residue or, alternatively, by insertion of an additional amino acid residue.

[0056] The term "remove" refers to removal of an amino acid residue by substitution of the amino acid residue to be removed by another amino acid residue or, alternatively, by deletion (without substitution) of the amino acid residue to be removed

[0057] The term "FVII" or "FVII polypeptide" refers to a FVII molecule provided in single chain form. One example of a FVII polypeptide is wild-type human FVII (hFVII) shown in SEQ ID NO:2. It should be understood, however, that the term "FVII polypeptide" also covers hFVII-like molecules, such as fragments or variants of SEQ ID NO:2, in particular variants where the sequence comprises at least one, such as 1-15, e.g., 1-10, amino acid modifications as compared to SEQ ID NO:2.

[0058] The term "FVIIa" or "FVIIa polypeptide" refers to a FVIIa molecule provided in its activated two-chain form. When the amino acid sequence of SEQ ID NO:2 is used to describe the amino acid sequence of FVIIa it will be understood that the peptide bond between R152 and I153 of the single-chain form has been cleaved, and that one of the chains comprises amino acid residues 1-152, the other chain amino acid residues 153406.

[0059] The terms "rFVII" and "rFVIIa" refer to FVII and FVIIa polypeptides produced by recombinant techniques.

[0060] The terms "hFVII" and "hFVIIa" refer to human wild-type FVII and FVIIa, respectively, having the amino acid sequence shown in SEQ ID NO:2

[0061] The terms "rhFVII" and "rhFVIIa" refer to human wild-type FVII and FVIIa, having the amino acid sequence shown in SEQ ID NO:2, produced by recombinant means. An example of rhFVIIa is NovoSeven®.

[0062] When used herein, the term "Gla domain" is intended to cover amino acid residues no. 1 to 45 of SEQ ID NO:2.

[0063] Accordingly, the term "located outside the Gla domain" covers amino acid residue no. 46-406 of SEQ ID NO:2.

[0064] The abbreviations "TF" and "TFPI, mean Tissue Factor and Tissue Factor Pathway Inhibitor, respectively.

[0065] The term "protease domain" is used about residues 153-406 counted from the N-terminus.

[0066] The term "catalytic site" is used to mean the catalytic triad consisting of S344, D242 and H193 of the polypeptide variant.

[0067] The term "parent" is intended to indicate the molecule to be modified/improved in accordance with the present invention. Although the parent polypeptide to be modified by the present invention may be any FVII or FVIIa polypeptide, and thus be derived from any origin, e.g. a non-human mammalian origin, it is preferred that the parent polypeptide is hFVII or hFVIIa

[0068] A "variant" is a polypeptide, which differs in one or more amino acid residues from its parent polypeptide, normally in 3-15 amino acid residues (e.g. in 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acid residues), such as in 3-10 amino acid residues, e.g. in 3-8 or 3-5 amino acid residues. In other words, a "variant" typically contains 3-15 amino acid modifications (for example 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acid modifications), such as 3-10 amino acid modifications, e.g. 3-8 or 3-5 amino acid modifications relative to the parent polypeptide. In the present context, the term "modification" encompasses insertions, deletions, substitutions and combinations thereof. It will be understood that a polypeptide variant according to the present invention will be modified in at least three positions, namely in at least position 10 and 32 (located in the Gla domain) and in at least one position located outside the Gla domain, where said at least one modification creates an in vivo N-glycosylation site.

[0069] The term "clotting activity" is used to mean the activity measured in the "Clotting Assay" described herein. In order to exhibit "clotting activity" a variant of the invention, in its activated form, should have at least 10% of the clotting acitivty of rhFVIIa when assayed in the "Clotting Assay" described herein. In a preferred embodiment of

the invention the variant, in its activated form, has at least 20% of the clotting activity of rhFVIIa, such as at least 30%, e.g. at least 40%, more preferably at least 50%, such as at least 60%, e.g. at least 70%, even more preferably at least 80%, such as at least 90% of the clotting activity of rhFVIIa when assayed in the "Clotting Assay" described herein. In an interesting embodiment the variant, in its activated form, has substantially the same clotting activity as rhFVIIs, such as a clotting activity of 75-125% of the clotting acitivity of rhFVIIa.

[0070] The term "amidolytic activity" is used to mean the activity measured in the "Amidolytic Assay" described herein. In order to exhibit "amidolytic activity" a variant of the invention, in its activated form, should have at least 10% of the amidolytic acitivty of rhFVIIa when assayed in the "Amidolytic Assay" described herein. In a preferred embodiment of the invention the variant, in its activated form, has at least 20% of the amidolytic activity of rhFVIIa, such as at least 30%, e.g. at least 40%, more preferably at least 50%, such as at least 60%, e.g. at least 70%, even more preferably at least 80%, such as at least 90% of the amidolytic activity of rhFVIIa when assayed in the "Amidolytic Assay" described herein. In an interesting embodiment the variant, in its activated form, has substantially the same amidolytic is activity as rhFVIIs, such as an amidolytic activity of 75-125% of the amidolytic acitivity of rhFVIIa.

[0071] In the present context, the term "activity" is also used in connection with the variants capability of activating FX to FXa. This activity is also denoted "FX activation activity" or "FXa generation activity".

[0072] The term "increased FX activation activity" or "increased FXa generation activity" is used to indicate that a variant of the invention, in its activated form, has a statistically significantly increased capability to activate FX to FXa as compared to rhFVIIa or. To what extent a variant of the invention (in its activated form) has an increased FX activation activity may conveniently be determined in the "TF-independent Factor X Activation Assay" described herein.

[0073] The term "stronger clot" or "increased clot strength" is used to indicate that the strength of the clot generated by the polypeptide variant is statistically significantly increased relative to that generated by rhFVIIa as determined under comparable conditions. This effect may be determined as the Area Under the Curve (AUC_{throm}) generated by the variant of the invention, in its activated form, when assayed in the "Thrombogram Assay" disclosed herein. In a similar way, the term "increased AUC_{throm}" is used to indicate that the Area Under the Curve generated by the variant (in its activated form) is statistically significantly increased relative to that generated by rhFVIIa as determined under comparable conditions and when measured in the "Thrombogram Assay" described herein

[0074] The term "T_{max}" is used about the time it takes to obtain the maximum thrombin activity level in the "Thrombogram Assay".

[0075] The term "immunogenicity" as used in connection with a given substance is intended to indicate the ability of the substance to induce a response from the immune system. The immune response may be a cell or antibody mediated response (see, e.g., Roitt: Essential Immunology (8th Edition, Blackwell) for further definition of immunogenicity). Normally, reduced antibody reactivity will be an indication of reduced immunogenicity. The reduced immunogenicity may be determined by use of any suitable method known in the art, e.g. in vivo or in vitro.

[0076] The term "functional in vivo half-life" is used in its normal meaning, i.e. the time at which 50% of the biological activity of the polypeptide is still present in the body/target organ, or the time at which the activity of the polypeptide is 50% of the initial value.

[0077] As an alternative to determining functional in vivo half-life, "serum half-life" may be determined, i.e. the time at which 50% of the polypeptide circulates in the plasma or bloodstream prior to being cleared. Determination of serum half-life is often more simple than determining the functional in vivo half-life and the magnitude of serum half-life is usually a good indication of the magnitude of functional in vivo half-life. Alternatively terms to serum half-life include "plasma half-life", "circulating half-life", "serum clearance", "plasma clearance" and "clearance half-life". The polypeptide is cleared by the action of one or more of the reticuloendothelial systems (RES), kidney, spleen or liver, by tissue factor, SEC receptor or other receptor mediated elimination, or by specific or unspecific proteolysis. Normally, clearance depends on size (relative to the cutoff for glomerular filtration), charge, attached carbohydrate chains, and the presence of cellular receptors for the protein. The functionality to be retained is normally selected from procoagulant, proteolytic or receptor binding activity. The functional in vivo half-life and the serum half-life may be determined by any suitable method known in the art.

[0078] The term "increased" as used about the functional in vivo half-life or serum half-life is used to indicate that the relevant half-life of the polypeptide variant is statistically significantly increased relative to that of rhFVIIa as determined under comparable conditions (typically determined in an experimental animal, such as rats, rabbits, pigs or monkeys).

[0079] The term "AUC_{iv}" or "Area Under the Curve when administered intravenously" is used in its normal meaning, i.e. as the area under the activity in serum-time curve, where the polypeptide variant has been administered intravenously, in particular when administered intravenously in rats. Typically, the activity measured is the "clotting activity" as defined hereinbefore. Once the experimental activity-time points have been determined, the AUC_{iv} may conveniently be calculated by a computer program, such as GraphPad Prism 3.01.

[0080] It will be understood that in order to make a direct comparison between the AUC_{iv}-values of different molecules (e.g. between the variants of the invention and a reference molecule such as rhFVIIa) the same amount of activity should be administered. Consequently, the AUC_{iv}-values are typically normalized (i.e. corrected for differences in the injected dose) and expressed as AUC_{iv}/dose administered.

[0081] The term "reduced sensitivity to proteolytic degradation" is primarily intended to mean that the polypeptide variant has reduced sensitivity to proteolytic degradation in comparison to hFVIIa or rhFVIIa as determined under comparable conditions. Preferably, the proteolytic degrada-

tion is reduced by at least 10% (e.g. by 10-25% or by 10-50%), such as at least 25% (e.g. by 25-50%, by 25-75% or by 25-100%), more preferably by at least 35%, such as at least 50%, (e.g. by 50-75% or by 50-100%) even more preferably by at least 60%, such as by at least 75% (e.g. by 75-100%) or even at least 90%. Most preferably, the proteolytic degradation is reduced by at least 100%.

[0082] The term "renal clearance" is used in its normal meaning to indicate any clearance taking place by the kidneys, e.g. by glomerular filtration, tubular excretion or degradation in the tubular cells. Renal clearance depends on physical characteristics of the polypeptide, including size (diameter), hydrodynamic volume, symmetry, shape/rigidity, and charge. Normally, a molecular weight of about 67 kDa is considered to be a cut-off-value for renal clearance. Renal clearance may be established by any suitable assay, e.g. an established in vivo assay. Typically, renal clearance is determined by administering a labelled (e.g. radiolabelled or fluorescence labelled) polypeptide to a patient and measuring the label activity in urine collected from the patient. Reduced renal clearance is determined relative to a corresponding reference polypeptide, e.g. rhFVIa, under comparable conditions. Preferably, the renal clearance rate of the polypeptide variant is reduced by at least 50%, preferably by at least 75%, and most preferably by at least 90% compared to rhFVIIa.

[0083] The terms "at least 25% of its side chain exposed to the surface of the molecule" and "at least 50% of its side chain exposed to the surface of the molecule" are defined with reference to Example 1, where the calculations, etc. are described in detail.

[0084] It should be noted that when the terms "at least 25% of its side chain exposed to the surface of the molecule" and "at least 50% of its side chain exposed to the surface of the molecule" are used in connection with introduction of an in vivo N-glycosylation site these terms refer to the surface accessibility of the amino acid side chain in the position where the sugar moiety is actually attached. In many cases it will be necessary to introduce a serine or a hreonine residue in position +2 relative to the asparagine residue to which the sugar moiety is actually attached (unless, of course, this position is already occupied by a serine or a threonine residue) and these positions, where the serine or threonine residues are introduced, are allowed to be buried, i.e. to have less than 25% or 50% of their side chains exposed to the surface of the molecule.

[0085] The terms "tissue factor binding site", "active site region" and "ridge of the active site binding cleft" are defined with reference to Example 1 herein, wherein the above-mentioned sites/regions are determined.

[0086] The term "hydrophobic amino acid residue" includes the following amino acid residues: isoleucine (I), leucine (L), methionine (M), valine (V), phenylalanine (F), tyrosine (Y) and tryptophan (W).

[0087] The term "negatively charged amino acid residue" includes the following amino acid residues: Aspartic acid (D) and glutamic acid (E).

[0088] The term "positively charged amino acid residue" includes the following amino acid residues: Lysine (K), arginine (R) and histidine (H).

VARIANTS OF THE INVENTION

[0089] In its broadest aspect the present invention relates to a FVII or FVIIa polypeptide variant having an amino acid sequence comprising 3-15 amino acid modifications relative to hFVII or hFVIIa having the amino acid sequence shown in SEQ ID NO:2, wherein said amino acid sequence of the variant comprises an amino acid substitution in position 10 and 32 and wherein a sugar moiety is covalently attached to an introduced in vivo N-glycosylation site located outside the Gla domain.

[0090] The modifications performed in the two above-indicated regions of the parent FVII polypeptide serve the following purposes:

[0091] The modifications performed in positions located outside the Gla domain (introduction of in vivo N-glycosylation site(s)) are preferably of such nature that the AUC_{iv}, the functional in vivo half-life and/or the serum half-life of the resulting variant is increased as compared to rhFVIIa.

[0092] The modifications performed in the Gla domain of the parent polypeptide are preferably of such nature that an increased phospholipid membrane binding affinity of the resulting molecule is achieved and/or of such nature the resulting molecule has an improved capability to activate FX to FXa and/or of such nature that a stronger clot is formed.

[0093] Without being limited by any particular theory, it is presently believed that the enhanced membrane affinity results in a higher local concentration of the activated polypeptide variants in close proximity to the other coagulation factors, particularly FX. Thus, the rate of activation of FX to FXa will be higher, simply due to a higher molar ratio of the activated FVII variant to FX. The increased activation rate of FX then results in a higher amount of active thrombin, and thus a higher rate of cross-linking of fibrin.

[0094] Thus, in preferred embodiments of the invention the parent FVII or FVIIa polypeptide has been modified so that the resulting activated polypeptide variant has (as compared to rhFVIIa):

[0095] i) an increased bioavailability (AUC_{iv}) and an increased phospholipid membrane binding affinity;

[0096] ii) an increased bioavailability (AUC_{iv}) and an increased capability to activate FX to FXa;

[0097] iii) an increased bioavailability (AUC_{iv}) and is capable of generating a stronger clot (increased AUC_{throm});

[0098] iv) an increased bioavailability (AUC_{iv}) and a reduced T_{max};

[0099] v) an increased functional in vivo half-life and an increased phospholipid membrane binding affinity;

[0100] vi) an increased functional in vivo half-life and an increased capability to activate FX to FXa;

[0101] vii) an increased functional in vivo half-life and is capable of generating a stronger clot (increased AUC_{throm});

[0102] viii) an increased functional in vivo half-life and a reduced T_{max};

[0103] ix) an increased serum half-life and an increased phospholipid membrane binding affinity;

[0104] x) an increased serum half-life and an increased capability to activate FX to FXa;

[0105] xi) an increased serum half-life and is capable of generating a stronger clot (increased AUC_{throm}); and/or

[0106] xii) an increased serum half-life and a reduced T_{max}.

[0107] Consequently, medical treatment with a polypeptide variant according to the invention offers a number of advantages over the currently available rhFVIIa compound (NovoSeven®), such as administration of lower dosage, longer duration between injections, increased clot strength and/or faster action.

[0108] Thus, preferred variants of the invention are such variants which, in their activated forms and when compared to rhFVIIa, generates in increased Area Under the Curve when administered intravenously (AUC_{iv}), in particular when administered intravenously in rats. More particularly, variants of the present invention, which are preferred, are such variants where the ratio between the AUC_{iv} of said variant, in its activated form, and the AUC_{iv} of rhFVIIa is at least 1.25, such as at least 1.5, e.g. at least 1.75, more preferably at least 2, such as at least 3, even more preferably at least 4, such as at least 5, in particular when administered (intravenously) in rats.

[0109] This effect may in turn (but do not necessarily do so) correspond to an increased functional in vivo half-life and/or an increased serum half-life as compared to rhFVIIa. Accordingly, in another preferred embodiment of the invention, the ratio between the functional in vivo half-life or the serum half-life for the variant, in its activated form, and the functional in vivo half-life or the serum half-life for rhFVIIa is at least 1.25. More preferably, the ratio between the relevant half-life for the variant, in its activated form, and the relevant half-life for hFVIIa or rhFVIIa is at least 1.5, such as at least 1.75, e.g. at least 2, even more preferably at least 3, such as at least 4, e.g. at least 5.

[0110] As will be understood, the variants of the invention also possess, in addition to the above-mentioned functionality (i.e. increased AUC_{iv}, increased functional in vivo half-life and/or increased serum half-life), an increased phospholipid membrane binding affinity as compared to rhFVII, an an increased capability to activate FX to Fxa, a capability of generating a stronger clot (increased AUC_{throm}) and/or a reduced T_{max}.

[0111] Thus, in one preferred embodiment of the invention, the polypeptide variant has (in addition to an increased AUC_{iv}, increased functional in vivo half-life and/or increased serum half-life) an increased phospholipid membrane binding affinity relative to rhFVIIa Membrane binding affinity may be measured by methods known in the art, such as by the Biacore® assay described in K Nagata and H. Handa (Ads.), Real-Time Analysis of Biomolecular Interactions, Springer-Verlag, Tokyo, 2000, Chapter 6 entitled "Lipid-Protein Interactions". Alternatively, the membrane binding affinity may be measured as described in Example 1 in WO 99/20767.

[0112] In another preferred embodiment of the invention, the polypeptide variant (in addition to an increased AUC_{iv}, increased functional in vivo half-life and/or increased serum half-life), has an increased FX activation activity as compared to rhFVIIa, in particular when assayed in a TF-independent assay, such as the "TF-independent Factor X Activation Assay" disclosed herein. More particularly, it is preferred that the ratio between the FX activation activity of the polypeptide variant, in its activated form, and the FX activation activity of rhFVIIa is at least 1.25 when assayed in the "TF-independent Factor X Activation Assay" disclosed herein. More preferably, the ratio between the FX activation activity of the variant, in its activated form, and the FX activation activity of rhFVIIa is at least 1.5, such as at least 1.75, e.g. at least 2, even more preferably at least 3, such as at least 4, e.g. at least 5, still more preferably at least 6, such as at least 7, e.g. at least 8, most preferably at least 9, such as at least 10, when assayed in the "TF-independent Factor X Activation Assay" described herein.

[0113] In still another preferred embodiment of the invention, the polypeptide variant is (in addition to an increased AUC_{iv}, increased functional in vivo half-life and/or increased serum half-life), in its activated form, capable of generating a stronger clot as compared to rhFVIIa. This effect may be determined in the "Thrombogram Assay" described herein as an increase in the Area Under the Curve (AUC_{throm}). The AUG_{throm} is also sometimes denoted "total thrombin work" and constitutes a measure for the strength of the clot formed. More particularly, it is preferred that the ratio between the AUC_{throm}, generated by the variant in its activated form, and the AUC_{throm} generated by rhFVIIa is at least 1.15 when assayed in the "Thrombogram Assay" described herein. More preferably, the ratio is at least 1.2, such as at least 1.25, e.g. at least 1.3, even more preferably at least 1.4, such as at least 1.5, e.g. at least 1.6, most preferably at least 1.7, such as at least 1.8, e.g. at least 1.9 or at least 2.

[0114] In even another preferred embodiment of the invention the polypeptide variant has (in addition to an increased AUC_{iv}, increased functional in vivo half-life and/or increased serum half-life), in its activated form, a faster action. This effect may be determined in the "Thrombogram Assay" described herein as a reduction in the time needed to reach maximum thrombin level (T_{max}). Accordingly, preferred variants are such variants where the ratio between T_{max} for the variant, in its activated form, and T_{max} for rhFVIIa is at the most 0.95 when assayed in the "Thrombogram Assay" described herein. Preferably, the ratio is at the most 0.9, such as at the most 0.8, e.g. at the most 0.7, more preferably at the most 0.6, such as at the most 0.5.

Introduction of In Vivo N-glycosylation Sites Located Outside the Gla Domain

[0115] A number of suitable modifications leading to an increase in AUC_{iv}, functional in vivo half-life and/or serum half-life is disclosed in WO 01/58935. The variants disclosed in WO 01/58935 are the result of a generally new strategy for developing improved FVII or FVIIa molecules, which may also be used for the parent FVII or FVIIa polypeptide of the present invention.

[0116] The position to be modified is preferably selected from a part of the FVII or FVIIa molecule that is located outside the tissue factor binding site, and/or outside the active site region, and/or outside the ridge of the active site binding cleft. These sites/regions are identified in Example 1 herein. It should be emphasized, however, that in certain situations, e.g. in case an inactivated polypeptide variant is

desired, it may be advantageous to perform modifications in or close to such regions. For example, it is contemplated that one or more in vivo N-glycosylation sites may advantageously be introduced in the active site region or at the ridge of the active site binding cleft of the FVII or FVIIa molecule. The active site region, the tissue factor binding site and the ridge of the active site binding cleft are defined in Example 1 herein and are constituted by the following residues:

[0117] I153, Q167, V168, L169, L170, L171, Q176, L177, C178, G179, G180, T181, V188, V189, S190, A191, A192, H193, C194, F195, D196, K197, I198, W201, V228, I229, I230, P231, S232, T233, Y234, V235, P236, G237, T238, T239, N240, H241, D242, I243, A244, L245, L246, V281, S282, G283, W284, G285, Q286, T293, T324, E325, Y326, M327, F328, D338, S339, C340, K341, G342, D343, S344, G345, G346, P347, H348, L358, T359, G360, I361, V362, S363, W364, G365, C368, V376, Y377, T378, R379, V380, Q382, Y383, W386, L387, L400 and F405 (active site region);

[0118] L13, K18, F31, E35, R36, L39, F40, I42, S43, S60, K62, D63, Q64, L65, I69, C70, F71, C72, L73, P74, F76, E77, G78, R79, E82, K85, Q88, I90, V92, N93, E94, R271, A274, F275, V276, R277, F278, R304, L305, M306, T307, Q308, D309, Q312, Q313, E325 and R379 (tissue factor binding site); and

[0119] N173, A175, K199, N200, N203, D289, R290, G291, A292, P321 and T370 (the ridge of the active site binding cleft).

[0120] The total number of amino acid residues to be modified outside the Gla domain in the parent FVII or FVIIa polypeptide (as compared to the amino acid sequence shown in SEQ ID NO:2) will typically not exceed 10. Preferably, the FVII or FVIIa variant comprises an amino acid sequence which differs in 1-10 amino acid residues from amino acid residues 46-406 shown in SEQ ID NO:2, typically in 1-8 or in 2-8 amino acid residues, e.g. in 1-5 or in 2-5 amino acid residues, such as in 1-4 or in 1-3 amino acid residues, e.g. in 1, 2 or 3 amino acid residues from amino acid residues 46-406 shown in SEQ ID NO:2.

[0121] Thus, the polypeptide variant of the invention may contain 1-10 (additional or introduced) in vivo N-glycosylation sites, typically 1-8 or 2-8 (additional or introduced) in vivo N-glycosylation sites, preferably 1-5 or 2-5 (additional or introduced) in vivo N-glycosylation sites, such as 1-4 or 1-3 (additional or introduced) in vivo N-glycosylation sites, e.g. 1, 2 or 3 (additional or introduced) in vivo N-glycosylation sites. Analogously, the polypeptide variant of the invention may contain 1-10 (additional or introduced) sugar moieties, typically 1-8 or 2-8 (additional or introduced) sugar moieties, preferably 1-5 or 2-5 (additional or introduced) sugar moieties, such as 14 or 1-3 (additional or introduced) sugar moieties, e.g. 1, 2 or 3 (additional or introduced) sugar moieties. It will be understood that the introduced sugar moiety/moieties will be covalently attached to the introduced in vivo N-glycosylation site(s)

[0122] When used in the present context, the term "naturally occurring glycosylation site" covers the glycosylation sites at positions N145, N322, S52 and S60. In a similar way, the term "naturally occurring in vivo O-glycosylation site" includes the positions S52 and S60, whereas the term "naturally occurring in vivo N-glycosylation site" includes positions N145 and N322.

[0123] It will be understood that in order to prepare a polypeptide variant, wherein the polypeptide variant comprises one or more sugar moieties covalently attached to one or more in vivo N-glycosylation sites, the polypeptide variant must be expressed in a host cell capable of attaching sugar (oligosaccharide) moieties at the glycosylation site(s) or alternatively subjected to in vitro glycosylation. Examples of glycosylating host cells are given in the section further below entitled "*Coupling to a sugar moiety*".

[0124] Examples of positions, wherein the in vivo N-glycosylation sites may be introduced include, but is not limited to, positions comprising an amino acid residue having an amino acid residue having at least 25% of its side chain exposed to the surface (as defined in Example 1 herein), such as in a position comprising an amino acid residue having at least 50% of its side chain exposed to the surface (as defined in Example 1 herein). In general, it is preferred that the in vivo N-glycosylation site is introduced by substitution, although insertion is also contemplated. The position is preferably selected from a part of the molecule that is located outside the tissue factor binding site and/or the active site region and/or outside the ridge of the active site cleft. These sites/regions are identified in Example 1 herein. It should be understood that when the term "at least 25% (or at least 50%) of its side chain exposed to the surface" is used in connection with introduction of an in vivo N-glycosylation site this term refers to the surface accessibility of the amino acid side chain in the position where the sugar moiety is actually attached. In many cases it will be necessary to introduce a serine or a threonine residue in position +2 relative to the asparagine residue to which the sugar moiety is actually attached (unless, of course, this position is already occupied by a serine or a threonine residue) and these positions, where the serine or threonine residues are introduced, are allowed to be buried, i.e. to have less than 25% of their side chains exposed to the surface.

[0125] Specific and preferred examples of such substitutions creating an in vivo N-glycosylation site include a substitution selected from the group consisting of A51N, G58N, T106N, K109N, G124N, K143N+N145T, A175T, I205S, I205T, V253N, T267N, T267N+S269T, S314N+K316S, S314N+K316T, R315N+V317S, R315N+V317T, K316N+G318S, K316N+G318T, G318N, D334N and combinations thereof More preferably, the in vivo N-glycosylation site is introduced by a substitution selected from the group consisting of A51N, G58N, T106N, K109N, G124N, K143N+N145T, A175T, I205T, V253N, T267N+S269T, S314N+K316T, R315N+V317T, K316N+G318T, G318N, D334N and combinations thereof. Even more preferably, the in vivo N-glycosylation site is introduced by a substitution selected from the group consisting of T106N, A175T, I205T, V253N, T267N+S269T and combinations thereof, in particular I205T.

[0126] In one embodiment, only one in vivo N-glycosylation site has been introduced by substitution. In another embodiment, two or more (such as two) in vivo N-glycosylation sites have been introduced by substitution. Examples of preferred substitutions creating two in vivo N-glycosylation sites include substitutions selected from the group consisting of A51N+G58N, A51N+T106N, A51N+K109N, A51N+G124N, A51N+K143N+N145T, A51N+A175T, A51N+I205T, A51N+V253N, A51N+T267N+S269T, A51N+S314N+K316T, A51N+R315N+V317T,

A51N+K316N+G318T, A51N+G318N, A51N+D334N, G58N+T106N, G58N+K109N, G58N+G124N, G58N+K143N+N145T, G58N+A175T, G58N+I205T, G58N+V253N, G58N+T267N+S269T, G58N+S314N+K316T, G58N+R315N+V317T, G58N+K316N+G318T, G58N+G318N, G58N+D334N, T106N+K109N, T106N+G124N, T106N+K143N+N145T, T106N+A175T, T106N+I205T, T106N+V253N, T106N+T267N+S269T, T106N+S314N+K316T, T106N+R315N+V317T, T106N+K316N+G318T, T106N+G318N, T106N+D334N, K109N+G124N, K109N+K143N+N145T, K109N+A175T, K109N+I205T, K109N+V253N, K109N+T267N+S269T, K109N+S314N+K316T, K109N+R315N+V317T, K109N+K316N+G318T, K109N+G318N, K109N+D334N, G124N+K143N+N145T, G124N+A175T, G124N+I205T, G124N+V253N, G124N+T267N+S269T, G124N+S314N+K316T, G124N+R315N+V317T, G124N+K316N+G318T, G124N+G318N, G124N+D334N, K143N+N145T+A175T, K143N+N145T+I205T, K143N+N145T+V253N, K143N+N145T+T267N+S269T, K143N+N145T+S314N+K316T, K143N+N145T+R315N+V317T, K143N+N145T+K316N+G318T, K143N+N145T+G318N, K143N+N145T+D334N, A175T+I205T, A175T+V253N, A175T+T267N+S269T, A175T+S314N+K316T, A175T+R315N+V317T, A175T+K316N+G318T, A175T+G318N, A175T+D334N, I205T+V253N, I205T+T267N+S269T, I205T+S314N+K316T, I205T+R315N+V317T, I205T+K316N+G318T, I205T+G318N, I205T+D334N, V253N+T267N+S269T, V253N+S314N+K316T, V253N+R315N+V317T, V253N+K316N+G318T, V253N+G318N, V253N+D334N, T267N+S269T+S314N+K316T, T267N+S269T+R315N+V317T, T267N+S269T+K316N+G318T, T267N+S269T+G318N, T267N+S269T+D334N, S314N+K316T+R315N+V317T, S314N+K316T+G318N, S314N+K316T+D334N, R315N+V317T+K316N+G318T, R315N+V317T+G318N, R315N+V317T+D334N and G318N+D334N. More preferably, the substitutions are selected from the group consisting of T106N+A175T, T106N+I205T, T106N+V253N, T106N+T267N+S269T, A175T+I205T, A175T+V253N, A175T+T267N+S269T, I205T+V253N, I205T+T267N+S269T and V253N+T267N+S269T, even more preferably from the group consisting of T106N+I205T, T106N+V253N and I205T+T267N+S269T.

[0127] In an even further embodiment, three or more (such as three) in vivo N-glycosylation sites have been introduced by substitution. Examples of preferred substitutions creating three in vivo N-glycosylation sites include substitutions selected from the group consisting of I205T+V253N+T267N+S269T and T 06N+I205T+V253N.

[0128] As discussed above, it is preferred that the in vivo N-glycosylation site is introduced in a position which does neither form part of the tissue factor binding site nor form part of the active site region and the ridge of the active site binding cleft as defined herein. It is envisaged that such glycosylation variants will primarily belong to the class of active polypeptide variants as defined hereinbefore.

[0129] It will be understood that as an alternative to introduction of in vivo N-glycosylation sites in the above-discussed positions one may introduce (either by substitution or by insertion) cysteine residues in the same positions, where the introduced cysteine residue is then subsequently covalently attached to a non-polypeptide moiety, such as PEG, in particular mPEG. Thus, examples of positions, wherein a cysteine residue may be introduced include, but is not limited to, positions comprising an amino acid residue having an amino acid residue having at least 25% of its side chain exposed to the surface (as defined in Example 1 herein), such as in a position comprising an amino acid residue having at least 50% of its side chain exposed to the surface (as defined in Example 1 herein). The position is preferably selected from a part of the molecule that is located outside the tissue factor binding site and/or the active site region and/or outside the ridge of the active site cleft. These sites/regions are identified in Example 1 herein. Thus, the above disclosure concerning introduction of in vivo N-glycosylation sites applies mutatis mutandis to introduction of cysteine residues.

[0130] It will be understood that the modifications in positions located outside the Gla domain discussed in the above section should be combined with one or more modifications in the Gla domain (see the section entitled "*Modifications in the Gla domain*" below).

Modifications in the Gla Domain

[0131] As will be understood the variants of the present invention comprises, in addition to at least one introduced in vivo N-glycosylation site located outside the Gla domain (cf. above), at least two substitutions in the Gla domain, namely a substitution in position 10 and position 32.

[0132] A number of suitable modifications leading to increased phospholipid membrane binding affinity is disclosed in WO 99/20767 and WO 00/66753.

[0133] In a preferred embodiment of the invention the substitution in position 10 is P10Q. In another preferred embodiment of the invention the substitution in position 32 is K32E. In a particularly preferred embodiment of the invention the variant comprises the following substitutions P10Q+K32E.

[0134] In an interesting embodiment of the invention the variant comprises, in addition to the substitutions in position 10 and 32, such as in addition to the substitutions P10Q+K32E, at least one further modification in the Gla domain.

[0135] In one preferred embodiment of the invention the further modification in the Gla domain comprises an amino acid substitution in position 33. Preferably, a hydrophobic amino acid residue is introduced by substitution in position 33, such as D33I, D33L, D33M, D33V, D33F, D33Y or D33W, in particular D33F. Accordingly, in one very interesting embodiment of the invention, the variant comprises the following substitutions P10Q+K32+D33F.

[0136] In another preferred embodiment of the invention the further modification in the Gla domain comprises an insertion of at least one (such as one) amino acid residue between position 3 and 4. It is preferred that the inserted amino acid residue is a hydrophobic amino acid residue. Most preferably the insertion is A3AY. Accordingly, in another very interesting embodiment of the invention, the variant comprises the following modifications A3AY+P10Q+K32E or A3AY+P10Q+K32E+D33F.

[0137] In still another preferred embodiment of the invention the further modification in the Gla domain comprises a substitution in position 34. It is preferred that a negatively charged amino acid residue is introduced by substitution in position 34. Most preferably the substitution is A34E. Accordingly, in still another very interesting embodiment of

the invention, the variant comprises the following modifications P10Q+K32E+A34E, P10Q+K32E+D33F+A34E, A3AY+P10Q+K32E+A34E or A3AY+P10Q+K2E+D33F+A34E.

[0138] The Gla domain may also contain modifications in other positions, in particular in positions 8, 11 and 28, such as R28F or R28E. On the other hand it should be understood that the Gla domain should not be modified to such an extent that the membrane binding properties are impaired. Accordingly, it is preferred that no modifications are made in the residues that become γ-carboxylated, i.e. it is preferred that no modifications are made in residues 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35. In a similar way, it is in general not preferred that non-polypeptide moieties, such as sugar moieties and/or PEG groups, are introduced in the Gla domain. Consequently, it is preferred that no modifications are made in the Gla domain that creates an in vivo N-glycosylation site.

[0139] Finally, it will be understood that the modifications in the Gla domain discussed in this section must be combined with one or more of the modifications disclosed in the section entitled "*Introduction of in vivo N-glycosylation sites located outside the Gla domain*" above.

[0140] Specific examples of such "combined" variants are given below.

[0141] In one embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+T106N.

[0142] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A175T.

[0143] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+I205T.

[0144] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+V253N.

[0145] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+T267+S269T.

[0146] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+T106N+I205T.

[0147] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+T106N+V253N.

[0148] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+I205T+T267N+S269T.

[0149] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+T106N.

[0150] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A175T.

[0151] In a further embodiment of the invention said FV" or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+I205T.

[0152] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+V253N.

[0153] In a further preferred embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+T267+S269T.

[0154] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+T106N+I205T.

[0155] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+T106N+V253N.

[0156] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+I205T+T267N+S269T.

[0157] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+T106N.

[0158] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A175T.

[0159] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+I205T.

[0160] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+V253N.

[0161] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+T267+S269T.

[0162] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+T106N+I205T.

[0163] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+T106N+V253N.

[0164] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+I205T+T267N+S269T.

[0165] In a further embodiment of the invention said FVII or FVIIa variant comprises the 30 following modifications: A3AY+P10Q+K32E+D33F+T106N.

[0166] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A175T.

[0167] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+I205T.

[0168] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+V253N.

[0169] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+T267+S269T.

[0170] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+T106N+I205T.

[0171] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+T106N+V253N.

[0172] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+I205T+T267N+S269T.

[0173] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+T106N.

[0174] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+A175T.

[0175] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+I205T.

[0176] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+V253N.

[0177] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+T267+S269T.

[0178] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+T106N+I205T.

[0179] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+T106N+V253N.

[0180] In a further embodiment of the invention said FVII or FVIIIa variant comprises the following modifications: P10Q+K32E+A34E+I205T+T267N+S269T.

[0181] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E+T106N.

[0182] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E A175T.

[0183] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E I205T.

[0184] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E V253N.

[0185] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E T267+S269T.

[0186] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E T106N+I205T.

[0187] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E T106N+V253N.

[0188] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E I205T+T267N+S269T.

[0189] In a further embodiment of the invention said FVII or FVIIa variant comprises the lo following modifications: A3AY+P10Q+K32E+A34E+T106N.

[0190] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E A175T.

[0191] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E I205T.

[0192] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E V253N.

[0193] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E T267+S269T.

[0194] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E T106N+I205T.

[0195] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E T106N+V253N.

[0196] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E I205T+T267N+S269T.

[0197] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E+T106N.

[0198] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E A175T.

[0199] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+B2E+D33F+A34E I205T.

[0200] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E V253N.

[0201] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E T267+S269T.

[0202] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E T106N+I205T.

[0203] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E T106N+V253N.

[0204] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E I205T+T267N+S269T.

Other Modifications Outside the Gla Domain

[0205] In a further embodiment of the present invention, the FVII or FVIIa variant may, in addition to the modifications described in the sections above, also contain mutations,

which are already known to increase the intrinsic activity of the polypeptide, e.g. such as those described in WO 02/22776

[0206] Examples of preferred substitutions include substitutions selected from the group consisting of V158D, E296D, M298Q, L305V and K337A. More preferably, said substitutions are selected from the group consisting of V158D+E296D+M298Q+L305V+K337A, V158D+E296D+M298Q+K337A, V158D+E296D+M298Q+L305V, V158D+E296D+M298Q, M298Q, L305V+K337A, L305V and K337A.

[0207] In a further embodiment of the present invention, the FVII or FVIIa variant may, in addition to the modifications described in the sections above, also contain mutations, which are already known to cause a decreased inhibition by TFPI. One example includes the substitution K341Q disclosed by Neuenschwander et al, Biochemistry, 1995; 34:8701-8707.

[0208] Moreover, the variant may contain modifications which are believed to increase the TF binding affinity. Examples of such modifications include substitutions selected from the group consisting of L39E, L39Q, L39H, I42K, I42R, S43H, S43Q, K62E, K62R, L65Q, L65Q, L65S, F71D, F71Y, F71E, F71Q, F71N, E82Q, E82N, E82K and F275H As already indicated above, the variant may also contain conservative amino acid substitutions.

The Non-Polypeptide Moiety

[0209] Based on the present disclosure the skilled person will be aware that amino acid residues comprising other attachment groups may be introduced by substitution into the parent polypeptide, using the same approach as that illustrated above with in vivo N-glycosylation sites. For instance, one or more amino acid residues comprising an acid group (glutamic acid or aspartic acid), tyrosine or lysine may be introduced into the positions discussed above. In particular, one or more cysteine residues may be introduced in the positions discussed above.

[0210] As indicated further above the non-polypeptide moiety of the conjugated variant is preferably selected from the group consisting of a polymer molecule, a lipophilic compound, a sugar moiety (by way of in vivo glycosylation) and an organic derivatizing agent. All of these agents may confer desirable properties to the variant polypeptide, in particular increased AUC_{iv}, increased functional in vivo half-life and/or increased plasma half-life. The variant polypeptide is normally conjugated to only one type of non-polypeptide moiety, but may also be conjugated to two or more different types of non-polypeptide moieties, e.g. to a polymer molecule and a sugar moiety, to a lipophilic group and a sugar moiety, to an organic derivatizing agent and a sugar moiety, to a lipophilic group and a polymer molecule, etc. The conjugation to two or more different non-polypeptide moieties may be done simultaneous or sequentially.

Methods of Preparing a Conjugated Variant of the Invention

[0211] In the following sections "Conjugation to a lipophilic compound", "Conjugation to a polymer molecule", "Conjugation to a sugar moiety" and "Conjugation to an organic derivatizing agent" conjugation to specific types of non-polypeptide moieties is described. In general, a conjugated variant according to the invention may be produced by culturing an appropriate host cell under conditions conducive for the expression of the variant polypeptide, and recovering the variant polypeptide, wherein a) the variant polypeptide comprises at least one N- or O-glycosylation site and the host cell is an eukaryotic host cell capable of in vivo glycosylation, and/or b) the variant polypeptide is subjected to conjugation to a non-polypeptide moiety in vitro.

[0212] It will be understood that the conjugation should be designed so as to produce the optimal molecule with respect to the number of non-polypeptide moieties attached, the size and form of such molecules (e.g. whether they are linear or branched), and the attachment site(s) in the polypeptide. The molecular weight of the non-polypeptide moiety to be used may, e.g., be chosen on the basis of the desired effect to be achieved. For instance, if the primary purpose of the conjugation is to achieve a conjugated variant having a high molecular weight (e.g. to reduce renal clearance) it is usually desirable to conjugate as few high molecular weight non-polypeptide moieties as possible to obtain the desired molecular weight. When a high degree of shielding is desirable this may be obtained by use of a sufficiently high number of low molecular weight non-polypeptide moieties (e.g. with a molecular weight of from about 300 Da to about 5 kDa, such as a molecular weight of from 300 Da to 2 kDa).

Conjugation to a Polymer Molecule

[0213] The polymer molecule to be coupled to the variant polypeptide may be any suitable polymer molecule, such as a natural or synthetic homo-polymer or hetero-polymer, typically with a molecular weight in the range of about 300-100,000 Da, such as about 500-20,000 Da, more preferably in the range of about 500-15,000 Da, even more preferably in the range of about 2-12 kDa, such as in the range of about 3-10 kDa When the term "about" is used herein in connection with a certain molecular weight, the word "about" indicates an approximate average molecular weight and reflects the fact that there will normally be a certain molecular lo weight distribution in a given polymer preparation.

[0214] Examples of homo-polymers include a polyol (i.e. poly-OH), a polyamine (i.e. poly-NH_2) and a polycarboxylic acid (i.e. poly-COOH). A hetero-polymer is a polymer comprising different coupling groups, such as a hydroxyl group and an amine group.

[0215] Examples of suitable polymer molecules include polymer molecules selected from the group consisting of polyalkylene oxide (PAO), including polyalkylene glycol (PAG), such as polyethylene glycol (PEG) and polypropylene glycol (PPG), branched PEGs, poly-vinyl alcohol (PVA), poly-carboxylate, poly-(vinylpyrolidone), polyethylene-co-maleic acid anhydride, polystyrene-co-maleic acid anhydride, dextran, including carboxymethyl-dextran, or any other biopolymer suitable for reducing immunogenicity and/or increasing functional in vivo half-life and/or serum half-life. Another example of a polymer molecule is human albumin or another abundant plasma protein. Generally, polyalkylene glycol-derived polymers are biocompatible, non-toxic, non-antigenic, non-immunogenic, have various water solubility properties, and are easily excreted from living organisms.

[0216] PEG is the preferred polymer molecule, since it has only few reactive groups capable of cross-linking compared

to, e.g., polysaccharides such as dextran. In particular, mono-functional PEG, e.g. methoxypolyethylene glycol (mPEG), is of interest since its coupling chemistry is relatively simple (only one reactive group is available for conjugating with attachment groups on the polypeptide). Consequently, as the risk of cross-linking is eliminated, the resulting conjugated variants are more homogeneous and the reaction of the polymer molecules with the variant polypeptide is easier to control.

[0217] To effect covalent attachment of the polymer molecule(s) to the variant polypeptide, the hydroxyl end groups of the polymer molecule must be provided in activated form, i.e. with reactive functional groups (examples of which include primary amino groups, hydrazide (HZ), thiol, succinate (SUC), succiniidyl succinate (SS), succinimidyl succinamide (SSA), succinimidyl propionate (SPA), succinimidyl butyrate (SBA), succinimidy carboxymethylate (SCM), benzotriazole carbonate (BTC), N-hydroxysuccinimide (NHS), aldehyde, nitrophenylcarbonate (NPC), and tresylate (TRES)). Suitable activated polymer molecules are commercially available, e.g. from Shearwater Polymers, Inc., Huntsville, Ala., USA, or from PolyMASC Pharmaceuticals plc, UK.

[0218] Alternatively, the polymer molecules can be activated by conventional methods known in the art, e.g. as disclosed in WO 90/13540. Specific examples of activated linear or branched polymer molecules for use in the present invention are described in the Shearwater Polymers, Inc. 1997 and 2000 Catalogs (Functionalized Biocompatible Polymers for Research and pharmaceuticals, Polyethylene Glycol and Derivatives, incorporated herein by reference).

[0219] Specific examples of activated PEG polymers include the following linear PEGs: NHS-PEG (e.g. SPA-PEG, SSPA-PEG, SBA-PEG, SS-PEG, SSA-PEG, SC-PEG, SG-PEG, and SCM-PEG), and NOR-PEG, BTC-PEG, EPOX-PEG, NCO-PEG, NPC-PEG, CDI-PEG, ALD-PEG, TRES-PEG, VS-PEG, IODO-PEG, and MAL-PEG, and branched PEGs such as PEG2-NHS and those disclosed in U.S. Pat. No. 5,932,462 and U.S. Pat. No. 5,643,575, both of which are incorporated herein by reference. Furthermore, the following publications, incorporated herein by reference, disclose useful polymer molecules and/or PEGylation chemistries: U.S. Pat. No. 5,824,778, U.S. Pat. No. 5,476,653, WO 97/32607, EP 229,108, EP 402,378, U.S. Pat. No. 4,902,502, U.S. Pat. No. 5,281,698, U.S. Pat. No. 5,122,614, U.S. Pat. No. 5,219,564, WO 92/16555, WO 94/04193, WO 94/14758, WO 94/17039, WO 94/18247, WO 94/28024, WO 95/00162, WO 95/11924, WO95/13090, WO 95/33490, WO 96/00080, WO 97/18832, WO 98/41562, WO 98/48837, WO 99/32134, WO 99/32139, WO 99/32140, WO 96/40791, WO 98/32466, WO 95/06058, EP 439 508, WO 97/03106, WO 96/21469, WO 95/13312, EP 921 131, U.S. Pat. No. 5,736,625, WO 98/05363, EP 809 996, U.S. Pat. No. 5,629,384, WO 96/41813, WO 96/07670, U.S. Pat. No. 5,473,034, U.S. Pat. No. 5,516,673, EP 605 963, U.S. Pat. No. 5,382,657, EP 510 356, EP 400 472, EP 183 503 and EP 154 316.

[0220] Specific examples of activated PEG polymers particularly preferred for coupling to cysteine residues, include the following linear PEGs: vinylsulfone-PEG (VS-PEG), preferably vinylsulfone-mPEG (VS-mPEG); maleimide-PEG (MAL-PEG), preferably maleimide-mPEG (MAL-mPEG) and orthopyridyl-disulfide-PEG (OPSS-PEG), preferably orthopyridyl-disulfide-mPEG (OPSS-mPEG). Typically, such PEG or mPEG polymers will have a size of about 5 kDa, about 10 kD, about 12 kDa or about 20 kDa.

[0221] The conjugation of the polypeptide variant and the activated polymer molecules is conducted by use of any conventional method, e.g. as described in the following references (which also describe suitable methods for activation of polymer molecules): Harris and Zalipsky, eds., Poly(ethylene glycol) Chemistry and Biological Applications, AZC, Washington; R. F. Taylor, (1991), "Protein immobilisation. Fundamental and applications", Marcel Dekker, N.Y.; S. S. Wong, (1992), "Chemistry of Protein Conjugation and Crosslinking", CRC Press, Boca Raton; G. T. Hermanson et al., (1993), "Immobilized Affinity Ligand Techniques", Academic Press, N.Y.).

[0222] The skilled person will be aware that the activation method and/or conjugation chemistry to be used depends on the attachment group(s) of the variant polypeptide (examples of which are given further above), as well as the functional groups of the polymer (e.g. being amine, hydroxyl, carboxyl, aldehyde, sulfydryl, succinimidyl, maleimide, vinysulfone or haloacetate). The PEGylation may be directed towards conjugation to all available attachment groups on the variant polypeptide (i.e. such attachment groups that are exposed at the surface of the polypeptide) or may be directed towards one or more specific attachment groups, e.g. the N-terminal amino group as described in U.S. Pat. No. 5,985,265 or to cysteine residues. Furthermore, the conjugation may be achieved in one step or in a stepwise manner (e.g. as described in WO 99/55377).

[0223] For PEGylation to cysteine residues (see above) the FVII or FVIIa variant is usually treated with a reducing agent, such as dithiothreitol (DDI) prior to PEGylation. The reducing agent is subsequently removed by any conventional method, such as by desalting. Conjugation of PEG to a cysteine residue typically takes place in a suitable buffer at pH 6-9 at temperatures varying from 4° C. to 25° C. for periods up to 16 hours.

[0224] It will be understood that the PEGylation is designed so as to produce the optimal molecule with respect to the number of PEG molecules attached, the size and form of such molecules (e.g. whether they are linear or branched), and the attachment site(s) in the variant polypeptide. The molecular weight of the polymer to be used may e.g. be chosen on the basis of the desired effect to be achieved.

[0225] In connection with conjugation to only a single attachment group on the protein (e.g. the N-terminal amino group), it may be advantageous that the polymer molecule, which may be linear or branched, has a high molecular weight, preferably about 10-25 kDa, such as about 15-25 kDa, e.g. about 20 kDa.

[0226] Normally, the polymer conjugation is performed under conditions aimed at reacting as many of the available polymer attachment groups with polymer molecules. This is achieved by means of a suitable molar excess of the polymer relative to the polypeptide. Typically, the molar ratios of activated polymer molecules to polypeptide are up to about 1000-1, such as up to about 200-1, or up to about 100-1. In some cases the ration may be somewhat lower, however, such as up to about 50-1, 10-1, 5-1, 2-1 or 1-1 in order to obtain optimal reaction.

[0227] It is also contemplated according to the invention to couple the polymer molecules to the polypeptide through a linker. Suitable linkers are well known to the skilled person. A preferred example is cyanuric chloride (Abuchowski et al., (1977), J. Biol. Chem., 252, 3578-3581; U.S. Pat. No. 4,179,337; Shafer et al., (1986), J. Polym. Sci. Polym. Chem. Ed., 24, 375-378).

[0228] Subsequent to the conjugation, residual activated polymer molecules are blocked according to methods known in the art, e.g. by addition of primary amine to the reaction mixture, and the resulting inactivated polymer molecules are removed by a suitable method.

[0229] It will be understood that depending on the circumstances, e.g. the amino acid sequence of the variant polypeptide, the nature of the activated PEG compound being used and the specific PEGylation conditions, including the molar ratio of PEG to polypeptide, varying degrees of PEGylation may be obtained, with a higher degree of PEGylation generally being obtained with a higher ratio of PEG to variant polypeptide. The PEGylated variant polypeptides resulting from any given PEGylation process will, however, normally comprise a stochastic distribution of conjugated polypeptide variants having slightly different degrees of PEGylation.

Coupling to a Sugar Moiety

[0230] In order to achieve in vivo glycosylation of a FVII molecule comprising one or more glycosylation sites the nucleotide sequence encoding the variant polypeptide must be inserted in a glycosylating, eucaryotic expression host. The expression host cell may be selected from fungal (filamentous fungal or yeast), insect or animal cells or from transgenic plant cells. In one embodiment the host cell is a mammalian cell, such as a CHO cell, BHK or HEK, e.g. HEK 293, cell, or an insect cell, such as an SF9 cell, or a yeast cell, e.g. *S. cerevisiae* or *Pichia pastoris*, or any of the host cells mentioned hereinafter.

[0231] Covalent in vitro coupling of sugar moieties (such as dextran) to amino acid residues of the variant polypeptide may also be used, e.g. as described, for example in WO 87/05330 and in Aplin etl al., CRC Crit Rev. Biochem, pp. 259-306, 1981. The in vitro coupling of sugar moieties or PEG to protein- and peptide-bound Gln-residues can be carried out by transglutaminases (TGases). Transglutaminases catalyse the transfer of donor amine-groups to protein- and peptide-bound Gln-residues in a so-called cross-linking reaction. The donor-amine groups can be protein- or peptide-bound, such as the ϵ-amino-group in Lys-residues or it can be part of a small or large organic molecule. An example of a small organic molecule functioning as amino-donor in TGase-catalysed cross-linking is putrescine (1,4-diaminobutane). An example of a larger organic molecule functioning as amino-donor in TGase-catalysed cross-linking is an amine-containing PEG (Sato et al., 1996, Biochemistry 35, 13072-13080).

[0232] TGases, in general, are highly specific enzymes, and not every Gln-residues exposed on the surface of a protein is accessible to TGase-catalysed cross-linking to amino-containing substances. On the contrary, only few Gin-residues are naturally functioning as TGase substrates but the exact parameters governing which Gln-residues are good TGase substrates remain unknown. Thus, in order to render a protein susceptible to TGase-catalysed cross-linking reactions it is often a prerequisite at convenient positions to add stretches of amino acid sequence known to function very well as TGase substrates. Several amino acid sequences are known to be or to contain excellent natural TGase substrates e.g. substance P, elafin, fibrinogen, fibronectin, α_2-plasmin inhibitor, α-caseins, and β-caseins.

Conjugation to an Organic Derivatizing Agent

[0233] Covalent modification of the variant polypeptide may be performed by reacting one or more attachment groups of the variant polypeptide with an organic derivatizing agent. Suitable derivatizing agents and methods are well known in the art. For example, cysteinyl residues most commonly are reacted with α-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, α-bromo-β-(4-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylnaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole. Histidyl residues are derivatized by reaction with diethylpyrocarbonateat pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful. The reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0. Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivating α-amino-containing residues include imidoesters such as methyl picolinimidate, pyridoxal phosphate, pyridoxal, chloroborohydride, tinitrobenzenesulfonic acid, O-methylisourea, 2,4-pentanedione and transaminase-catalyzed reaction with glyoxylate. Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction e performed in alkaline conditions because of the high pKa of the guanidine functional group.

[0234] Furthermore, these reagents may react with the groups of lysine as well as the arginine guanidino group. Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R—N═C═N—R'), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylphenyl)carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Conjugation to a Lipophilic Compound

[0235] The variant polypeptide and the lipophilic compound may be conjugated to each other, either directly or by use of a linker. The lipophilic compound may be a natural compound such as a saturated or unsaturated fatty acid, a fatty acid diketone, a terpene, a prostaglandin, a vitamine, a carotenoide or steroide, or a synthetic compound such as a carbon acid, an alcohol, an amine and sulphonic acid with one or more alkyl-, aryl-, alkenyl- or other multiple unsaturated compounds. The conjugation between the variant polypeptide and the lipophilic compound, optionally through a linker may be done according to methods known

in the art, e.g. as described by Bodanszky in Peptide Synthesis, John Wiley, New York, 1976 and in WO 96/12505.

Methods of Preparing a Polypeptide Variant of the Invention

[0236] The polypeptide variant of the present invention may be produced by any suitable method known in the art. Such methods include constructing a nucleotide sequence encoding the polypeptide variant and expressing the sequence in a suitable transformed or transfected host. Preferably, the host cell is a gammacarboxylating host cell such as a mammalian cell. However, polypeptide variants of the invention may be produced, albeit less efficiently, by chemical synthesis or a combination of chemical synthesis or a combination of chemical synthesis and recombinant DNA technology.

[0237] A nucleotide sequence encoding a polypeptide of the invention may be constructed by isolating or synthesizing a nucleotide sequence encoding the parent FVII, such as hFVII with the amino acid sequence shown in SEQ ID NO:2 and then changing the nucleotide sequence so as to effect introduction (i.e. insertion or substitution) or removal (i.e. deletion or substitution) of the relevant amino acid residue(s).

[0238] The nucleotide sequence is conveniently modified by site-directed mutagenesis in accordance with conventional methods. Alternatively, the nucleotide sequence is prepared by chemical synthesis, e.g. by using an oligonucleotide synthesizer, wherein oligonucleotides are designed based on the amino acid sequence of the desired polypeptide, and preferably selecting those codons that are favored in the host cell in which the recombinant polypeptide will be produced. For example, several small oligonucleotides coding for portions of the desired polypeptide may be synthesized and assembled by PCR, ligation or ligation chain reaction (LCR) (Barany, PNAS 88:189-193, 1991). The individual oligonucleotides typically contain 5' or 3' overhangs for complementary assembly.

[0239] Alternative nucleotide sequence modification methods are available for producing polypeptide variants for high throughput screening, for instance methods which involve homologous cross-over such as disclosed in U.S. Pat. No. 5,093,257, and methods which involve gene shuffling, i.e. recombination between two or more homologous nucleotide sequences resulting in new nucleotide sequences having a number of nucleotide alterations when compared to the starting nucleotide sequences. Gene shuffling (also known as DNA shuffling) involves one or more cycles of random fragmentation and reassembly of the nucleotide sequences, followed by screening to select nucleotide sequences encoding polypeptides with desired properties. In order for homology-based nucleic acid shuffling to take place, the relevant parts of the nucleotide sequences are preferably at least 50% identical, such as at least 60% identical, more preferably at least 70% identical, such as at least 80% identical. The recombination can be performed in vitro or in vivo.

[0240] Examples of suitable in vitro gene shuffling methods are disclosed by Stemmer et al. (1994), Proc. Natl. Acad. Sci. USA; vol. 91, pp. 10747-10751; Stemmer (1994), Nature, vol. 370, pp.389-391; Smith (1994), Nature vol. 370, pp.324-325; Zhao et al., Nat. Biotechnol. 1998, Mar; 16(3): 258-61; Zhao H. and Arnold, F B, Nucleic Acids Research, 1997, Vol. 25. No. 6 pp. 1307-1308; Shao et al., Nucleic Acids Research Jan. 15, 1998; 26(2): pp.681-83; and WO 95/17413.

[0241] An example of a suitable in vivo shuffling method is disclosed in WO 97/07205. Other techniques for mutagenesis of nucleic acid sequences by in vitro or in vivo recombination are disclosed e.g. in WO 97/20078 and U.S. Pat. No. 5,837,458. Examples of specific shuffling techniques include "family shuffling", "synthetic shuffling" and "in silico shuffling".

[0242] Family shuffling involves subjecting a family of homologous genes from different species to one or more cycles of shuffling and subsequent screening or selection. Family shuling techniques are disclosed e.g. by Crameri et al. (1998), Nature, vol. 391, pp. 288-291; Christians et al. (1999), Nature Biotechnology, vol. 17, pp. 259-264; Chang et al. (1999), Nature Biotechnology, vol. 17, pp. 793-797; and Ness et al. (1999), Nature Biotechnology, vol. 17, 893-896.

[0243] Synthetic shuffling involves providing libraries of overlapping synthetic oligonucleotides based e.g. on a sequence alignment of homologous genes of interest. The synthetically generated oligonucleotides are recombined, and the resulting recombinant nucleic acid sequences are screened and if desired used for further shuffling cycles. Synthetic shuffling techniques are disclosed in WO 00/42561.

[0244] In silico shuffling refers to a DNA shuffling procedure, which is performed or modelled using a computer system, thereby partly or entirely avoiding the need for physically manipulating nucleic acids. Techniques for in silico shuffling are disclosed in WO 00/42560.

[0245] Once assembled (by synthesis, site-directed mutagenesis or another method), the nucleotide sequence encoding the polypeptide is inserted into a recombinant vector and operably linked to control sequences necessary for expression of the FVII in the desired transformed host cell.

[0246] It should of course be understood that not all vectors and expression control sequences function equally well to express the nucleotide sequence encoding the polypeptide variants described herein. Neither will all hosts function equally well with the same expression system. However, one of skill in the art may make a selection among these vectors, expression control sequences and hosts without undue experimentation. For example, in selecting a vector, the host must be considered because the vector must replicate in it or be able to integrate into the chromosome. The vector's copy number, the ability to control that copy number, and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered. In selecting an expression control sequence, a variety of factors should also be considered. These include, for example, the relative strength of the sequence, its controllability, and its compatibility with the nucleotide sequence encoding the polypeptide, particularly as regards potential secondary structures. Hosts should be selected by consideration of their compatibility with the chosen vector, the toxicity of the product coded for by the nucleotide sequence, their secretion characteristics, their ability to fold

the polypeptide variant correctly, their fermentation or culture requirements, and the ease of purification of the products coded for by the nucleotide sequence.

[0247] The recombinant vector may be an autonomously replicating vector, i.e. a vector, which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector is one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

[0248] The vector is preferably an expression vector, in which the nucleotide sequence encoding the polypeptide variant of the invention is operably linked to additional segments required for transcription of the nucleotide sequence. The vector is typically derived from plasmid or viral DNA. A number of suitable expression vectors for expression in the host cells mentioned herein are commercially available or described in the literature. Useful expression vectors for eukaryotic hosts, include, for example, vectors comprising expression control sequences from SV40, bovine papilloma virus, adenovirus and cytomegalovirus. Specific vectors are, e.g., pCDNA3.1 (+)Hyg (Invitrogen, Carlsbad, Calif., USA) and pCI-neo (Stratagene, La Jola, Calif., USA). Useful expression vectors for yeast cells include the 2μ plasmid and derivatives thereof, the POT1 vector (U.S. Pat. No. 4,931,373), the pJSO37 vector described in Okkels, Ann. New York Acad. Sci. 782, 202-207, 1996, and pPICZ A, B or C (Invitrogen). Useful vectors for insect cells include pVL941, pBG311 (Cate et al., "Isolation of the Bovine and Human Genes for Mullerian Inhibiting Substance And Expression of the Human Gene In Animal Cells", Cell, 45, pp. 685-98 (1986), pBluebac 4.5 and pMelbac (both available from Invitrogen). Useful expression vectors for bacterial hosts include known bacterial plasmids, such as plasmids from *E. coli*, including pBR322, pET3a and pET12a (both from Novagen Inc., WI, USA), wider host range plasmids, such as RP4, phage DNAs, e.g., the numerous derivatives of phage lambda, e.g., NM989, and other DNA phages, such as M13 and filamentous single stranded DNA phages.

[0249] Other vectors for use in this invention include those that allow the nucleotide sequence encoding the polypeptide variant to be amplified in copy number. Such amplifiable vectors are well known in the art. They include, for example, vectors able to be amplified by DHFR amplification (see, e.g., Kaufman, U.S. Pat. No. 4,470,461, Kaufman and Sharp, "Construction Of A Modular Dihydrafolate Reductase cDNA Gene: Analysis Of Signals Utilized For Efficient Expression", Mol. Cell. Biol., 2, pp. 1304-19 (1982)) and glutamine synthetase ("GS") amplification (see, e.g., U.S. Pat. No. 5,122,464 and EP 338,841).

[0250] The recombinant vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. An example of such a sequence (when the host cell is a mammalian cell) is the SV40 origin of replication. When the host cell is a yeast cell, suitable sequences enabling the vector to replicate are the yeast plasmid 21 replication genes REP 1-3 and origin of replication.

[0251] The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or the *Schizosaccharomyces pombe* TPI gene (described by P. R. Russell, Gene 40, 1985, pp. 125-130), or one which confers resistance to a drug, e.g. ampicillin, kanamycin, tetracyclin, chloramphenicol, neomycin, hygromycin or methotrexate. For *Saccharomyces cerevisiae*, selectable markers include ura3 and leu2. For filamentous fungi, selectable markers include amdS, pyrG, arcB, niad and sC.

[0252] The term "control sequences" is defined herein to include all components, which are necessary or advantageous for the expression of the polypeptide variant of the invention. Each control sequence may be native or foreign to the nucleic acid sequence encoding the polypeptide variant. Such control sequences include, but are not limited to, a leader sequence, polyadenylation sequence, propeptide sequence, promoter, enhancer or upstream activating sequence, signal peptide sequence, and transcription terminator. At a minimum, the control sequences include a promoter.

[0253] A wide variety of expression control sequences may be used in the present invention. Such useful expression control sequences include the expression control sequences associated with structural genes of the foregoing expression vectors as well as any sequence known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof.

[0254] Examples of suitable control sequences for directing transcription in mammalian cells include the early and late promoters of SV40 and adenovirus, e.g. the adenovirus 2 major late promoter, the MT-1 (metallothionein gene) promoter, the human cytomegalovirus immediate-early gene promoter (CMV), the human elongation factor 1α (EF-1α) promoter, the *Drosophila* minimal heat shock protein 70 promoter, the Rous Sarcoma Virus (RSV) promoter, the human ubiquitin C (UbC) promoter, the human growth hormone terminator, SV40 or adenovirus Elb region polyadenylation signals and the Kozak consensus sequence (Kozak, M. *J Mol Biol Aug.* 20, 1987;196(4):947-50).

[0255] In order to improve expression in mammalian cells a synthetic intron may be inserted in the 5' untranslated region of the nucleotide sequence encoding the polypeptide. An example of a synthetic intron is the synthetic intron from the plasmid pCI-Neo (available from Promega Corporation, WI, USA).

[0256] Examples of suitable control sequences for directing transcription in insect cells include the polyhedrin promoter, the P10 promoter, the *Autographa californica* polyhedrosis virus basic protein promoter, the baculovirus immediate early gene 1 promoter and the baculovirus 39K delayed-early gene promoter, and the SV40 polyadenylation sequence. Examples of suitable control sequences for use in yeast host cells include the promoters of the yeast α-mating system, the yeast triose phosphate isomerase (TPI) promoter, promoters from yeast glycolytic genes or alcohol dehydrogenase genes, the ADH2-4c promoter, and the inducible GAL promoter. Examples of suitable control sequences for use in filamentous fungal host cells include the ADH3 promoter and terminator, a promoter derived from the genes encoding *Aspergillus oryzae* TAKA amylase triose phosphate isomerase or alkaline protease, an *A. niger* α-amylase, *A. niger* or *A. nidulans* glucoamylase, *A. nidulans* acetamidase, *Rhizomucor miehei* aspartic proteinase or

lipase, the Th11 terminator and the ADH3 terminator. Examples of suitable control sequences for use in bacterial host cells include promoters of the lac system, the trp system, the TAC or TRC system, and the major promoter regions of phage lambda.

[0257] The presence or absence of a signal peptide will, e.g., depend on the expression host cell used for the production of the polypeptide variant to be expressed (whether it is an intracellular or extracellular polypeptide) and whether it is desirable to obtain secretion. For use in filamentous fungi, the signal peptide may conveniently be derived from a gene encoding an *Aspergillus* sp. amylase or glucoamylase, a gene encoding a *Rhizomucor miehei* lipase or protease or a *Humicola lanuginosa* lipase. The signal peptide is preferably derived from a gene encoding *A. oryzae* TAKA amylase, *A. niger* neutral α-amylase, *A. niger* acid-stable amylase, or *A. niger* glucoamylase. For use in insect cells, the signal peptide may conveniently be derived from an insect gene (cf WO 90/05783), such as the *Lepidopteran manduca sexta* adipokinetic hormone precursor, (cf. U.S. Pat. No. 5,023,328), the honeybee melittin (Invitrogen), ecdysteroid UDP glucosyltransferase (egt) (Murphy et al., Protein Expression and Purification 4, 349-357 (1993) or human pancreatic lipase (hpl) (Methods in Enzymology 284, pp. 262-272, 1997). A preferred signal peptide for use in mammalian cells is that of hFVII or the murine Ig kappa light chain signal peptide (Coloma, M (1992) J. Imm. Methods 152:89-104). For use in yeast cells suitable signal peptides have been found to be the α-factor signal peptide from *S. cereviciae* (cf. U.S. Pat. No. 4,870,008), a modified carboxypeptidase signal peptide (cf. L. A. Valls et al., 30 Cell 48, 1987, pp. 887-897), the yeast BAR1 signal peptide (cf WO 87/02670), the yeast aspartic protease 3 (YAP3) signal peptide (cf. M. Egel-Mitani et al., Yeast 6, 1990, pp. 127-137), and the synthetic leader sequence TA57 (WO98/32867). For use in *E. coli* cells a suitable signal peptide have been found to be the signal peptide ompA (EP581821).

[0258] The nucleotide sequence of the invention encoding a polypeptide variant, whether prepared by site directed mutagenesis, synthesis, PCR or other methods, may optionally include a nucleotide sequence that encode a signal peptide. The signal peptide is present when the polypeptide variant is to be secreted from the cells in which it is expressed. Such signal peptide, if present, should be one recognized by the cell chosen for expression of the polypeptide variant. The signal peptide may, e.g. be that normally associated with hFVII) or, alternatively, the signal peptide may be from another source than hFVII, such as any of those normally associated with other human wild-type vitamin K-dependent polypeptides. Furthermore, the signal peptide may be a signal peptide normally expressed from the host cell or one which is not normally expressed from the host cell. Accordingly, the signal peptide may be prokaryotic, e.g. derived from a bacterium such as *E. coli*, or eukaryotic, e.g. derived from a mammalian, or insect or yeast cell.

[0259] Any suitable host may be used to produce the polypeptide variant, including bacteria (although not particularly preferred), fungi (including yeasts), plant, insect, mammal, or other appropriate animal cells or cell lines, as well as transgenic animals or plants. Examples of bacterial host cells include grampositive bacteria such as strains of *Bacillus*, e.g. *B. brevis* or *B. subtlis, Pseudomonas* or *Streptomyces*, or gramnegative bacteria, such as strains of *E. coli*. The introduction of a vector into a bacterial host cell may, for instance, be effected by protoplast transformation (see, e.g., Chang and Cohen, 1979, *Molecidar General Genetics* 168: 111-115), using competent cells (see, e.g., Young and Spizizin, 1961, *Journal of Bacteriology* 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, *Journal of Molecular Biology* 56: 209-221), electroporation (see, e.g., Shigekawa and Dower, 1988, *Biotechniques* 6: 742-751), or conjugation (see, e.g., Koehler and Thome, 1987, *Journal of Bacteriology* 169: 5771-5278). Examples of suitable filamentous fungal host cells include strains of *Aspergillus*, e.g. *A. oryzae, A. niger*, or *A. nidulans, Fusarinum* or *Trichoderma*. Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known per se. Suitable procedures for transformation of *Aspergillus* host cells are described in EP 238 023 and U.S. Pat. No. 5,679,543. Suitable methods for transforming *Fusarium* species are described by Malardier et al., 1989, *Gene* 78: 147-156 and WO 96/00787. Examples of suitable yeast host cells include strains of *Saccharomyces*, e.g. *S. cerevisiae, Schizosaccharomyces, Klyveromyces, Pichia*, such as *P. pastors* or *P. methanolica, Hansenula*, such as *H. Polymorpha* or *Yarrowia*. Yeast may be transformed using the procedures described by Becker and Guarente, In Abelson, J. N. and Simon, M. I., editors, *Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology*, Volume 194, pp 182-187, Academic Press, Inc., New York; Ito et al., 1983, *Journal of Bacteriology* 153: 163; Hinnen et al., 1978, *Proceedings of the National Academy of Sciences USA* 75: 1920: and as disclosed by Clontech Laboratories, Inc, Palo Alto, Calif., USA (in the product protocol for the Yeastmaker™ Yeast Transformation System Kit). Examples of suitable insect host cells include a *Lepidoptora* cell line, such as *Spodoptera frugiperda* (Sf9 or S121) or *Trichoplusioa ni* cells (High Five) (U.S. Pat. No. 5,077,214). Transformation of insect cells and production of heterologous polypeptides therein may be performed as described by Invitrogen. Examples of suitable mammalian host cells include Chinese hamster ovary (CHO) cell lines, (e.g. CHO-K1; ATCC CCL-61), Green Monkey cell lines (COS) (e.g. COS 1 (ATCC CRL-1650), COS 7 (ATCC CRL-1651)); mouse cells (e.g. NS/O), Baby Hamster Kidney (BHK) cell lines (e.g. ATCC CRL-1632 or ATCC CCL-10), and human cells (e.g. SEK 293 (ATCC CRL-1573)), as well as plant cells in tissue culture. Additional suitable cell lines are known in the art and available from public depositories such as the American Type Culture Collection, Rockville, Md. Also, the mammalian cell, such as a CHO cell, may be modified to express sialyltransferase, e.g. 1,6-sialyltransferase, e.g. as described in U.S. Pat. No. 5,047,335, in order to provide improved glycosylation of the polypeptide variant.

[0260] In order to increase secretion it may be of particular interest to produce the polypeptide variant of the invention together with an endoprotease, in particular a PACE paired basic amino acid converting enzyme) (e.g. as described in U.S. Pat. No. 5,986,079), such as a Kex2 endoprotease (e.g. as described in WO 00/28065).

[0261] Methods for introducing exogeneous DNA into mammalian host cells include calcium phosphate-mediated transfection, electroporation, DEAE-dextran mediated transfection, liposome-mediated transfection, viral vectors and the transfection method described by Life Technologies

Ltd, Paisley, UK using Lipofectamin 2000. These methods are well known in the art and e.g. described by Ausbel et al. (eds.), 1996, Current Protocols in Molecular Biology, John Wiley & Sons, New York, USA. The cultivation of mammalian cells are conducted according to established methods, e.g. as disclosed in (Animal Cell Biotechnology, Methods and Protocols, Edited by Nigel Jenkins, 1999, Human Press Inc, Totowa, N.J., USA and Harrison M A and Rae I F, General Techniques of Cell Culture, Cambridge University Press 1997).

[0262] In the production methods of the present invention, the cells are cultivated in a nutrient medium suitable for production of the polypeptide variant using methods known in the art. For example, the cell may be cultivated by shake flask cultivation, small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermenters performed in a suitable medium and under conditions allowing the polypeptide to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or may be prepared according to published compositions (e.g., in catalogues of the American Type Culture Collection). If the polypeptide variant is secreted into the nutrient medium, the polypeptide can be recovered directly from the medium. If the polypeptide variant is not secreted, it can be recovered from cell lysates.

[0263] The resulting polypeptide variant may be recovered by methods known in the art. For example, the polypeptide variant may be recovered from the nutrient medium by conventional procedures including, but not limited to, centrifugation, filtration, extraction, spray drying, evaporation, or precipitation.

[0264] The polypeptides may be purified by a variety of procedures known in the art including, but not limited to, chromatography (e.g., ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (e.g., preparative isoelectric focusing), differential solubility (e.g., ammonium sulfate precipitation), HPLC, or extraction (see, e.g., Protein Purification, J.-C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989).

[0265] Single chain polypeptide variants of the invention can be purified and activated to two-chain polypeptide variants by a number of methods as described in the literature (Broze and Majerus, 1980, J. Biol. Chem. 255:1242-47 and Hedner and Kisiel, 1983, J. Clin. Invest. 71:1836-41). Another method whereby single chain polypeptide variant can be purified is by incorporation of Zn ions during purification as described in U.S. Pat. No. 5,700,914. In a preferred embodiment the polypeptide variant is purified as a single chain polypeptide variant. The single chain polypeptide variant is activated by either use of an immobilized enzyme (e.g. factors IIa, IXa, Xa and XIIa) or by autoactivation using a positively charged ion exchange matrix or the like.

[0266] It is advantageous to first purify the polypeptide variant in its single chain form, then PEGylate (if desired) and last activate by one of the methods described above or by autoactivation as described by Pedersen et al, 1989, Biochemistry 28: 9331-36. The advantage of carrying out PEGylation before activation is that PEGylation of the new aminoterminal formed by cleavage of R152-1153 is avoided. PEGylation of this new amino terminal would render the molecule inactive since the formation of a hydrogen bond between D242 and the amino terminal of 1153 is necessary for activity.

PHARMACEUTICAL COMPOSITION OF THE INVENTION AND ITS USE

[0267] In a further aspect, the present invention relates to a composition, in particular to a pharmaceutical composition, comprising a polypeptide variant of the invention and a pharmaceutically acceptable carrier or excipient.

[0268] The polypeptide variant or the pharmaceutical composition according to the invention may be used as a medicament.

[0269] Due to the improved properties mentioned hereinbefore, the polypeptide variants of the invention, or the pharmaceutical composition of the invention, are particular useful for the treatment of uncontrollable bleeding events in trauma patients, thrombocytopenic patients, patients in anticoagulant treatment, and cirrhosis patients with variceal bleeds, or other upper gastrointestinal bleedings, and in patients undergoing orthotopic liver transplantation, or liver resection (allowing for transfusion free surgery).

[0270] Trauma is defined as an injury to living tissue caused by an extrinsic agent. It is the 4th leading cause of death in the US and places a large financial burden on the economy.

[0271] Trauma is classified as either blunt or penetrative. Blunt trauma results in internal compression, organ damage and internal haemorrhage whereas penetrative trauma (as the consequence of an agent penetrating the body and destroying tissue, vessels and organs) results in external haemorrhage.

[0272] Haemorrhage, as a result of trauma, can start a cascade of problems. For example physiological compensation mechanisms are initiated with initial peripheral and mesenteric vasoconstriction to shunt blood to the central circulation. If circulation is not restored, hypovolemia shock (multiple organ failure due to inadequate perfusion) ensues. Since tissues throughout the body become starved for oxygen, anaerobic metabolism begins. However, the concomitant lactic acid leads the blood pH to drop and metabolic acidosis develops. If acidosis is severe and uncorrected, the patient may develop multisystem failure and die.

[0273] Although the majority of trauma patients are hypothermic on arrival in the emergency room due to the environmental conditions at the scene, inadequate protection, intravenous fluid administration and ongoing blood loss worsen the hypothermic state. Deficiencies in coagulation factors can result from blood loss or transfusions. Meanwhile, acidosis and hypothermia interfere with blood clotting mechanisms. Thus coagulopathy develops, which in turn, may mask surgical bleeding sites and hamper the control of mechanical bleeding. Hypothermia, coagulopathy and acidosis are often characterised as the "tauma triad of death"

[0274] Trauma may be caused by several events. For example, road traffic accidents result in many different types of trauma Whilst some road traffic accidents are likely to

result in penetrative trauma, many road traffic accidents are likely to inflict blunt trauma to both head and body. However, these various types of trauma can all result in coagnlopathy in the patient. Road traffic accidents are the leading cause of accidental death in the US. There are over 42,000 deaths from them in the US each year. Many trauma patients die at the location of the accident either whilst being treated by the paramedics, before they arrive or in transit to the ER.

[0275] Another example includes gunshot wounds. Gunshot wounds are traumas that can result in massive bleeding. They are penetrative and destroy tissue as the bullet passes through the body, whether it be in the torso or a limb. In the US about 40,000 people a year die from gunshot wounds

[0276] A further example includes falls. Falls result in a similar profile of trauma type to road traffic accidents. By falling onto a solid object or the ground from height can cause both penetrative and decelerative blunt trauma. In the US, falls are a common cause of accidental death, numbering about 13,000.

[0277] A still further example includes machinery accidents. A smaller number of people die in the US from machinery accident related deaths, whether struck by, or entangled in machinery. The figures are small but significant—around 2,000.

[0278] A still further example includes stab wounds. Stab wounds are penetrative injuries that can also cause massive bleeding. The organs most likely to be damaged in a stab wound are the liver, small intestine and the colon.

[0279] Cirrhosis of the liver is the terminal sequel of prolonged repeated injury to the hepatic parenchyma The end result is the formation of broad bands of fibrous tissue separating regenerative nodules that do not maintain the normal organization of liver lobules and thus cause deteriorated liver function. Patients have prolonged prothrombin times as a result of the depletion of vitamin K-dependent coagulation factors. Pathogenetically, liver cirrhosis should be regarded as the final common pathway of chronic liver injury, which can result from any form of intense repeated prolonged liver cell injury. Cirrhosis of the liver may be caused by direct liver injury, including chronic alcoholism, chronic viral hepatitis (types B, C, and D), and auto immune hepatitis as well as by indirect injury by way of bile duct damage, including primary biliary cirrhosis, primary sclerosing cholangitis and biliary atresia. Less common causes of cirrhosis include direct liver injury from inherited disease such as cystic fibrosis, alpha-1-antitrypsin deficiency, hemochromatosis, Wilson's disease, galactosemia, and glycogen storage disease.

[0280] Transplantation is primarily reserved for late stage cirrhotic patients, where it is the key intervention for treating the disease. To be eligible for transplantation, a patient must be classified as Child's B or C, as well as meet additional criteria for selection. Last year, in the US alone, 4,954 transplants were performed.

[0281] It has been estimated that there are 6,000 bleeding episodes associated with patients undergoing resection each year. This correlates with the reserved position of this procedure although seems slightly high in comparison with transplantation numbers.

[0282] Accurate data on the incidence of variceal bleeding is hard to obtain. The key facts known are that at the time of diagnosis, varices are present in about 60% of decompensated and 30% of compensated patients and that about 30% of these patients with varices will experience a bleed and that each episode of variceal bleeding is associated with a 30% risk of mortality.

[0283] Thus, in a further aspect the present invention relates to a polypeptide variant of the invention for the manufacture of a medicament for the treatment of diseases or disorder wherein clot formation is desirable. A still further aspect of the present invention relates to a method for treating a mammal having a disease or disorder wherein clot formation is desirable, comprising administering to a mammal in need thereof an effective amount of the polypeptide variant or the pharmaceutical composition of the invention.

[0284] Thrombocytopenia is caused by one of three mechanisms-decreased bone marrow production, increased splenic sequestration, or accelerated destruction of platelets. Thrombocytopenia is a risk factor for hemorrhage, and platelet transfusion reduces the incidence of bleeding. The threshold for prophylactic platelet transfusion is 10,000/μl. In patients without fever or infections, a threshold of 5000/μl may be sufficient to prevent spontaneous hemorrhage. For invasive procedures, 50,000/μl platelets is the usual target level. In patients who develop antibodies to platelets following repeated transfusions, bleeding can be extremely difficult to control.

[0285] Examples of diseases/disorders wherein increased clot formation is desirable include, but is not limited to, hemorrhages, including brain hemorrhages, as well as patient with severe uncontrolled bleedings, such as trauma Further examples include patients undergoing living transplantations, patients undergoing resection and patients with variceal bleedings.

[0286] The polypeptide variants of the invention is administered to patients in a therapeutically effective dose, normally one approximately paralleling that employed in therapy with rFVII such as NovoSeven®, or at lower dosage. By "therapeutically effective dose"herein is meant a dose that is sufficient to produce the desired effects in relation to the condition for which it is administered. The exact dose will depend on the circumstances, and will be ascertainable by one skilled in the art using known techniques. Normally, the dose should be capable of preventing or lessening the severity or spread of the condition or indication being treated. It will be apparent to those of skill in the art that an effective amount of a polypeptide variant or composition of the invention depends, inter alia, upon the disease, the dose, the administration schedule, whether the polypeptide variant or composition is administered alone or in conjunction with other therapeutic agents, the plasma half-life of the compositions, and the general health of the patient. Preferably, the polypeptide variant or composition of the invention is administered in an effective dose, in particular a dose which is sufficient to normalize the coagulation disorder.

[0287] The polypeptide variant of the invention is preferably administered in a composition including a pharmaceutically acceptable carrier or excipient. "Pharmaceutically acceptable" means a carrier or excipient that does not cause any untoward effects in patients to whom it is administered. Such pharmaceutically acceptable carriers and excipients are well known in the art (see, for example, Remington's

Pharmaceutical Sciences, 18th edition, A. R. Gennaro, Ed., Mack Publishing Company [1990]; Pharmaceutical Formulation Development of Peptides and Proteins, S. Frokjaer and L. Hovgaard, Eds., Taylor & Francis [2000]; and Handbook of Pharmaceutical Excipients, 3rd edition, A. Kibbe, Ed., Pharmaceutical Press [2000]).

[0288] The polypeptide variant of the invention can be formulated into pharmaceutical compositions by well-known methods. Suitable formulations are described by Remington's Pharmaceutical Sciences by E. W. Martin (Mark Publ. Co., 16th Ed., 1980).

[0289] The polypeptide variant of the invention can be used "as is" and/or in a salt form thereof. Suitable salts include, but are not limited to, salts with alkali metals or alkaline earth metals, such as sodium, potassium, calcium and magnesium, as well as e.g. zinc salts. These salts or complexes may by present as a crystalline and/or amorphous structure.

[0290] The pharmaceutical composition of the invention may be administered alone or in conjunction with other therapeutic agents. These agents may be incorporated as part of the same pharmaceutical composition or may be administered separately from the polypeptide variant of the invention, either concurrently or in accordance with another treatment schedule. In addition, the polypeptide variant or pharmaceutical composition of the invention may be used as an adjuvant to other therapies.

[0291] A "patient" for the purposes of the present invention includes both humans and other mammals. Thus, the methods are applicable to both human therapy and veterinary applications. The pharmaceutical composition comprising the polypeptide variant of the invention may be formulated in a variety of forms, e.g. as a liquid, gel, lyophilized, or as a compressed solid. The preferred form will depend upon the particular indication being treated and will be apparent to one skilled in the art.

[0292] In particular, the pharmaceutical composition comprising the polypeptide variant of the invention may be formulated in lyophilised or stable soluble form. The polypeptide variant may be lyophilised by a variety of procedures known in the art. The polypeptide variant may be in a stable soluble form by the removal or shielding of proteolytic degradation sites as described herein. The advantage of obtaining a stable soluble preparation lies in easier handling for the patient and, in the case of emergencies, quicker action, which potentially can become life saving. The preferred form will depend upon the particular indication being treated and will be apparent to one of skill in the art.

[0293] The administration of the formulations of the present invention can be performed in a variety of ways, including, but not limited to, orally, subcutaneously, intravenously, intracerebrally, intranasally, transdermally, intraperitoneally, intramuscularly, intrapulmonary, vaginally, rectally, intraocularly, or in any other acceptable manner. The formulations can be administered continuously by infusion, although bolus injection is acceptable, using techniques well known in the art, such as pumps or implantation. In some instances the formulations may be directly applied as a solution or spray.

Parentals

[0294] A preferred example of a pharmaceutical composition is a solution designed for parenteral administration. Although in many cases pharmaceutical solution formulations are provided in liquid form, appropriate for immediate use, such parenteral formulations may also be provided in frozen or in lyophilized form. In the former case, the composition must be thawed prior to use. The latter form is often used to enhance the stability of the active compound contained in the composition under a wider variety of storage conditions, as it is recognized by those skilled in the art that lyophilized preparations are generally more stable than their liquid counterparts. Such lyophilized preparations are reconstituted prior to use by the addition of one or more suitable pharmaceutically acceptable diluents such as sterile water for injection or sterile physiological saline solution.

[0295] In case of parenterals, they are prepared for storage as lyophized formulations or aqueous solutions by mixing, as appropriate, the polypeptide variant having the desired degree of purity with one or more pharmaceutically acceptable carriers, excipients or stabilizers typically employed in the art (all of which are termed "excipients"), for example buffering agents, stabilizing agents, preservatives, isotonifiers, non-ionic surfactants or detergents, antioxidants and/or other miscellaneous additives.

[0296] Buffering agents help to maintain the pH in the range which approximates physiological conditions. They are typically present at a concentration ranging from about 2 mM to about 50 mM. Suitable buffering agents for use in the present invention include both organic and inorganic acids and salts thereof such as citrate buffers (e.g., monosodium citrate-disodium citrate mixture, citric acid-trisodium citrate mixture, citric acid-monosodium citrate mixture, etc.), succinate buffers (e.g., succinic acid-monosodium succinate mixture, succinic acid-sodium hydroxide mixture, succinic acid-disodium succinate mixture, etc.), tartrate buffers (e.g., tartaric acid-sodium tartrate mixture, tartaric acid-potassium tartrate mixture, tartaric acid-sodium hydroxide mixture, etc.), fumarate buffers (e.g., fumaric acid-monosodium fumarate mixture, fumaric acid-disodium fumarate mixture, monosodium fumarate-disodium fumarate mixture, etc.), gluconate buffers (e.g., gluconic acid-sodium glyconate mixture, gluconic acid-sodium hydroxide mixture, gluconic acid-potassium glyuconate mixture, etc.), oxalate buffer (e.g., oxalic acid-sodium oxalate mixture, oxalic acid-sodium hydroxide mixture, oxalic acid-potassium oxalate mixture, etc.), lactate buffers (e.g., lactic acid-sodium lactate mixture, lactic acid-sodium hydroxide mixture, lactic acid-potassium lactate mixture, etc.) and acetate buffers (e.g., acetic acid-sodium acetate mixture, acetic acid-sodium hydroxide mixture, etc.). Additional possibilities are phosphate buffers, histidine buffers and trimethylamine salts such as Tris.

[0297] Stabilizers refer to a broad category of excipients, which can range in function from a bulking agent to an additive which solubilizes the therapeutic agent or helps to prevent denaturation or adherence to the container wall. Typical stabilizers can be polyhydric sugar alcohols (enumerated above); amino acids such as arginine, lysine, glycine, glutamine, asparagine, histidine, alanine, ornithine, L-leucine, 2-phenylalanine, glutamic acid, threonine, etc., organic sugars or sugar alcohols, such as lactose, trehalose,

stachyose, mannitol, sorbitol, xylitol, ribitol, myoinisitol, galactitol, glycerol and the like, including cyclitols such as inositol; polyethylene glycol; amino acid polymers; sulfur-containing reducing agents, such as urea, glutathione, thioctic acid, sodium thioglycolate, thioglycerol, α-monothioglycerol and sodium thiosulfate; low molecular weight polypeptides (i.e. <10 residues); proteins such as human serun albumin, bovine serum albumin, gelatin or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; monosaccharides such as xylose, mannose, fructose and glucose; disaccharides such as lactose, maltose and sucrose; trisaccharides such as raffinose, and polysaccharides such as dextran. Stabilizers are typically present in the range of from 0.1 to 10,000 parts by weight based on the active protein weight.

[0298] Preservatives are added to retard microbial growth, and are typically added in amounts of about 0.2%-1% (w/v). Suitable preservatives for use with the present invention include phenol, benzyl alcohol, meta-cresol, methyl paraben, propyl paraben, octadecyldimethylbenzyl ammonium chloride, benzalkonium halides (e.g. benzalkonium chloride, bromide or iodide), hexamethonium chloride, alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol and 3-pentanol.

[0299] Isotonicifiers are added to ensure isotonicity of liquid compositions and include polyhydric sugar alcohols, preferably trihydric or higher sugar alcohols, such as glycerin, erythritol, arabitol, xylitol, sorbitol and mannitol. Polyhydric alcohols can be present in an amount between 0.1% and 25% by weight, typically 1% to 5%, taking into account the relative amounts of the other ingredients.

[0300] Non-ionic surfactants or detergents (also known as "wetting agents") may be present to help solubilizing the therapeutic agent as well as to protect the therapeutic polypeptide against agitation-induced aggregation, which also permits the formulation to be exposed to shear surface stress without causing denaturation of the polypeptide. Suitable non-ionic surfactants include polysorbates (20, 80, etc.), polyoxamers (184, 188 etc.), Pluronic® polyols, polyoxyethylene sorbitan monoethers (Tween®-20, Tween®-80, etc.).

[0301] Additional miscellaneous excipients include bulking agents or fillers (e.g. starch), chelating agents (e.g. EDTA), antioxidants (e.g., ascorbic acid, methionine, vitamin E) and cosolvents.

[0302] The active ingredient may also be entrapped in microcapsules prepared, for example, by coascervation techniques or by interfacial polymerization, for example hydroxymethylcellulose, gelatin or poly-(methylmethacylate) microcapsules, in colloidal drug delivery systems (for example liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, supra.

[0303] Parenteral formulations to be used for in vivo administration must be sterile. This is readily accomplished, for example, by filtration through sterile filtration membranes.

Sustained Release Preparations

[0304] Examples of sustained-release preparations include semi-permeable matrices of solid hydrophobic polymers containing the polypeptide variant, the matrices having a suitable form such as a film or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate) or poly(vinylalcohol)), polylactides, copolymers of L-glutamic acid and ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the ProLease® technology or Lupron Depot® (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(−)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for long periods such as up to or over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated polypeptides remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37° C., resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

[0305] The invention is further described in the following non-limiting examples.

MATERIALS AND METHODS

Accessible Surface Area (ASA)

[0306] The computer program Access (B. Lee and F. M. Richards, J. Mol. Biol. 55: 379-400 (1971)) version 2 (© 1983 Yale University) is used to compute the accessible surface area (ASA) of the individual atoms in the structure. This method typically uses a probe-size of 1.4 Å and defines the Accessible Surface Area (ASA) as the area formed by the center of the probe. Prior to this calculation all water molecules and all hydrogen atoms should be removed from the coordinate set, as should other atoms not directly related to the protein.

[0307] Fractional ASA of Side Chain

[0308] The fractional ASA of the side chain atoms is computed by division of the sum of the ASA of the atoms in the side chain with a value representing the ASA of the side chain atoms of that residue type in an extended Ala-x-Ala tripeptide (See Hubbard, Campbell & Thornton (1991) J. Mol. Biol. 220, 507-530). For this example the CA atom is regarded as a part of the side chain of Glycine residues but not for the remaining residues. The following table is used as standard 100% ASA for the side chain:

Ala	69.23 Å^2
Arg	200.35 Å^2
Asn	106.25 Å^2
Asp	102.06 Å^2
Cys	96.69 Å^2
Gln	140.58 Å^2
Glu	134.61 Å^2
Gly	32.28 Å^2
His	147.00 Å^2

-continued

Ile	137.91 Å^2
Leu	140.76 Å^2
Lys	162.50 Å^2
Met	156.08 Å^2
Phe	163.90 Å^2
Pro	119.65 Å^2
Ser	78.16 Å^2
Thr	101.67 Å^2
Trp	210.89 Å^2
Tyr	176.61 Å^2
Val	114.14 Å^2

[0309] Residues not detected in the structure are defined as having 100% exposure as they are thought to reside in flexible regions. The gamma-carboxy glutamic acids at positions 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35 are all defined as being 100% exposed.

Determining Distances Between Atoms

[0310] The distance between atoms is most easily determined using molecular graphics software e.g. Insight® v. 98.0, MSI INC.

Active Site Region

[0311] The active site region is defined as any residues having at least one atom within 10 Å of any atom in the catalytic triad (residues H193, D242, S344).

Determination of Tissue Factor Binding Site

[0312] The TF binding site is defined as comprising of all residues having their accessible surface area changed upon TF binding. This is determined by at least two ASA calculations; one on the isolated ligand(s) in the ligand(s)/receptor(s) complex and one on the complete ligand(s)/receptor(s) complex.

Measurement of Reduced Sensitivity to Proteolytic Degradation

[0313] Proteolytic degradation can be measured using the assay described in U.S. Pat. No. 5,580,560, Example 5, where proteolysis is autoproteolysis.

[0314] Furthermore, reduced proteolysis can be tested in an in vivo model using radiolabelled samples and comparing proteolysis of rhFVIIa and the polypeptide variant of the invention by withdrawing blood samples and subjecting these to SDS-PAGE and autoradiography.

[0315] Irrespectively of the assay used for determining proteolytic degradation, "reduced proteolytic degradation" is intended to mean a measurable reduction in cleavage compared to that obtained by rhFVIIa as measured by gel scanning of Coomassie stained SDS-PAGE gels, HPLC or as measured by conserved catalytic activity in comparison to wild type using the tissue factor independent activity assay decribed below.

Determination of the Molecular Weight of Polypeptide Variants

[0316] The molecular weight of polypeptide variants is determined by either SDS-PAGE, gel filtration, Western Blots, matrix assisted laser desorption mass spectrometry or equilibrium centrifugation, e.g. SDS-PAGE according to Laemmli, U.K, Nature Vol 227 (1970), pp. 680-85.

TF-Independent Factor X Activation Assay

[0317] This assay has been described in detail on page 39826 in Nelsestuen et al., J Biol Chem, 2001; 276:39825-39831.

[0318] Briefly, the molecule to be assayed (either hFVIIa, rhFVIIa or the polypeptide variant of the invention in its activated form) is mixed with a source of phospholipid (phosphatidylcholine and phosphatidylserine in a ratio of 8:2 or phosphatidylcholine, phosphatidylserine and phosphatidylethanol in a ratio of 4:2:4) and Factor X in Tris buffer containing BSA. After a specified incubation time the reaction is stopped by addition of excess EDTA. The concentration of factor Xa is then measured from absorbance change at 405 nm after addition of a chromogenic substrate (S-2222, Chromogenix). After correction from background the tissue factor independent activity of rhFVIIa (a_{wt}) is determined as the absorbance change after 10 minutes and the tissue factor independent activity of the polypeptide variant of the invention ($a_{variant}$) is also determined as the absorbance change after 10 minutes. The ratio between the activity of the polypeptide variant, in its activated form, and the activity of rhFVIIa is defined as $a_{variant}/a_{wt}$.

Clotting Assay

[0319] Clotting activity is measured in one-stage assays and clotting times are recorded on a Thrombotrack IV coagulometer (MEDINOR). FVII depleted human plasma (American Diagnostica) is reconstituted and equilibrated at room temperature for 15-20 minutes. 50 µl of plasma is then transferred to the coagulometer cups.

[0320] hFVIIa, rhFVIIa or variants are diluted in Glyoxaline Buffer (5.7 mM barbiturate, 4.3 mM sodium citrate, 117 mM NaCl, 1 mg/mL BSA, pH 7.35). The samples are added to the cup in 50 µl and incubated at 37° C. for 2 minutes.

[0321] Thromboplastin (MEDINOR) is reconstituted with water and $CaCl_2$ is added. The reaction is initiated by adding 0.1 ml thromboplastin containing 4.5 mM $CaCl_2$.

[0322] Data are analysed using PRISM software.

TF-Independent Clotting Assay

[0323] This assay is performed as described above under "Clotting Assay " but without addition of thromboplastin.

[0324] Amidolytic Assay

[0325] The ability of the variants to cleave small peptide substrates can be measured using the chromogenic substrate S-2288 (D-Ile-Pro-Arg-p-nitroanilide). The amidolytic activity may be measured both in the presence and absence of antithrombin III (ATIII).

[0326] HFVIIa, rhFVIIa or variant is diluted to 90 nM in assay buffer (50 mM Na-Hepes pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 0.1% BSA, 1 U/ml Heparin). Furthermore, soluble TF (sTF) is diluted to 450 nM in assay buffer. ATDI is diluted to 900 nM in assay buffer. 120 µl of assay buffer is mixed with 20 µl of the FVIIa sample, 20 µl sTF and 20 µl ATIII or assay buffer. The final concentrations of FVIIa, sTF and ATIII are 10, 50 and 100 nM, respectively. After 5 min incubation at room temperature with gentle shaking, followed by 10 min incubation at 37° C., the reaction is started by addition of the S-2288 substrate to 1 mM and the absorption at 405 nm is determined at several time points.

Thrombogram Assay

[0327] The effect of hFVIIa, rhFVIIa or variant on thrombin generation in human plasma is tested in a modified version of the assay described on page 589 in Hemker et al. in Thromb Haemost 2000; 83: 589-91. Briefly, the molecule to be assayed (either hFVIIa, rhFVIIa or variant) is mixed with normal platelet rich plasma (PRP), normal platelet poor plasma (PPP) or FVII depleted PPP with or without the addition of recombinant human tissue factor (rTF), relipidated rTF or another source of TF (such as thromboplastin). A source of phospholipid (phosphatidylcholine and phosphatidylserine in a ratio of 8:2 or phosphatidylcholine, phosphatidylserine and phosphatidylethanol in a ratio of 4:2:4) can be added.

[0328] The reaction is started by addition of a fluoregenic thrombin substrate and calcium chloride. The fluorescence is measured continuously and the thrombin amidolytic activity is calculated by calculating the slope of the fluorescence curve (the increase in fluorescence over time). In this way the time until maximum thrombin amidolytic activity is obtained (T_{max}) and total thrombin work (area under the curve (AUC)) can be calculated.

[0329] The following procedure is used: PRP is obtained by centrifuging freshly drawn blood at 250 g, 15° C. for 10 min. Blood coagulation is inhibited either by using citrate (13 mM tri-sodium citrate), corn trypsin inhibitor (50-100 μg/ml blood) or a combination of citrate and corn trypsin inhibitor. The platelet count is adjusted to 3×10^8/mL using buffer or autologous platelet poor plasma (PPP). PPP is obtained by double centrifugation of PRP at 1000 g, 15° C. for 10 min. FVII depletion is done by incubating PPP with a FVII specific monoclonal antibody coupled to a solid phase.

[0330] Per well of a 96-well microtiter plate 80 μl PRP is added and 20 μL buffer containing rhFVII or variant to be tested in final concentrations between 0.1 and 100 nM. rTF is added in 5 μL assay buffer to a final concentration of 1 μM. The assay buffer consists of 20 mM Hepes, 150 mM NaCl and 60 mg/ml BSA in distilled water. The reaction is started by adding 20 μL of the substrate solution containing 0.1 M calcium chloride. The assay plate and reagents are prewarmed to 37° C. and the reaction takes place at this temperature. The fluorimeter used is a BMG Fluormeter with a excitation filter at 390 nm and an emission filter at 460 mm. The fluorescence is measured in each well of 96-well clear bottom plates in 20-40 second interval over 30-180 minutes. Data are analyzed using PRISM Software.

ELISA Assay

[0331] FVII/FVIIa (or variant) concentrations are determined by ELISA. Wells of a microtiter plate are coated with an antibody directed against the protease domain using a solution of 2 μg/ml in PBS (100 μl per well). After 2 hours coating at R.T., the wells are washed 4 times with THT buffer (100 mM NaCl, 50 mM Tris-HCl pH 7.2 0.05% Tween-20). Subsequently, 200 μl of 1% Casein (diluted from 2.5% stock using 100 mM NaCl, 50 mM Tris-HCl pH 7.2) is added per well for blocking. After 1 hr incubation at R.T., the wells are emptied, and 100 μl of sample (optionally diluted in dilution buffer (THT+0.1% Casein)) is added. After another incubation of 1 hr at room temperature, the wells are washed 4 times with THF buffer, and 100 μl of a biotin-labelled antibody directed against the EGF-like domain (1 μg/ml) is added. After another 1 hr incubation at RT., followed by 4 more washes with THT buffer, 100 μl of streptavidin-horse radish peroxidase (DAKO A/S, Glostrup, Denmark, 1/10000 diluted) is added. After another 1 hr incubation at R.T., followed by 4 more washes with THT buffer, 100 μl of TMB (3,3',5,5'-tetramethylbenzidine, Kemen-Tech A/S, Denmark) is added. After 30 min incubation at R.T. in the dark, 100 μl of 1 M H_2SO_4 is added and OD_{450} is determined. A standard curve is prepared using rhFVIIa (NovoSeven®).

[0332] Alternatively, FVII/FVIIa or variants may be quantified through the Gla domain rather than through the protease domain. In this ELISA set-up, wells are coated overnight with an antibody directed against the EGF-ike domain and for detection, a calcium-dependent biotin-labelled monoclonal anti-Gla domain antibody is used (2 μg/ml,100 μl per well). In this set-up, 5 mM $CaCl_2$ is added to the THT and dilution buffers.

Whole Blood Assay

[0333] The whole blood clotting assay is performed as described by Elg et al. Thrombosis Res. 2001, 101(3):159-170.

Reconstituted Coagulation Assay

[0334] The reconstituted coagulation assay is performed as described by Van't Veer et al. Blood 2000, 95(4), 1330-1335.

EXAMPLES

Example 1

[0335] The X-ray structure of hFVIIa in complex with soluble tissue factor by Banner et. al., J Mol Biol, 1996; 285:2089 is used for this example. It is noted that the numbering of residues in the reference does not follow the sequence. Here we have used the sequential numbering according to SEQ ID NO:2. The gamma-carboxy glutamic acids at positions 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35 are all here named GLU (three letter abbreviation) or E (one letter abbreviation). Residues 143-152 are not present in the structure.

Surface Exposure

[0336] Performing fractional ASA calculations on FVII fragments alone combined with the definition of accessibilities of non standard and/or missing residues described in the methods resulted in the following residues having more than 25% of their side chain exposed to the surface: A1, N2, A3, F4, L5, E6, E7, L8, R9, P10, S12, L13, E14, E16, K18, E19, E20, Q21, S23, F24, E25, E26, R28, E29, F31, K32, D33, A34, E35, R36, K38, L39, W41, I42, S43, S45, G47, D48, Q49, A51, S52, S53, Q56, G58, S60, K62, D63, Q64, L65, Q66, S67, I69, F71, L73, P74, A75, E77, G78, R79, E82, T83, H84, K85, D86, D87, Q88, L89, I90,V92, N93, E94, G97, E99, S103, D104, H105, T106, G107, T108, K109, S111, R113, E116, G117, S119, L120, L121, A122, D123, G124, V125, S126, T128, P129, T130, V131, E132, I140, L141, E142, K143, R144, N145, A146, S147, K148, P149, Q150, G151, R152, G155, K157, V158, P160, K161, E163, L171, N173, G174, A175, N184, T185, I186, H193, K197, K199, N200, R202, N203, I205, S214, E215, H216, D217, G218, D219, S222, R224, S232, T233, V235, P236, G237, T238, T239, N240, H249, Q250, P251, V253, T255, D256, E265, R266, T267, E270, R271, F275, V276, R277, F278,

L280, L287, L288, D289, R290, G291, A292, T293, L295, E296, N301, M306, T307, Q308, D309, L311, Q312, Q313, R315, K316, V317, G318, D319, S320, P321, N322, T324, E325, Y326, Y332, S333, D334, S336, K337, K341, G342, H351, R353, G354, Q366, G367, T370, V371, G372, R379, E385, Q388, K389, R392, E394, P395, R396, P397, G398, V399, L400, L401, R402, P404 and P406 (A1-S45 are located in the Gla domain, the remaining positions are located outside the Gla domain).

[0337] The following residues had more than 50% of their side chain exposed to the surface: A1, A3, F4, L5, E6, E7, L8, R9, P10, E14, E16, K18, E19, E20, Q21, S23, E25, E26, E29, K32, A34, E35, R36, K38, L39, 142, S43, G47, D48, A51, S52, S53, Q56, G58, S60, K62, L65, Q66, S67, 169, F71, L73, P74, A75, E77, G78, R79, E82, H84, K85, D86, D87, Q88, L89, I90, V92, N93, E94, G97,T106, G107,T108, K109, SI i l, E116, Sll9, L121, A122,D123, G124, V131, E132, L141, E142, K143, R144, N145, A146, S147, K148, P149, Q150, G151, R152, G155, K157, P160, N173, G174, A175, K197, K199, N200, R202, S214, E215, H216, G218, R224, V235, P236, G237, T238, H249, Q250, V253, D256, T267, F275, R277, F278, L288, D289, R290, G291, A292, T293, L295, N301, M306, Q308, D309, L311, Q312, Q313, R315, K316, G318, D319, N322, E325, D334, K341, G354, G367, V371, E385, K389, R392, E394, R396, P397, G398, R402, P404 and P406 (A1-S43 are located in the Gla domain, the remaining positions are located outside the Gla domain).

Tissue Factor Binding Site

[0338] Performing ASA calculations the following residues in human FVII change their ASA in the complex. These residues were defined as constituting the receptor binding site: L13, K18, F31, E35, R36, L39, F40, I42, S43, S60, K62, D63, Q64, L65, I69, C70, F71, C72, L73, P74, F76, E77, G78, R79, E82, K85, Q88, I90, V92, N93, E94, R271, A274, F275, V276, R277, F278, R304, L305, M306, T307, Q308, D309, Q312, Q313, E325 and R379.

Active Site Region

[0339] The active site region is defined as any residue having at least one atom within a distance of 10 Å from any atom in the catalytic triad (residues H1 93, D242, S344): 1153, Q167, V168, L169, L170, L171, Q176, L177, C178, G179, G180, T181, V188, V189, S190, A191, A192, H193, C194, F195, D196, K197, I198, W201, V228, I229, I230, P231, S232, T233, Y234, V235, P236, G237, T238, T239, N240, H241, D242, I243, A244, L245, L246, V281, S282, G283, W284, 0285, Q286, T293, T324, E325, Y326, M327, F328, D338, S339, C340, K341, G342, D343, S344, G345, G346, P347, H348, L358, T359, G360, I361, V263, S363, W364, G365, C368, V376, Y377, T378, R379, V380, Q382, Y383, W386, L387, L400 and F405.

The Ridge of the Active Site Binding Cleft

[0340] The ridge of the active site binding cleft region was defined by visual inspection of the FVIIa structure lFAK pdb as: N173, A175, K199, N200, N203, D289, R290, G291, A292, P321 and T370.

Example 2

[0341] Design of an expression Cassette for Expression of rhFVII in Mammalian Cells

[0342] The DNA sequence shown in SEQ ID NO:1, encompassing the short form of the full length cDNA encoding hFVII with its native short signal peptide (Hagen et al., 1986. PNAS 83:2412), was synthesized in order to facilitate high expression in mammalian cells. First the ATG start codon context was modified according to the Kozak consensus sequence (Kozak, M. *J Mol Biol* Aug 20, 1987;196(4):947-50), so that there is a perfect match to the consensus sequence upstream of the ATG start codon. Secondly the open reading frame of the native cDNA was modified by making a bias in the codon usage towards the codons frequently used in highly expressed human genes. Further, two translational stop codons were inserted at the end of the open reading frame in order to facilitate efficient translational stop. The fully synthetic and expression optimized hFVII gene was assembled from 70-mer DNA oligonucleotides and finally amplified using end primers inserting BamHI and HindIII sites at the 5' and 3' ends respectively using standard PCR techniques, which resulted in the following sequence:

```
ggatcccgccaccatggtcagccaggccctccgcctcctgtgcctgctcctggggctgcagggctgcctggctgccgtcttcgtcaccca

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gcgtctacacgcgcgtcagccagtacattgagtggctgcagaagctcatgcggagcgaaccccggcccggggtgctcctgcgggccc

ctttcccttgataaaagctt

[0343] A vector for the cloning of the generated PCR product encompassing the expression cassette for hFVII was prepared by cloning the intron from pCINeo (Promega). The synthetic intron from pCI-Neo was amplified using standard PCR conditions and the primers:

CBProFpr174:
5'- AGCTGGCTAGCCACTGGGCAGGTAAGTATCA -3'
and

CBProFpr175:
5'- TGGCGGGATCCTTAAGAGCTGTAATTGAACT -3'

resulting in a 332 bp PCR fragment. The fragment was cut with NheI and BamHI before cloning into pCDNA3.1/HygR (obtained from Invitrogen) resulting in PF#34.

[0344] The expression cassette for hFVII was cloned between the BamHI and HindIII sites of PF#34, resulting in plasmid PF#226.

Example 3

Expression of Polypeptide Variants in CHO K1 Cells

[0345] The cell line CHO K1 (ATCC # CCL-61) is seeded at 50% confluence in T-25 flasks using MEMA, 10% FCS (Gibco/BRL Cat # 10091), P/S and 5 μg/nil phylloquinone and allowed to grow until confluent. The confluent mono cell layer is transfected with 5 μg of the relevant plasmid described above using the Lipofectamine 2000 transfection agent (Life technologies) according to the manufacturer's instructions. Twenty four hours post transfection a sample is drawn and quantified using e.g. an ELISA recognizing the EGF1 domain of hFVII. At this time point relevant selection (e.g. Hygromycin B) may be applied to the cells with the purpose of generating a pool of stable transfectants. When using CHO K1 cells and the Hygromycin B resistance gene as selectable marker on the plasmid, this is usually achieved within one week.

Example 4

Generation of CHO K1 Cells Stably Expressing Polypeptide Variants.

[0346] A vial of CHO-K1 transfectant pool is thawed and the cells seeded in a 175 cm^2 tissue flask containing 25 ml of MEMα, 10% FCS, phylloquinone (5 μg/ml), 100 U/l penicillin, 100 μg/l streptomycin and grown for 24 hours. The cells are harvested, diluted and plated in 96 well microtiter plates at a cell density of ½-1 cell/well. After a week of growth, colonies of 20-100 cells are present in the wells and those wells containing only one colony are labelled. After a further two weeks, the media in all wells containing only one colony is substituted with 200 μl fresh medium. After 24 hours, a medium sample is withdrawn and analysed by e.g. ELISA. High producing clones are selected and used to produce FVII or variant on large scale.

Example 5

Purification of Polypeptide Variants and Subsequent Activation

[0347] FVII and FVII variants are purified as follows: The procedure is performed at 4° C. The harvested culture media from large-scale production is ultrafiltered using a Millipore TFF system with 30 KDa cut-off Pellicon membranes. After concentration of the medium, citrate is added to 5 mM and the pH is adjusted to 8.6. If necessary, the conductivity is lowered to below 10 mS/cm. Subsequently, the sample is applied to a Q-sepharose FF column, equilibrated with 50 mM NaCl, 10 mM Tris pH 8.6. After washing the column with 100 mM NaCl, 10 mM Tris pH 8.6, followed by 150 mM NaCl, 10 mM Tris pH 8.6, FVII is eluted using 10 mM Tris, 25 mM NaCl, 35 mM $CaCl_2$, pH 8.6.

[0348] For the second chromatographic step, an affinity column is prepared by coupling of a monoclonal Calcium-dependent antiGla-domain antibody to CNBr-activated Sepharose FF. About 5.5 mg antibody is coupled per ml resin. The column is equilibrated with 10 mM Tris, 100 mM NaCl, 35 mM $CaCl_2$, pH 7.5. NaCl is added to the sample to a concentration of 100 mM NaCl and the pH is adjusted to 7.4 -7.6. After O/N application of the sample, the column is washed with 100 mM NaCl, 35 mM $CaCl_2$, 10 mM Tris pH 7.5, and the FVII protein is eluted with 100 mM NaCl, 50 mM citrate, 75 mM Tris pH 7.5.

[0349] For the third chromatographic, the conductivity of the sample is lowered to below 10 mS/cm, if necessary, and the pH is adjusted to 8.6. The sample is then applied to a Q-sepharose column (equilibrated with 50 mM NaCl, 10 mM Tris pH 8.6) at a density around 3-5 mg protein per ml gel to obtain efficient activation. After application, the column is washed with 50 mM NaCl, 10 mM Tris pH 8.6 for about 4 hours with a flow of 34 column volumes (cv) per hour. The FVII protein is eluted using a gradient of 0-100% of 500 mM NaCl, 10 mM Tris pH 8.6 over 40 cv. FVII containing fractions are pooled.

[0350] For the final chromatographic step, the conductivity is lowered to below 10 mS/cm. Subsequently, the sample is applied to a Q-sepharose column (equilibrated with 140 mM NaCl, 10 mM glycylglycine pH 8.6) at a concentration of 3-5 mg protein per ml gel. The column is then washed with 140 mM NaCl, 10 mM glycylglycine pH 8.6 and FVII is eluted with 140 mM NaCl, 15 mM $CaCl_2$, 10 mM glycylglycine pH 8.6. The eluate is diluted to 10 mM $CaCl_2$ and the pH is adjusted 6.8-7.2. Finally, Tween-80 is added to 0.01% and the pH is adjusted to 5.5 for storage at −80° C.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 4

<210> SEQ ID NO 1
<211> LENGTH: 1338
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
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<222> LOCATION: (115)..(1335)

<400> SEQUENCE: 1

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                                                            Ala
                                                             1

aat gcc ttt ctg gaa gag ctc cgc cct ggc tcc ctg gaa cgc gaa tgc      165
Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu Cys
              5                  10                  15

aaa gag gaa cag tgc agc ttt gag gaa gcc cgg gag att ttc aaa gac      213
Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys Asp
         20                  25                  30

gct gag cgg acc aaa ctg ttt tgg att agc tat agc gat ggc gat cag      261
Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp Gln
     35                  40                  45

tgc gcc tcc agc cct tgc cag aac ggg ggc tcc tgc aaa gac cag ctg      309
Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln Leu
 50                  55                  60                  65

cag agc tat atc tgc ttc tgc ctg cct gcc ttt gag ggg cgc aat tgc      357
Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn Cys
                 70                  75                  80

gaa acc cat aag gat gac cag ctg att tgc gtc aac gaa aac ggg ggc      405
Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly Gly
             85                  90                  95

tgc gag cag tac tgc agc gat cac acg ggc acg aag cgg agc tgc cgc      453
Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys Arg
        100                 105                 110

tgc cac gaa ggc tat agc ctc ctg gct gac ggg gtg tcc tgc acg ccc      501
Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr Pro
    115                 120                 125

acg gtg gaa tac cct tgc ggg aag att ccc att cta gaa aag cgg aac      549
Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg Asn
130                 135                 140                 145

gct agc aaa ccc cag ggc cgg atc gtc ggc ggg aag gtc tgc cct aag      597
Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro Lys
                150                 155                 160

ggg gag tgc ccc tgg cag gtc ctg ctc ctg gtc aac ggg gcc cag ctg      645
Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln Leu
            165                 170                 175

tgc ggc ggg acc ctc atc aat acc att tgg gtc gtg tcc gcc gct cac      693
Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala His
        180                 185                 190

tgc ttc gat aag att aag aat tgg cgg aac ctc atc gct gtg ctc ggc      741
Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu Gly
    195                 200                 205

gaa cac gat ctg tcc gag cat gac ggg gac gaa cag tcc cgc cgg gtg      789
Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg Val
210                 215                 220                 225
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                230                 235                 240

gat atc gct ctg ctc cgc ctc cac cag ccc gtc gtg ctc acc gat cac      885
Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp His
            245                 250                 255

gtc gtg cct ctg tgc ctg cct gag cgg acc ttt agc gaa cgc acg ctg      933
Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr Leu
        260                 265                 270

gct ttc gtc cgc ttt agc ctc gtg tcc ggc tgg ggc cag ctg ctc gac      981
Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu Asp
    275                 280                 285

cgg ggc gct acc gct ctc gag ctg atg gtg ctc aac gtc ccc cgg ctg     1029
Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg Leu
290                 295                 300                 305

atg acc cag gac tgc ctg cag cag tcc cgc aaa gtg ggg gac tcc   ccc   1077
Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser Pro
                310                 315                 320

aat atc acg gag tat atg ttt tgc gct ggc tat agc gat ggc   tcc aag   1125
Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser Lys
            325                 330                 335

gat agc tgc aag ggg gac tcc ggc ggg ccc cat gcc acg   cac tat cgc   1173
Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr Arg
        340                 345                 350

ggg acc tgg tac ctc acc ggg atc gtc agc tgg ggc   cag ggc tgc gcc   1221
Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys Ala
    355                 360                 365

acg gtg ggg cac ttt ggc gtc tac acg cgc gtc agc cag tac att gag     1269
Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile Glu
370                 375                 380                 385

tgg ctg cag aag ctc atg cgg agc gaa ccc cgg ccc ggg gtg ctc   ctg   1317
Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu Leu
                390                 395                 400

cgg gcc cct ttc   cct tga taa                                      1338
Arg Ala Pro Phe Pro
            405


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  1               5                  10                  15

Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys
             20                  25                  30

Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp
         35                  40                  45

Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln
     50                  55                  60

Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn
 65                  70                  75                  80

Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly
                 85                  90                  95

Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys
```

-continued

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            100                 105                 110

Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr
        115                 120                 125

Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg
    130                 135                 140

Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro
145                 150                 155                 160

Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln
                165                 170                 175

Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala
            180                 185                 190

His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu
        195                 200                 205

Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg
    210                 215                 220

Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn
225                 230                 235                 240

His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp
                245                 250                 255

His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr
            260                 265                 270

Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu
        275                 280                 285

Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg
    290                 295                 300

Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser
305                 310                 315                 320

Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser
                325                 330                 335

Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr
            340                 345                 350

Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys
        355                 360                 365

Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile
    370                 375                 380

Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu
385                 390                 395                 400

Leu Arg Ala Pro Phe Pro
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1. A Factor VII (FVII) or Factor VIIa (FVIIa) polypeptide variant having an amino acid sequence comprising 3-15 amino acid modifications relative to human Factor VII (hFVII) or human Factor VIIa (hFVIIa) having the amino acid sequence shown in SEQ ID NO:2, wherein said amino acid sequence of the variant comprises an amino acid substitution in position 10 and 32 and wherein a sugar moiety is covalently attached to an introduced in vivo N-glycosylation site located outside the Gla domain.

2. The variant according to claim 1, wherein said substitution in position 10 is P10Q.

3. The variant according to claim 1, wherein said substitution in position 32 is K32E.

4. The variant according to claim 1, wherein said substitution in position 10 is P10Q and said substitution in position 32 is K32E.

5. The variant according to claim 1, wherein said variant comprises at least one further amino acid modification in the Gla domain.

6.-**9**. (canceled)

10. The variant according to claim 5, wherein said further modification in the Gla domain comprises insertion of at least one amino acid residue between position 3 and 4.

11. The variant according to claim 10, wherein said further modification in the Gla domain comprises insertion of one amino acid residue between position 3 and 4.

12. The variant according to claim 10 or 11, wherein a hydrophobic amino acid residue is inserted between position 3 and 4.

13. The variant according to claim 12, wherein said insertion is A3AY.

14. The variant according to claim 13, wherein said variant comprises the modifications A3AY+P10Q+K32E.

15. (canceled)

16. The variant according to claim 5, wherein said further modification in the Gla domain comprises an amino acid substitution in position 34.

17. The variant according to claim 16, wherein a negatively charged amino acid residue is introduced by substitution in position 34.

18. The variant according to claim 17, wherein said substitution is A34E.

19. The variant according to claim 18, wherein said variant comprises the substitutions P10O+K32E+A34E.

20. (canceled)

21. The variant according to claim 18, wherein said variant comprises the modifications A3AY+P10Q+K32E+A34E.

22.-**24**. (canceled)

25. The variant according to claim 1, wherein no modifications are made in residues 6, 7, 14, 16, 19, 20; 25, 26, 29 and 35.

26. The variant according to claim 1, wherein said in vivo N-glycosylation site is introduced by substitution.

27. The variant according to claim 26, wherein said in vivo N-glycosylation site is introduced into a position comprising an amino acid residue having at least 25% of its side chain exposed to the surface (as defined in Example 1 herein).

28. The variant according to claim 27, wherein said in vivo N-glycosylation site is introduced into a position comprising an amino acid residue having at least 50% of its side chain exposed to the surface (as defined in Example 1 herein).

29. The variant according to claim 26, wherein said in vivo N-glycosylation site is introduced by a substitution selected from the group consisting of A51N, G58N, T106N, K109N, G124N, K143N+N145T, A175T, I205S, I205T, V253N, T267N, T-267N+S269T, S314N+K316S, S314N+K316T, R315N+V317S, R315N+V317T, K316N+G318S, K316N+G318T, G318N, D334N and combinations thereof.

30. The variant according to claim 29, wherein said in vivo N-glycosylation site is introduced by a substitution selected from the group consisting of A51N, G58N, T106N, K109N, G124N, K143N+N145T, A175T, I205T, V253N, T267N+S269T, S314N+K316T, R315N+V317T, K316N+G318T, G318N, D334N and combinations thereof.

31. The variant according to claim 30, wherein said in vivo N-glycosylation site is introduced by a substitution selected from the group consisting of T106N, A175T, I205T, V253N, T267N+S269T and combinations thereof.

32. The variant according to claim 16, wherein one in vivo N-glycosylation site has been introduced by substitution.

33. The variant according to claim 16, wherein two or more in vivo N-glycosylation sites have been introduced by substitution.

34. The variant according to claim 33, wherein two in vivo N-glycosylation sites have been introduced by substitution.

35. The variant according to claim 33, wherein said in vivo N-glycosylation sites have been introduced by substitutions selected from the group consisting of A51N+G58N, A51N+T106N, A51N+K109N, A51N+G124N, A51N+K143N+N145T, A51N+A175T, A51N+I205T, A51N+V253N, A51N+T267N+S269T, A51N+S314N+K316T, A51N+R315N+V317T, A51N+K316N+G318T, A51N+G318N, A51N+D334N, G58N+T106N, G58N+K109N, G58N+G124N, G58N+K143N+N145T, G58N+A175T, G58N+I205T, G58N+V253N, G58N+T267N+S269T, G58N+S314N+K316T, G58N+R315N+V317T, G58N+K316N+G318T, G58N+G318N, G58N+D334N, T106N+K109N, T106N+G124N, T106N+K143N+N145T, T106N+A175T, T106N+I205T, T106N+V253N, T106N+T267N+S269T, T106N+S314N+K316T, T106N+R315N+V317T, T106N+K316N+G318T, T106N+G318N, T106N+D334N,

K109N+G124N, K109N+K143N+N145T, K109N+A175T, K109N+I205T, K109N+V253N, K109N+T267N+S269T, K109N+S314N+K316T, K109N+R315N+V317T, K109N+K316N+G318T, K109N+G318N, K109N+D334N, G124N+K143N+N145T, G124N+A175T, G124N+I205T, G124N+V253N, G124N+T267N+S269T, G124N+S314N+K316T, G124N+R315N+V317T, G124N+K316N+G318T, G124N+G318N, G124N+D334N, K143N+N145T+A175T, K143N+N145T+I205T, K143N+N145T+V253N, K143N+N145T+T267N+S269T, K143N+N145T+S314N+K316T, K143N+N145T+R315N+V317T, K143N+N145T+K 316N+G318T, K143N+N145T+G318N, K143N+N145T+D334N, A175T+I205T, A175T+V253N, A175T+T267N+S269T, A175T+S314N+K316T, A175T+R315N+V317T, A175T+K 316N+G318T, A175T+G318N, A175T+D334N, I205T+V253N, I205T+T267N+S269T, I205T+S314N+K316T, I205T+R31SN+V317T, I205T+K316N+G318T, I205T+G318N, I205T+D334N, V253N+T267N+S269T, V253N+S314N+K316T, V253N+R315N+V317T, V253N+K 316N+G318T, V253N+G318N, V253N+D334N, T267N+S269T+S314N+K316T, T267N+S269T+R315N+V317T, T267N+S269T+K316N+G318T, T267N+S269T+G318N, T267N+S269T+D334N, S314N+K316T+R315N+V317T, S314N+K316T+G318N S314N+K316T+D334N, R315N+V317T+K316N+G318T, R315N+V317T+G318N, R315N+V317T+D334N and G318N+D334N.

36. The variant according to claim 35, wherein said in vivo N-glycosylation sites have been introduced by substitutions selected from the group consisting of T106N+A175T, T106N+I205T, T106N+V253N, T106N+T267N+S269T, A175T+I205T, A175T+V253N, A175T+T267N+S269T, I205T+V253N, I205T+T267N+S269T and V253N+T267N+S269Y.

37. The variant according to claim 36, wherein said in vivo N-glycosylation sites have been introduced by substitutions selected from the group consisting of T106N+I205T, T106N+V253N and I205T+T267N+S269T.

38. (canceled)

39. The variant according to claim 26, wherein three in vivo N-glycosylation sites have been introduced by substitution.

40. (canceled)

41. The variant according to claim 1, wherein said variant is in its activated form.

42. The variant according to claim 1, wherein said variant, in its activated form, has at least 10% of the amidolytic activity of rhFVIIa when assayed in the "Amidolytic Assay" described herein.

43. The variant according to claim 1, wherein said variant, in its activated form, has at least 10% of the clotting activity of rhFVIIa when assayed in the "Clotting Assay" described herein.

44. A nucleotide sequence encoding the variant of claim 1.

45. An expression vector comprising the nucleotide sequence of claim 44.

46. A host cell comprising the nucleotide sequence claim 44.

47. The host cell according to claim 46, wherein said host cell is a gammacarboxylating cell capable of in vivo glycosylation.

48. A pharmaceutical composition comprising the variant of claim 1, and a pharmaceutical acceptable carrier or excipient.

49.-**54**. (canceled)

55. A method for treating a mammal having a disease or a disorder wherein clot formation is desirable, comprising administering to a mammal in need thereof an effective amount of the variant of claim 1.

56. The method according to claim 55, wherein said disease or disorder is selected from the group consisting of hemorrhages, including brain hemorrhages, severe uncontrolled bleedings, such as trauma, bleedings in patients undergoing living transplantations, bleeding in patients undergoing resection and variceal bleedings.

57. The method according to claim 56, wherein said disease or disorder is trauma.

58. The method according to claim 57, wherein said disease or disorder is blunt trauma.

59. The method according to claim 57, wherein said disease or discorder is penetrative trauma.

* * * * *

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(54) FVII OR FVIIA VARIANTS HAVING INCREASED CLOTTING ACTIVITY

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C07K 14/755	(2006.01)

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(57) **ABSTRACT**

The present invention relates to novel Factor VII or VIIa variants comprising a substitution in at least one position selected from the group consisting of L39, 142, S43, K62, L65, F71, E82 and F275. Such variants exhibit increased clotting activity as compared to human wild-type Factor VIIa. The present invention also relates to use of such Factor VII or VIIa variants in therapy, in particular for the treatment of a variety of coagulation-related disorders.

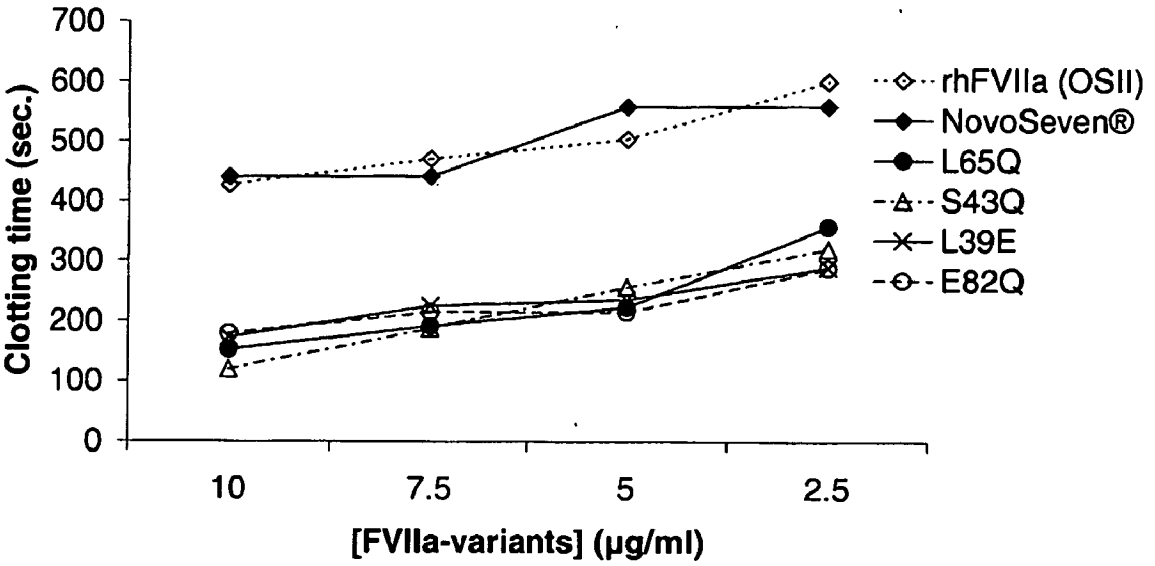

700
600
500
400
300
200
100
0
Clotting time (sec.)
10
7.5
5
2.5
[FVIIa-variants] (µg/ml)
rhFVIIa (OSII)
NovoSeven®
L65Q
S43Q
L39E
E82Q

Fig. 1

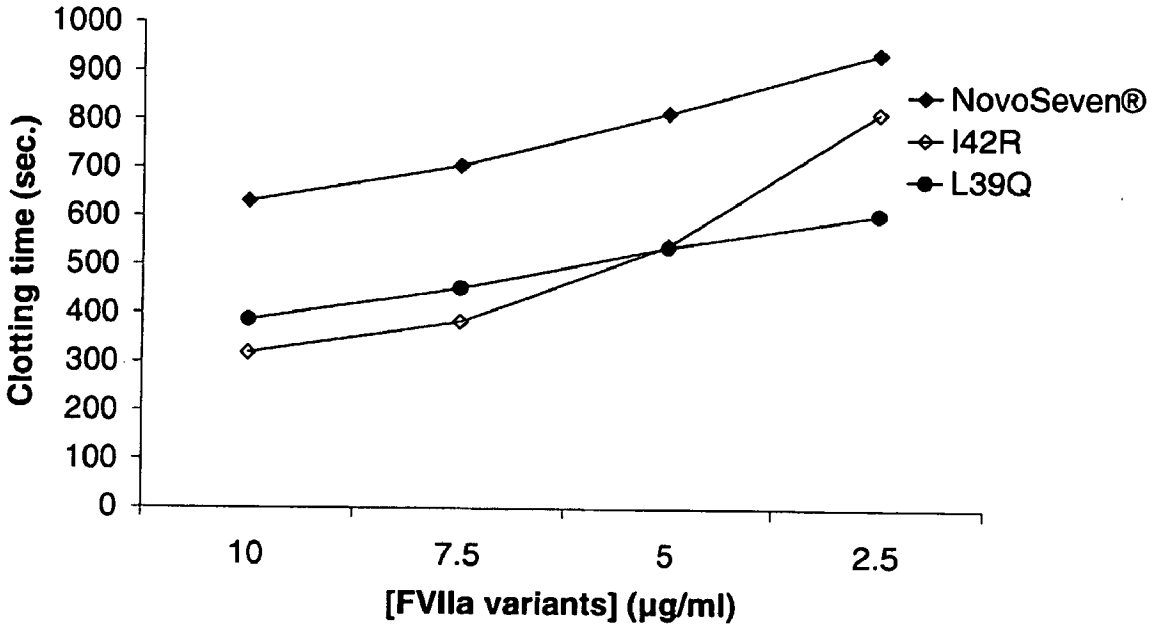

1000
900
800
700
600
500
400
300
200
100
0
Clotting time (sec.)
10
7.5
5
2.5
[FVIIa variants] (µg/ml)
NovoSeven®
I42R
L39Q

Fig. 2

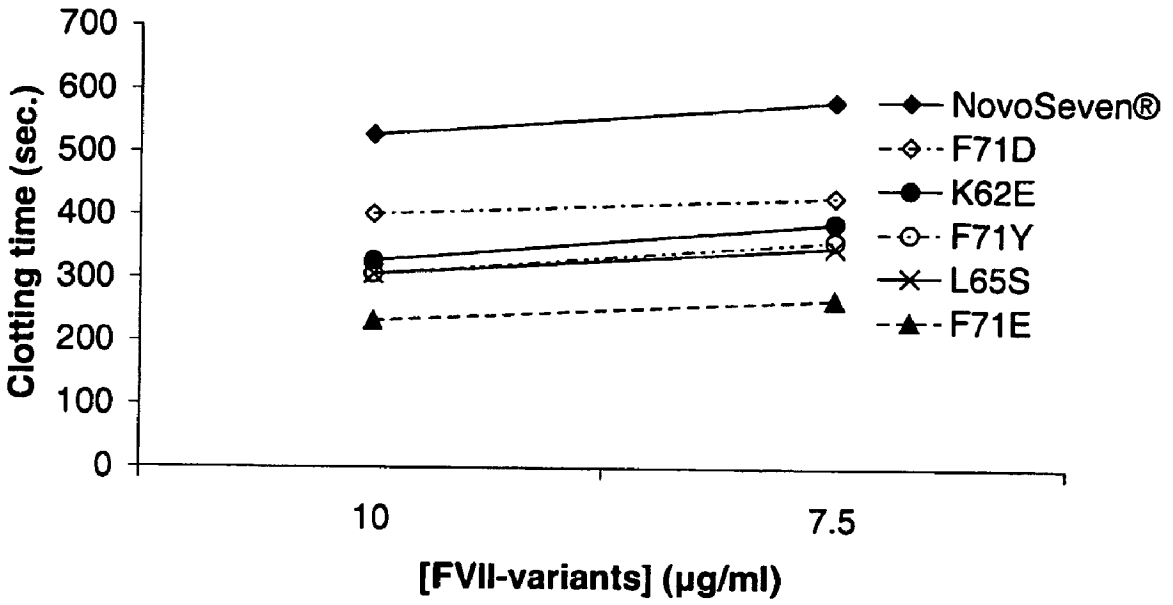

700
600
500
400
300
200
100
0
Clotting time (sec.)
10
7.5
[FVII-variants] (µg/ml)
NovoSeven®
F71D
K62E
F71Y
L65S
F71E

Fig. 3

FVII OR FVIIA VARIANTS HAVING INCREASED CLOTTING ACTIVITY

FIELD OF THE INVENTION

[0001] The present invention relates to novel FVII or FVIIa variants comprising a substitution in a position selected from the group consisting of L39, I42, S43, K62, L65, F71, E82 and F275. Such variants exhibit increased clotting activity. The present invention also relates to use of such polypeptide variants in therapy, in particular for the treatment of a variety of coagulation-related disorders.

BACKGROUND OF THE INVENTION

[0002] Blood coagulation is a process consisting of a complex interaction of various blood components (or factors) that eventually results in a fibrin clot. Generally, the blood components participating in what has been referred to as the "coagulation cascade" are proenzymes or zymogens, i.e. enzymatically inactive proteins that are converted into an active form by the action of an activator. One of these coagulation factors is FVII.

[0003] FVII is a vitamin K-dependent plasma protein synthesized in the liver and secreted into the blood as a single-chain glycoprotein with a molecular weight of 53 kDa (Broze & Majerus, *J Biol Chem* 1980; 255:1242-1247). The FVII zymogen is converted into an activated form (FVIIa) by proteolytic cleavage at a single site, R152-I153, resulting in two chains linked by a single disulfide bridge. FVIIa in complex with tissue factor (TF), the FVIIa complex, is able to convert both factor IX and factor X into their activated forms, followed by reactions leading to rapid thrombin production and fibrin formation (Østerud & Rapaport, *Proc Natl Acad Sci USA* 1977; 74:5260-5264).

[0004] FVII undergoes post-translational modifications, including vitamin K-dependent carboxylation resulting in ten γ-carboxyglutamic acid residues in the N-terminal region of the molecule. Thus, residue number 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35 shown in SEQ ID NO:1 are γ-carboxyglutamic acids residues in the Gla domain important for FVII activity. Other post-translational modifications include sugar moiety attachment at two naturally occurring N-glycosylation sites at position 145 and 322, respectively, and at two naturally occurring O-glycosylation sites at position 52 and 60, respectively.

[0005] The gene coding for human FVII (hFVII) has been mapped to chromosome 13 at q34-qter 9 (de Grouchy et al., *Hum Genet* 1984; 66:230-233). It contains nine exons and spans 12.8 Kb (O'Hara et al., *Proc Natl Acad Sci USA* 1987; 84:5158-5162). The gene organisation and protein structure of FVII are similar to those of other vitamin K-dependent procoagulant proteins, with exons 1a and 1b encoding for signal sequence; exon 2 the propeptide and Gla domain; exon 3 a short hydrophobic region; exons 4 and 5 the epidermal growth factor-like domains; and exon 6 through 8 the serine protease catalytic domain (Yoshitake et al., *Biochemistry* 1985; 24: 3736-3750).

[0006] Reports exist on experimental three-dimensional structures of hFVIIa (Pike et al., *PNAS USA* 1999; 96:8925-30 and Kemball-Cook et al., *J Struct Biol* 1999; 127:213-223), of hFVIIa in complex with soluble tissue factor using X-ray crystallographic methods (Banner et al., *Nature* 1996; 380:41 and Zhang et al., *J Mol Biol* 1999; 285: 2089), and of smaller fragments of hFVII (Muranyi et al., *Biochemistry* 1998; 37:10605 and Kao et al., *Biochemistry* 1999; 38:7097).

[0007] Some protein-engineered variants of FVII have been reported (Dickinson & Ruf, *J Biol Chem* 1997; 272:19875-19879; Kemball-Cook et al., *J Biol Chem* 1998; 273:8516-8521; Bharadwaj et al., *J Biol Chem* 1996; 271:30685-30691; Ruf et al., *Biochemistry* 1999; 38:1957-1966, U.S. Pat. No. 5,560,580; U.S. Pat. No. 5,288,629; WO 01/83725; WO 02/22776; WO 02/077218; WO 03/027147; WO 02/38162; WO 03/037932; WO 99/20767; WO 00/66753 and WO 01/58935).

[0008] Reports exist on expression of FVII in BHK or other mammalian cells (WO 92/15686, WO 91/11514 and WO 88/10295) and co-expression of FVII and kex2 endoprotease in eukaryotic cells (WO 00/28065).

[0009] Commercial preparations of human recombinant FVIIa are sold as NovoSeven®. NovoSeven® is indicated for the treatment of bleeding episodes in hemophilia A or B patients. NovoSeven® is the only rFVIIa for effective and reliable treatment of bleeding episodes available on the market.

[0010] An inactive form of FVII in which arginine 152 and/or isoleucine 153 is/are modified has been reported in WO 91/11514. These amino acids are located at the activation site. WO 96/12800 describes inactivation of FVIIa by a serine proteinase inhibitor. Inactivation by carbamylation of FVIIa at the α-amino acid group I153 has been described by Petersen et al., *Eur J Biochem* 1999; 261:124-129. The inactivated form is capable of competing with wild-type FVII or FVIIa for binding to TF and inhibiting clotting activity. The inactivated form of FVIIa is suggested to be used for treatment of patients being in hypercoagulable states, such as patients with sepsis, in risk of myocardial infarction or of thrombotic stroke.

[0011] WO 98/32466 suggests that FVII, among many other proteins, may be PEGylated but does not contain any further information in this respect.

[0012] WO 01/58935 discloses a new strategy for developing FVII or FVIIa molecules having inter alia an increased half-life.

[0013] A circulating rFVIIa half-life of 2.3 hours was reported in "Summary Basis for Approval for NovoSeven®", FDA reference number 96-0597. Relatively high doses and frequent administration are necessary to reach and sustain the desired therapeutic or prophylactic effect. As a consequence adequate dose regulation is difficult to obtain and the need of frequent intravenous administrations imposes restrictions on the patient's way of living.

[0014] rFVIIa treatment could be rendered more efficient if a FVIIa form could be used which is engineered in such way that its binding to TF is improved. Without being limited to a specific theory, such a FVIIa variant could improve treatment by the following mechanism: A modified FVIIa molecule with increased affinity for TF would enable a more efficient treatment of haemorrhage due to its ability to replace inactive FVII or inactivated FVIIa on TF hereby mediating a stronger amplification of the coagulation pathway and hence speed up the clot formation process and

possibly even mediate formation of a stronger clot. Thus, such an improved FVIIa would be more efficient in stopping uncontrolled bleedings, for example in trauma patients.

[0015] This increased efficiency will be localized to places of tissue damage since this is the only place where cells (endothelial cells) bearing active TF are present. Thus, in addition to increased efficiency, a modified FVIIa with increased affinity for TF will constitute a safer procoagulant treatment due to the localization of the activity to sites of tissue injury, i.e. to the cells that are exposed from the endothelium, i.e. at sites where increased procoagulant activity is desirable.

[0016] Accordingly, the main object of the present invention is to provide FVII/FVIIa variants with an increased clotting efficiency, such as an increased clotting activity (reduced clotting time) and/or an ability to generate stronger clots. The variants may be further engineered to obtain an increased phospholipid membrane binding affinity. Such variants will increase the efficiency of FVIIa even further since such a molecule might target the TF present on platelets through their fusion with the so-called microparticles budded from e.g. TF-producing monocytes. This targeting will co-localize the increase in Factor X generation with the remainder of the clotting cascade i.e. at the site of thrombin and fibrin formation.

[0017] Another problem in current rFVIIa treatment is the relative instability of the molecule with respect to proteolytic degradation. Proteolytic degradation is a major obstacle for obtaining a preparation in solution as opposed to a lyophilized product. The advantage of obtaining a stable soluble preparation lies in easier handling for the patient, and, in the case of emergencies, quicker action, which potentially can become life saving. Attempts to prevent proteolytic degradation by site directed mutagenesis at major proteolytic sites have been disclosed in WO 88/10295. Another attempt to prepare stabilized liquid formulations of FVII/FVIIa is described in WO 03/055512.

[0018] Thus, a further object of the present invention is to provide FVII/FVIIa variants which, in addition to the above-mentioned improved properties, are more stable towards proteolytic degradation, i.e. possess reduced sensitivity to proteolytic degradation.

[0019] A molecule with a longer circulation half-life would decrease the number of necessary administrations. Given the association of current FVIIa product with frequent injections, and the potential for obtaining more optimal therapeutic FVIIa levels with concomitant enhanced therapeutic effect, there is a clear need for FVII or FVIIa molecules with an increased circulating half-life. One way to increase the circulation half-life of a protein is to ensure that renal clearance of the protein is reduced. This may be achieved by conjugating the protein to a chemical moiety which is capable of conferring reduced renal clearance to the protein. Furthermore, attachment of a chemical moiety to the protein or substitution of amino acids exposed to proteolysis may effectively block a proteolytic enzyme from contact leading to proteolytic degradation of the protein. Polyethylene glycol (PEG) is one such chemical moiety that has been used in the preparation of therapeutic protein products.

[0020] Thus, a still further objective of the present invention is to provide FVII/FVIIa variants which, in addition to the above-mentioned improved properties, have an increased functional in vivo half-life and/or an increased serum half-life.

[0021] The above-mentioned objectives are met by the improved FVII/VIIa variants disclosed herein.

BRIEF DISCLOSURE OF THE INVENTION

[0022] The present invention provides improved recombinant FVII or FVIIa variants comprising a substitution in at least one position selected from the group consisting of L39, I42, S43, K62, L65, F71, E82 and F275. These amino acid substitutions in the TF binding site of the FVII molecule result in an improved clotting activity.

[0023] In interesting embodiments, the FVII or FVIIa variant has been further modified so that the resulting variant has an enhanced phospholipid membrane binding affinity, increased functional in vivo half-life and/or increased plasma half-life. In still other embodiments, the variant has been further modified so as to possess increased bioavailability and/or reduced sensitivity to proteolytic degradation. Consequently, medical treatment with such a variant offers a number of advantages over the currently available rFVIIa compound, such as lower dosage, faster action in uncontrolled bleedings and, optionally, longer duration between injections.

[0024] Accordingly, in a first aspect the invention relates to a Factor VII (FVII) or Factor VIIa (FVIIa) polypeptide variant having an amino acid sequence comprising 1-15 amino acid modifications relative to human Factor VII (hFVII) or human Factor Vila (hFVIIa) having the amino acid sequence shown in SEQ ID NO:1, wherein said variant sequence comprises a substitution in at least one position selected from the group consisting of L39, I42, S43, K62, L65, F71, E82 and F275, with the proviso that said variant is not

[0025] [K32E+D33N+A34T+K38T+L39E]hFVII or

[0026] [A1Y+K32E+D33N+A34T+K38T+L39E]hFVII or

[0027] [A1Y+A3S+F4GK+K32E+D33N+A34T+K38T+L39E]hFVII or

[0028] [A1Y+L8F+R9V+P10Q+K32E+D33N+A34T+K38T+L39E]hFVII or

[0029] [A1Y+A3S+F4GK+L8F+R9V+P10Q+K32E+D33N+A34T+K38T+L39E]hFVII or

[0030] [A3S+F4GK+K32E+D33N+A34T+K38T+L39E]hFVII or

[0031] [A3S+F4GK+L8F+R9V+P10Q+K32E+D33N+A34T+K38T+L39E]hFVII or

[0032] [L8F+R9V+P10Q+K32E+D33N+A34T+K38T+L39E]hFVII or

[0033] [I42N]hFVII/hFVIIa or [I42S]hFVII/hFVIIa or [I42A]hFVII/hFVIIa or

[0034] [I42Q]hFVII/hFVIIa.

[0035] In a second aspect the invention relates to a Factor VIIa (FVIIa) polypeptide variant having an amino acid sequence comprising 1-15 amino acid modifications relative to human Factor Vila (hFVIIa) having the amino acid

sequence shown in SEQ ID NO:1, wherein said variant sequence comprises a substitution in at least one position selected from the group consisting of L39, I42, S43, K62, L65, F71, E82 and F275. The FVII variants comprising the L39E mutation disclosed by Cheung and Stafford *Throm Res* 1995; 79; 199-206 are not within the scope of the present application. Likewise, the FVII/FVIIa variants comprising the I42N/S/A/Q mutations disclosed in U.S. Pat. No. 5,580, 560 are not within the scope of the present application.

[0036] Another aspect of the invention relates to a nucleotide sequence encoding the variant of the invention.

[0037] In a further aspect the invention relates to an expression vector comprising the nucleotide sequence of the invention.

[0038] In a still further aspect the invention relates to a host cell comprising the nucleotide sequence of the invention or the expression vector of the invention.

[0039] In an even further aspect the invention relates to a pharmaceutical composition comprising variant of the invention, and a pharmaceutical acceptable carrier or excipient.

[0040] Still another aspect of the invention relates to the variant of the invention, or the pharmaceutical composition of the invention, for use as a medicament.

[0041] Further aspects of the present invention will be apparent from the below description as well as from the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0042] **FIG. 1** shows the clotting time vs. concentration for [L65Q]rhFVIIa, [S43Q]rhFVIIa, [L39E]rhFVIIa and [E82Q]rhFVIIa when assayed in the "Whole Blood Assay". For comparison, the results for rhFVIIa (OSII-activated) and NovoSeven® are included. ◇ rhFVIIa (OSII-activated); ◆ NovoSeven®; ●[L65Q]rhFVIIa; Δ[S43Q]rhFVIIa; x [L39E]rhFVIIa; o [E82Q]rhFVIIa.

[0043] **FIG. 2** shows the clotting time vs. concentration for [I42R]rhFVIIa and [L39Q]rhFVIIa when assayed in the "Whole Blood Assay". For comparison, the result for NovoSeven® is included. ◆ NovoSeven®; ◇[I42R] rhFVIIa; ●[L39Q]rhFVIIa.

[0044] **FIG. 3** shows the clotting time vs. concentration for [F71D]rhFVIIa, [K62E]rhFVIIa, [F71Y]rhFVIIa, [L65S]rhFVIIa and [F71E]rhFVIIa when assayed in the "Whole Blood Assay". For comparison, the result for NovoSeven® is included. ◆ NovoSeven®; ◇[F71D] rhFVIIa; ●[K62E]rhFVIIa; o [F71Y]rhFVIIa; x [L65S] rhFVIIa; ▲[F71E]rhFVIIa.

DETAILED DISCLOSURE OF THE INVENTION

Definitions

[0045] In the context of the present application and invention the following definitions apply:

[0046] The term "conjugate" (or interchangeably "conjugated polypeptide variant") is intended to indicate a heterogeneous (in the sense of composite or chimeric) molecule formed by the covalent attachment of one or more polypeptide(s) to one or more non-polypeptide moieties such as polymer molecules, lipophilic compounds, sugar moieties or organic derivatizing agents. Preferably, the conjugate is soluble at relevant concentrations and conditions, i.e. soluble in physiological fluids such as blood. Examples of conjugated polypeptide variants of the invention include glycosylated and/or PEGylated polypeptides.

[0047] The term "covalent attachment" or "covalently attached" means that the polypeptide variant and the non-polypeptide moiety are either directly covalently joined to one another, or else are indirectly covalently joined to one another through an intervening moiety or moieties, such as a bridge, spacer, or linkage moiety or moieties.

[0048] When used herein, the term "non-polypeptide moiety" means a molecule that is capable of conjugating to an attachment group of the polypeptide variant of the invention. Preferred examples of such molecules include polymer molecules, sugar moieties, lipophilic compounds, or organic derivatizing agents, in particular sugar moieties. When used in the context of a polypeptide variant of the invention it will be understood that the non-polypeptide moiety is linked to the polypeptide part of the polypeptide variant through an attachment group of the polypeptide variant. As explained above, the non-polypeptide moiety may be directly covalently joined to the attachment group or it may be indirectly covalently joined to the attachment group through an intervening moiety or moieties, such as a bridge, spacer, or linkage moiety or moieties.

[0049] A "polymer molecule" is a molecule formed by covalent linkage of two or more monomers, wherein none of the monomers is an amino acid residue. The term "polymer" may be used interchangeably with the term "polymer molecule". The term is also intended to cover carbohydrate molecules attached by in vitro glycosylation, i.e. a synthetic glycosylation performed in vitro normally involving covalently linking a carbohydrate molecule to an attachment group of the polypeptide variant, optionally using a cross-linking agent. In vitro glycosylation is discussed in detail further below.

[0050] The term "sugar moiety" is intended to indicate a carbohydrate-containing molecule comprising one or more monosaccharide residues, capable of being attached to the polypeptide variant (to produce a polypeptide variant conjugate in the form of a glycosylated polypeptide variant) by way of in vivo glycosylation. The term "in vivo glycosylation" is intended to mean any attachment of a sugar moiety occurring in vivo, i.e. during posttranslational processing in a glycosylating cell used for expression of the polypeptide variant, e.g. by way of N-linked or O-linked glycosylation. The exact oligosaccharide structure depends, to a large extent, on the glycosylating organism in question.

[0051] An "N-glycosylation site" has the sequence N-X-S/T/C, wherein X is any amino acid residue except proline, N is asparagine and S/T/C is either serine, threonine or cysteine, preferably serine or threonine, and most preferably threonine. Preferably, the amino acid residue in position +3 relative to the asparagine residue is not a proline residue.

[0052] An "O-glycosylation site" is the OH-group of a serine or threonine residue.

[0053] The term "attachment group" is intended to indicate a functional group of the polypeptide variant, in particular of an amino acid residue thereof or a carbohydrate

moiety, capable of attaching a non-polypeptide moiety such as a polymer molecule, a lipophilic molecule, a sugar moiety or an organic derivatizing agent. Useful attachment groups and their matching non-polypeptide moieties are apparent from the table below.

Attachment group	Amino acid	Examples of non-polypeptide moiety	Conjugation method/-Activated PEG	Reference
—NH_2	N-terminal, Lys	Polymer, e.g. PEG, with amide or imine group	mPEG-SPA Tresylated mPEG	Shearwater Inc. Delgado et al., Critical Reviews in Therapeutic Drug Carrier Systems 9(3, 4): 249–304 (1992)
—COOH	C-terminal, Asp, Glu	Polymer, e.g. PEG, with ester or amide group	mPEG-Hz	Shearwater Inc.
		Carbohydrate moiety	In vitro coupling	
—SH	Cys	Polymer, e.g. PEG, with disulfide, maleimide or vinyl sulfone group	PEG-vinylsulphone PEG-maleimide	Shearwater Inc. Delgado et al, critical reviews in Therapeutic Drug Carrier Systems 9(3, 4): 249–304 (1992)
		Carbohydrate moiety	In vitro coupling	
—OH	Ser, Thr, Lys, OH—	Sugar moiety	In vivo O-linked glycosylation	
		PEG with ester, ether, carbamate, carbonate		
—$CONH_2$	Asn as part of an N-glycosylation site	Sugar moiety	In vivo N-glycosylation	
		Polymer, e.g. PEG		
Aromatic residue	Phe, Tyr, Trp	Carbohydrate moiety	In vitro coupling	
—$CONH_2$	Gln	Carbohydrate moiety	In vitro coupling	Yan and Wold, Biochemistry, 1984, Jul 31; 23(16): 3759–65
Aldehyde Ketone	Oxidized oligo-saccharide	Polymer, e.g. PEG, PEG-hydrazide	PEGylation	Andresz et al., 1978, Makromol. Chem. 179: 301, WO 92/16555, WO 00/23114
Guanidino	Arg	Carbohydrate moiety	In vitro coupling	Lundblad and Noyes, Chimical Reagents for Protein Modification, CRC Press Inc., Florida, USA
Imidazole ring	His	Carbohydrate moiety	In vitro coupling	As for guanidine

[0054] For in vivo N-glycosylation, the term "attachment group" is used in an unconventional way to indicate the amino acid residues constituting a N-glycosylation site (with the sequence N-X-S/T/C, wherein X is any amino acid residue except proline, N is asparagine and S/T/C is either serine, threonine or cysteine, preferably serine or threonine, and most preferably threonine). Although the asparagine residue of the N-glycosylation site is the one to which the sugar moiety is attached during glycosylation, such attachment cannot be achieved unless the other amino acid residues of the N-glycosylation site are present.

[0055] Accordingly, when the non-polypeptide moiety is a sugar moiety and the conjugation is to be achieved by in vivo N-glycosylation, the term "amino acid residue comprising an attachment group for the non-polypeptide moiety" as used in connection with alterations of the amino acid sequence of the polypeptide variant is to be understood as meaning that one or more amino acid residues constituting an in vivo N-glycosylation site are to be altered in such a manner that either a functional in vivo N-glycosylation site is introduced into the amino acid sequence or removed from said sequence.

[0056] In the present application, amino acid names and atom names (e.g. CA, CB, CD, CG, SG, NZ, N, O, C, etc) are used as defined by the Protein DataBank (PDB) (www.pdb.org) based on the IUPAC nomenclature (IUPAC Nomenclature and Symbolism for Amino Acids and Peptides (residue names, atom names, etc.), *Eur. J. Biochem.*, 138, 9-37 (1984) together with their corrections in *Eur. J. Biochem.*, 152, 1 (1985)).

[0057] The term "amino acid residue" is intended to indicate an amino acid residue contained in the group consisting of alanine (Ala or A), cysteine (Cys or C), aspartic

acid (Asp or D), glutamic acid (Glu or E), phenylalanine (Phe or F), glycine (Gly or G), histidine (His or H), isoleucine (Ile or I), lysine (Lys or K), leucine (Leu or L), methionine (Met or M), asparagine (Asn or N), proline (Pro or P), glutamine (Gln or Q), arginine (Arg or R), serine (Ser or S), threonine (Thr or T), valine (Val or V), tryptophan (Trp or W), and tyrosine (Tyr or Y) residues.

[0058] The terminology used for identifying amino acid positions is illustrated as follows: G124 indicates that position 124 is occupied by a glycine residue in the amino acid sequence shown in SEQ ID NO:1. G124R indicates that the glycine residue of position 124 has been substituted with an arginine residue. Alternative substitutions are indicated with a "/", e.g. N145S/T means an amino acid sequence in which asparagine in position 145 is substituted with either serine or threonine. Multiple substitutions are indicated with a "+", e.g. K143N+N145S/T means an amino acid sequence which comprises a substitution of the lysine residue in position 143 with an asparagine residue and a substitution of the asparagine residue in position 145 with a serine or a threonine residue. Insertion of an additional amino acid residue, such as insertion of an alanine residue after G124 is indicated by G124GA. Insertion of two additional alanine residues after G124 is indicated by G124GAA, etc. When used herein, the term "inserted in position X" or "inserted at position X" means that the amino acid residue(s) is (are) inserted between amino acid residue X and X+1. A deletion of an amino acid residue is indicated by an asterix. For example, deletion of the glycine residue in position 124 is indicated by G124*.

[0059] Unless otherwise indicated, the numbering of amino acid residues made herein is made relative to the amino acid sequence of human wild-type FVII/FVIIa (SEQ ID NO:1).

[0060] The term "differs from" as used in connection with specific mutations is intended to allow for additional differences being present apart from the specified amino acid difference. For instance, in addition to the specified substitutions in positions 39, 42, 43, 62, 65, 71, 82 and/or 275, the FVII or FVIIa polypeptide variant may comprise other substitutions. Examples of such additional modifications or differences may include truncation of the N- and/or C-terminus by one or more amino acid residues (e.g. by 1-10 amino acid residues), or addition of one or more extra residues at the N- and/or C-terminus, e.g. addition of a methionine residue at the N-terminus as well as "conservative amino acid substitutions", i.e. substitutions performed within groups of amino acids with similar characteristics, e.g. small amino acids, acidic amino acids, polar amino acids, basic amino acids, hydrophobic amino acids and aromatic amino acids.

[0061] Examples of such conservative substitutions are shown in the below table.

1	Alanine (A)	Glycine (G)	Serine (S)	Threonine (T)
2	Aspartic acid (D)	Glutamic acid (E)		
3	Asparagine (N)	Glutamine (Q)		
4	Arginine (R)	Histidine (H)	Lysine (K)	
5	Isoleucine (I)	Leucine (L)	Methionine (M)	Valine (V)
6	Phenylalanine (F)	Tyrosine (Y)	Tryptophan (W)	

[0062] Still other examples of additional modifications include modifications giving rise to an increased functional in vivo half-life or an increased serum half-life. Specific examples of such modifications are discussed further below. Moreover, the polypeptide variant of the invention may contain additional modifications giving rise to an enhanced phospholipid membrane binding affinity. Specific examples are given below.

[0063] The term "nucleotide sequence" is intended to indicate a consecutive stretch of two or more nucleotide molecules. The nucleotide sequence may be of genomic, cDNA, RNA, semisynthetic, synthetic origin, or any combinations thereof.

[0064] The term "polymerase chain reaction" or "PCR" generally refers to a method for amplification of a desired nucleotide sequence in vitro, as described, for example, in U.S. Pat. No. 4,683,195. In general, the PCR method involves repeated cycles of primer extension synthesis, using oligonucleotide primers capable of hybridising preferentially to a template nucleic acid.

[0065] The term "vector" refers to a plasmid or other nucleotide sequences that are capable of replicating within a host cell or being integrated into the host cell genome, and as such, are useful for performing different functions in conjunction with compatible host cells (a vector-host system): to facilitate the cloning of the nucleotide sequence, i.e. to produce usable quantities of the sequence, to direct the expression of the gene product encoded by the sequence and to integrate the nucleotide sequence into the genome of the host cell. The vector will contain different components depending upon the function it is to perform.

[0066] "Cell", "host cell", "cell line" and "cell culture" are used interchangeably herein and all such terms should be understood to include progeny resulting from growth or culturing of a cell.

[0067] "Transformation" and "transfection" are used interchangeably to refer to the process of introducing DNA into a cell.

[0068] "Operably linked" refers to the covalent joining of two or more nucleotide sequences, by means of enzymatic ligation or otherwise, in a configuration relative to one another such that is the normal function of the sequences can be performed. For example, the nucleotide sequence encoding a presequence or secretory leader is operably linked to a nucleotide sequence coding for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide: a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the nucleotide sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. Linking is accomplished by ligation at convenient restriction

sites. If such sites do not exist, then synthetic oligonucleotide adaptors or linkers are used, in conjunction with standard recombinant DNA methods.

[0069] In the context of the present invention the terms "modification" or "amino acid modification" is intended to cover replacement of an amino acid side chain, substitution of an amino acid residue, deletion of an amino acid residue and/or insertion of an amino acid residue of interest.

[0070] The terms "mutation" and "substitution" are used interchangeably herein.

[0071] The term "introduce" refers to introduction of an amino acid residue by substitution of an existing amino acid residue, or alternatively by insertion of an additional amino acid residue.

[0072] The term "remove" refers to removal of an amino acid residue by substitution of the amino acid residue to be removed by another amino acid residue, or alternatively by deletion (without substitution) of the amino acid residue to be removed.

[0073] The term "FVII" or "FVII polypeptide" refers to a FVII molecule provided in single chain form. One example of a FVII polypeptide is the wild-type human FVII (hFVII) having the amino acid sequence shown in SEQ ID NO:1. It should be understood, however, that the term "FVII polypeptide" also covers hFVII-like molecules, such as fragments or variants of SEQ ID NO:1, in particular variants where the sequence comprises at least one, such as 1-15, preferably 1-10, amino acid modifications as compared to SEQ ID NO:1

[0074] The term "FVIIa" or "FVIIa polypeptide" refers to a FVIIa molecule provided in its activated two-chain form. When the amino acid sequence of SEQ ID NO:1 is used to describe the amino acid sequence of FVIIa it will be understood that the peptide bond between R152 and I153 of the single-chain form has been cleaved, and that one of the chains comprises amino acid residues 1-152, the other chain amino acid residues 153-406.

[0075] The terms "rFVII" and "rFVIIa" refer to FVII and FVIIa molecules produced by recombinant techniques, respectively.

[0076] The terms "hFVII" and "hFVIIa" refer to wild-type human FVII and FVIIa, respectively, having the amino acid sequence shown in SEQ ID NO:1.

[0077] The terms "rhFVII" and "rhFVIIa" refer to human wild-type FVII and FVIIa, having the amino acid sequence shown in SEQ ID NO:1, produced by recombinant means. An example of rhFVIIa is NovoSeven®.

[0078] When used herein, the term "Gla domain" is intended to cover amino acid residues no. 1 to 45 of SEQ ID NO:1. Accordingly, the term "position located outside the Gla domain" covers amino acid residue no. 46-406 of SEQ ID NO:1.

[0079] The abbreviations "FX", "TF" and "TFPI" mean Factor X, Tissue Factor and Tissue Factor Pathway Inhibitor, respectively.

[0080] The term "protease domain" is used about residues 153-406 counted from the N-terminus.

[0081] The term "catalytic site" is used to mean the catalytic triad consisting of S344, D242 and H193 of the FVII/FVIIa molecule.

[0082] The term "parent" is intended to indicate the molecule to be modified/improved in accordance with the present invention. Although the parent polypeptide to be modified by the present invention may be any FVII or FVIIa polypeptide, and thus be derived from any origin, e.g. a non-human mammalian origin, it is preferred that the parent polypeptide is hFVII or hFVIIa.

[0083] A "variant" is a polypeptide, which differs in one or more amino acid residues from its parent polypeptide, normally in 1-15 amino acid residues (e.g. in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acid residues), such as in 1-10 amino acid residues, e.g. in 1-8, 1-6, 1-5 or 1-3 amino acid residues. Normally, the parent polypeptide is hFVII or hFVIIa. Thus, a "variant" typically contains 1-15 amino acid modifications (for example 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acid modifications), such as 1-10 amino acid modifications, e.g. 1-8, 1-6, 1-5 or 1-3 amino acid modifications relative to the parent polypeptide. As explained above, the parent polypeptide is normally hFVII or hFVIIa. It will be understood that a polypeptide variant according to the present invention will differ from the SEQ ID NO:1 in at least one of the following positions: L39, I42, S43, K62, L65, F71, E82 and/or F275.

[0084] In the present context, the term "modification" encompasses insertions, deletions, substitutions and combinations thereof. It will be understood that a polypeptide variant according to the present invention will be modified in at least one position relative to the parent polypeptide.

[0085] The term "amidolytic activity" is intended to mean the activity measured in the "Amidolytic Assay" described herein. In order to exhibit "amidolytic activity" a variant of the invention, in its activated form, should have at least 10% of the amidolytic acitivty of rhFVIIa when assayed in the "Amidolytic Assay" described herein. In a preferred embodiment of the invention the variant, in its activated form, has at least 20% of the amidolytic activity of rhFVIIa, such as at least 30%, e.g. at least 40%, more preferably at least 50%, such as at least 60%, e.g. at least 70%, even more preferably at least 80%, such as at least 90% of the amidolytic activity of rhFVIIa when assayed in the "Amidolytic Assay" described herein. In an interesting embodiment the variant, in its activated form, has substantially the same amidolytic activity as rhFVIIa, such as an amidolytic activity of 75-125% of the amidolytic acitivity of rhFVIIa.

[0086] The term "clotting activity" is used to mean the activity measured in the "Whole Blood Assay" described herein. It will be understood that the activity measured in the "Whole Blood Assay" is the time needed to obtain clot formation. Thus, a lower clotting time corresponds to a higher clotting activity. The term "increased clotting activity" is used to indicate that the clotting time of the polypeptide variant is statistically significantly decreased relative to that generated by rhFVIIa as determined under comparable conditions and when measured in the "Whole Blood Assay" described herein.

[0087] The term "immunogenicity" as used in connection with a given substance is intended to indicate the ability of the substance to induce a response from the immune system.

The immune response may be a cell or antibody mediated response (see, e.g., Roitt: Essential Immunology (8th Edition, Blackwell) for further definition of immunogenicity). Normally, reduced antibody reactivity will be an indication of reduced immunogenicity. The reduced immunogenicity may be determined by use of any suitable method known in the art, e.g. in vivo or in vitro.

[0088] The term "functional in vivo half-life" is used in its normal meaning, i.e. the time at which 50% of the biological activity of the polypeptide variant is still present in the body/target organ, or the time at which the amidolytic or clotting activity of the polypeptide variant is 50% of the initial value.

[0089] As an alternative to determining functional in vivo half-life, "serum half-life" may be determined, i.e. the time at which 50% of the polypeptide variant circulates in the plasma or bloodstream prior to being cleared. Determination of serum half-life is often more simple than determining the functional in vivo half-life and the magnitude of serum half-life is usually a good indication of the magnitude of functional in vivo half-life. Alternatively terms to serum half-life include "plasma half-life", "circulating half-life", "serum clearance", "plasma clearance" and "clearance half-life". The polypeptide variant is cleared by the action of one or more of the reticuloendothelial systems (RES), kidney, spleen or liver, by tissue factor, SEC receptor or other receptor mediated elimination, or by specific or unspecific proteolysis. Normally, clearance depends on size (relative to the cutoff for glomerular filtration), charge, attached carbohydrate chains, and the presence of cellular receptors for the protein. The functionality to be retained is normally selected from procoagulant, proteolytic or receptor binding activity. The functional in vivo half-life and the serum half-life may be determined by any suitable method known in the art.

[0090] The term "increased" as used about the functional in vivo half-life or serum half-life is used to indicate that the relevant half-life of the polypeptide variant is statistically significantly increased relative to that of a reference molecule, such as a hFVIIa or rhFVIIa (e.g. NovoSeven®) as determined under comparable conditions (typically determined in an experimental animal, such as rats, rabbits or pigs).

[0091] The term "AUC_{iv}" or "Area Under the Curve when administered intravenously" is used in its normal meaning, i.e. as the area under the activity in serum-time curve, where the polypeptide variant has been administered intravenously, in particular when administered intravenously in rats. Typically, the activity measured is the "clotting activity" as defined hereinbefore. Once the experimental activity-time points have been determined, the AUC_{iv} may conveniently be calculated by a computer program, such as GraphPad Prism 3.01.

[0092] It will be understood that in order to make a direct comparison between the AUC_{iv}-values of different molecules (e.g. between the variants of the invention and rhFVIIa the same amount of activity should be administered. Consequently, the AUC_{iv}-values are typically normalized (i.e. corrected for differences in the injected dose) and expressed as AUC_{iv}/dose administered.

[0093] The term "reduced sensitivity to proteolytic degradation" is primarily intended to mean that the polypeptide variant has reduced sensitivity to proteolytic degradation in comparison to hFVIIa or rhFVIIa (e.g. NovoSeven®) as determined under comparable conditions. Preferably, the proteolytic degradation is reduced by at least 10% (e.g. by 10-25% or by 10-50%), such as at least 25% (e.g. by 25-50%, by 25-75% or by 25-100%), more preferably by at least 35%, such as at least 50%, (e.g. by 50-75% or by 50-100%) even more preferably by at least 60%, such as by at least 75% (e.g. by 75-100%) or even at least 90%. Most preferably, the proteolytic degradation is reduced by at least 99%.

[0094] The term "renal clearance" is used in its normal meaning to indicate any clearance taking place by the kidneys, e.g. by glomerular filtration, tubular excretion or degradation in the tubular cells. Renal clearance depends on physical characteristics of the polypeptide, including size (diameter), hydrodynamic volume, symmetry, shape/rigidity, and charge. Normally, a molecular weight of about 67 kDa is considered to be a cut-off-value for renal clearance. Renal clearance may be established by any suitable assay, e.g. an established in vivo assay. Typically, renal clearance is determined by administering a labelled (e.g. radiolabelled or fluorescence labelled) polypeptide to a patient and measuring the label activity in urine collected from the patient. Reduced renal clearance is determined relative to a corresponding reference polypeptide, e.g. human wild-type FVIIa, under comparable conditions. Preferably, the renal clearance rate of the polypeptide variant is reduced by at least 50%, preferably by at least 75%, and most preferably by at least 90% compared to hFVIIa or rhFVIIa (e.g. NovoSeven®).

[0095] The terms "at least 25% of its side chain exposed to the surface of the molecule" and "at least 50% of its side chain exposed to the surface of the molecule" are defined with reference to Example 1, where the calculations, etc. are described in detail.

[0096] It should be noted that when the terms "at least 25% of its side chain exposed to the surface of the molecule" and "at least 50% of its side chain exposed to the surface of the molecule" are used in connection with introduction of an in vivo N-glycosylation site these terms refer to the surface accessibility of the amino acid side chain in the position where the sugar moiety is actually attached. In many cases it will be necessary to introduce a serine or a threonine residue in position +2 relative to the asparagine residue to which the sugar moiety is actually attached (unless, of course, this position is already occupied by a serine or a threonine residue) and these positions, where the serine or threonine residues are introduced, are allowed to be buried, i.e. to have less than 25% or 50% of their side chains exposed to the surface of the molecule.

[0097] In the present description and claims, any reference to "a" component, e.g. in the context of a non-polypeptide moiety, an amino acid residue, a substitution, a buffer, etc., is intended to refer to one or more of such components, unless stated otherwise or unless it is clear from the particular context that this is not the case. For example, the expression "a component selected from the group consisting of A, B and C" is intended to include all combinations of A, B and C, i.e. A, B, C, A+B, A+C, B+C or A+B+C.

[0098] A polypeptide, nucleotide sequence or other component is "isolated" when it is partially or completely

separated from components with which it is normally associated (other peptides, polypeptides, proteins (including complexes, e.g., polymerases and ribosomes which may accompany a native sequence), nucleic acids, cells, synthetic reagents, cellular contaminants, cellular components, etc.), e.g., such as from other components with which it is normally associated in the cell from which it was originally derived. A polypeptide, nucleotide sequence, or other component is isolated when it is partially or completely recovered or separated from other components of its natural environment such that it is the predominant species present in a composition, mixture, or collection of components (i.e., on a molar basis it is more abundant than any other individual species in the composition). In some instances, the preparation consists of more than about 60%, more than about 70% or more than about 75%, typically more than about 80%, or preferably more than about 90% of the isolated species.

[0099] The terms "tissue factor binding site", "active site region" and "ridge of the active site binding cleft" are defined with reference to Example 1, wherein the above-mentioned sites/regions are determined.

[0100] The term "hydrophobic amino acid residue" includes the following amino acid residues: Ile, Leu, Met, Val, Phe, Tyr and Trp.

[0101] The term "charged amino acid residue" encompasses the following amino acid residues: Lys, Arg, His, Asp and Glu.

[0102] The term "negatively charged amino acid residue" includes the following amino acid residues: Asp and Glu.

[0103] The term "positively charged amino acid residue" includes the following amino acid residues: Lys, Arg and His.

[0104] The term "polar amino acid residue" encompasses the following amino acid residues: Gly, Ser, Thr, Cys, Tyr, Asn and Gin.

[0105] The term "mammal" as used herein includes humans, non-human primates (e.g., baboons, orangutans, monkeys), mice, pigs, cows, goats, cats, rabbits, rats, guinea pigs, hamsters, horses, monkeys, sheep, or other non-human mammal.

[0106] The term "effective amount" means a dosage or amount sufficient to produce a desired result. The desired result may comprise an objective or subjective improvement in the recipient of the dosage or amount.

Variants of the Invention

[0107] In its broadest aspect the present invention relates to a Factor VII (FVII) or Factor VIIa (FVIIa) polypeptide variant having an amino acid sequence comprising 1-15 amino acid modifications relative to human Factor VII (hFVII) or human Factor Vila (hFVIIa) having the amino acid sequence shown in SEQ ID NO:1, wherein said variant sequence comprises a substitution in at least one position selected from the group consisting of L39, I42, S43, K62, L65, F71, E82 and F275,

[0108] with the proviso that said variant is not

[0109] [K32E+D33N+A34T+K38T+L39E]hFVII/hFVIIa or

[0110] [A1Y+K32E+D33N+A34T+K38T+L39E]hFVII/hFVIIa or

[0111] [A1Y+A3S+F4GK+K32E+D33N+A34T+K38T+L39E]hFVII/hFVIIa or

[0112] [A1Y+L8F+R9V+P10Q+K32E+D33N+A34T+K38T+L39E]hFVII/hFVIIa or

[0113] [A1Y+A3S+F4GK+L8F+R9V+P10Q+K32E+D33N+A34T+K38T+L39E]hFVII/hFVIIa or

[0114] [A3S+F4GK+K32E+D33N+A34T+K38T+L39E]hFVII/hFVIIa or

[0115] [A3S+F4GK+L8F+R9V+P10Q+K32E+D33N+A34T+K38T+L39E]hFVII/hFVIIa or

[0116] [L8F+R9V+P 10Q+K32E+D33N+A34T+K38T+L39E]hFVII/hFVIIa or

[0117] [I42N]hFVII/hFVIIa or [I42S]hFVII/hFVIIa or [I42A]hFVII hFVIIa or [I42Q]hFVII/hFVIIa.

[0118] In a further interesting embodiment of the invention, the FVII or FVIIa variant comprises the substitution L39E.

[0119] In a further interesting embodiment of the invention, the FVII or FVIIa variant comprises the substitution L39Q.

[0120] In a further interesting embodiment of the invention, the FVII or FVIIa variant comprises the substitution L39H.

[0121] In a further interesting embodiment of the invention, the FVII or FVIIa variant comprises the substitution I42R.

[0122] In a further interesting embodiment of the invention, the FVII or FVIIa variant comprises the substitution S43Q.

[0123] In a further interesting embodiment of the invention, the FVII or FVIIa variant comprises the substitution K62E.

[0124] In a further interesting embodiment of the invention, the FVII or FVIIa variant comprises the substitution K62R.

[0125] In a further interesting embodiment of the invention, the FVII or FVIIa variant comprises the substitution L65Q.

[0126] In a further interesting embodiment of the invention, the FVII or FVIIa variant comprises the substitution L65S.

[0127] In a further interesting embodiment of the invention, the FVII or FVIIa variant comprises the substitution F71D.

[0128] In a further interesting embodiment of the invention, the FVII or FVIIa variant comprises the substitution F71Y.

[0129] In a further interesting embodiment of the invention, the FVII or FVIIa variant comprises the substitution F71E.

[0130] In a further interesting embodiment of the invention, the FVII or FVIIa variant comprises the substitution F71Q.

[0131] In a further interesting embodiment of the invention, the FVII or FVIIa variant comprises the substitution F71N.

[0132] In a further interesting embodiment of the invention, the FVII or FVIIa variant comprises the substitution E82Q.

[0133] In a further interesting embodiment of the invention, the FVII or FVIIa variant comprises the substitution E82N.

[0134] In a further interesting embodiment of the invention, the FVII or FVIIa variant comprises the substitution F275H.

[0135] In a highly interesting embodiment of the invention, the FVII or FVIIa variant of the invention comprises a substitution selected from the group consisting of L65Q, F71Y, K62E and S43Q, in particular selected from the group consisting of L65Q, K62E and S43Q.

[0136] It will be understood that it may be advantageous to combine one or more of the above-mentioned substitutions. Accordingly, in a further interesting embodiment of the invention, the variant comprises at least two (such as two) substitutions in positions selected from the group consisting of L39, I42, S43, K62, L65, F71, E82 and F275, such as substitutions in positions selected from the group consisting of L39+I42, L39+S43, L39+K62, L39+L65, L39+F71, L39+E82, L39+F275, I42+S43, I42+K62, I42+L65, I42+F71, I42+F71, I42+E82, I42+F275, S43+K62, S43+L65, S43+F71, S43+E82, S43+F275, K62+L65, K62+F71, K62+E82, K62+F275, L65+F71, L65+E82, L65+F275, F71+E82, F71+F275 and E82+F275. According to this embodiment of the invention, it is preferred that the variant comprises at least two (such as two) substitutions in positions selected from the group consisting of L65+F71, L65+K62, L65+S43, F71+K62, F71+S43 and K62+S43, in particular at least two (such as two) substitutions in positions selected from the group consisting of L65+K62, L65+S43 and K62+S43. More particularly, the variant of the invention may comprise at least two (such as two) substitutions selected from the group consisting of L39E, L39Q, L39H, I42R, S43Q, K62E, K62R, L65Q, L65S, F71D, F71Y, F71E, F71Q, F71N, E82Q, E82N and F275H, preferably at least two (such as two) substitutions selected from the group consisting of L65Q, F71Y, K62E and S43Q, more preferably at least two (such as two) substitutions selected from the group consisting of L65Q, K62E and S43Q. Specific examples include L65Q+F71Y, L65Q+K62E, L65Q+S43Q, F71Y+K62E, F71Y+S43Q and K62E+S43Q, in particular L65Q+K62E, L65Q+S43Q and K62E+S43Q.

[0137] In a still further interesting embodiment of the invention, the variant comprises at least three (such as three) substitutions in positions selected from the group consisting of L39, I42, S43, K62, L65, F71, E82 and F275, such as substitutions in positions selected from the group consisting of L39+I42+S43, L39+I42+K62, L39+I42+L65, L39+I42+F71, L39+I42+E82, L39+I42+F275, L39+S43+K62, L39+S43+L65, L39+S43+F71, L39+K62+E82, L39+S43+F275, L39+K62+L65, L39+K62+F71, L39+K62+E82, L39+K62+F275, L39+L65+F71, L39+L65+E82, L39+L65+F275, L39+F71+E82, L39+F71+F275, L39+E82+F275, I42+S43+K62, I42+S43+L65, I42+S43+F71, I42+S43+E82, I42+S43+F275, 142+K62+L65, I42+K62+F71, I42+K62+E82, I42+K62+F275, I42+L65+F71, I42+L65+E82, I42+L65+F275, I42+F71+E82, I42+F71+F275, I42+E82+F275, S43+K62+L65, S43+K62+F71, S43+K62+E82, S43+K62+F275, S43+L65+F71, S43+L65+E82, S43+L65+F275, S43+F71+E82, S43+F71+F275, S43+E82+F275, K62+L65+F71, K62+L65+E82, K62+L65+F275, K62+F71+E82, K62+F71F275, K62+E82+F275, L65+F71+E82, L65+F71+F275, L65+E82+F275 and F71+E82+F275, preferably substitutions in positions selected from the group consisting of K62+L65+F71, S43+L65+F71, S43+K62+L65 and S43+K62+F71, in particular S43+K62+L65.

[0138] More particularly, the variant of the invention may comprise at least three (such as three) substitutions selected from the group consisting of L65Q, F71Y, K62E and S43Q. Specific examples include L65Q+F71Y+K62E, L65Q+F71Y+S43Q, L65Q+K62E+S43Q and F71Y+K62E+S43Q, in particular L65Q+K62E+S43Q.

[0139] The variants of the invention possess an increased clotting activity (or a reduced clotting time) as compared to hFVIIa or rhFVIIa. In a preferred embodiment of the invention the ratio between the time to reach clot formation for the variant ($t_{variant}$) and the time to reach clot formation for hFVIIa or rhFVIIa (t_{wt}) is at the most 0.9 when assayed in the "Whole Blood Assay" described herein. More preferably the ratio ($t_{variant}/t_{wt}$) is at the most 0.75, such as 0.7, even more preferably the ratio ($t_{variant}/t_{wt}$) is at the most 0.6, most preferably the ratio ($t_{variant}/t_{wt}$) is at the most 0.5 when assayed in the "Whole Blood Assay" described herein.

[0140] In a further interesting embodiment of the invention, the variant comprises 1-10 amino acid modifications (e.g. substitutions), such as 1-5 amino acid modifications (e.g. substitutions), e.g. 1-3 amino acid modifications (e.g. substitutions) relative to SEQ ID NO:1.

[0141] For example, the variant may contain at least one amino acid modification made in the Gla domain as explained in the section entitled "Modifications in the Gla domain" below, and/or at least one amino acid modification which leads to introduction of an N-glycosylation site as explained in the section entitled "Introduction of additional sugar moieties" below, and/or at least one amino acid modification which decreases the TFPI-binding affinity. Examples of the latter modifications are described in the section entitled "Other modifications" below.

Further Modifications

[0142] As indicated above the FVII or FVIIa variant of the invention may comprise further modifications, in particular further modifications which confer additional advantageous properties to the FVII or FVIIa molecule. Thus, in addition to one or more of the substitutions mentioned above, i.e. a substitution in one or more of the positions L39, I42, S43, K62, L65, F71, E82, F275H, the variant may comprise at least one further amino acid modification, in particular at least one further amino acid substitution.

[0143] In order to avoid too much disruption of the structure and function of the FVII or FVIIa polypeptide, the FVII or FVIIa polypeptide variant of the invention typically comprises an amino acid sequence having at least 95% identity with SEQ ID NO:1, such as at least 96% identity

with SEQ ID NO:1, e.g. at least 97% identity with SEQ ID NO:1, at least 98% identity with SEQ ID NO:1, or at least 99% identity with SEQ ID NO:1. Amino acid sequence identity is conveniently determined from aligned sequences, using e.g. the ClustalW program, version 1.8, June 1999, using default parameters (Thompson et al., 1994, ClustalW: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, Nucleic Acids Research, 22: 4673-4680) or from the PFAM families database version 4.0 (http://pfam.wustl.edu/) (*Nucleic Acids Res.* 1999 Jan. 1; 27(1):260-2) by use of GENEDOC version 2.5 (Nicholas, K. B., Nicholas H. B. Jr., and Deerfield, D. W. II. 1997 GeneDoc: Analysis and Visualization of Genetic Variation, EMBNEW.NEWS 4:14; Nicholas, K. B. and Nicholas H. B. Jr. 1997 GeneDoc: Analysis and Visualization of Genetic Variation).

Modifications in the Gla Domain

[0144] In an interesting embodiment of the invention, the variant further comprises at least one amino acid modification (such as at least one amino acid substitution and/or insertion) in the Gla domain. Preferably, no modifications are made in position 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35.

[0145] Without being limited by any particular theory, it is presently believed that an increased clotting activity may be achieved by an enhanced binding affinity of the FVIIa molecule to the phospholipid membranes present on the surface of activated platelets. This enhanced affinity is believed to result in a higher local concentration of the activated FVIIa polypeptide in close proximity to the other coagulation factors, particularly FX. Thus, the rate of activation of FX to FXa will be higher, simply due to a higher molar ratio of the activated FVII polypeptide to FX. The increased activation rate of FX then results in a higher amount of active thrombin, and thus a higher rate of cross-linking of fibrin.

[0146] Thus, in a preferred embodiment according to this aspect of the invention, the polypeptide variant has, in its activated form, an enhanced phospholipid membrane binding affinity relative to the rhFVIIa polypeptide. Phospholipid membrane binding affinity may be measured by methods known in the art, such as by the assays described in Nelsestuen et al., *Biochemistry* 1977; 30; 10819-10824 or as described in Example 1 in U.S. Pat. No. 6,017,882.

[0147] Modifications in the FVII Gla domain leading to an increased phospholipid membrane binding affinity have been described in the art (see, for example, WO 99/20767 and WO 00/66753). Particular interesting positions in the Gla domain to be modified are positions P10, K32, D33, A34 as well as insertion of an amino acid residue between A3 and F4.

[0148] Thus, in a preferred embodiment of the invention, the variant comprises, in addition to one or more of the modifications mentioned above, a substititution in a position selected from the group consisting of P10, K32, D33 and A34 and combinations thereof.

[0149] In another interesting embodiment at least one of said substitutions are combined with an insertion of an amino acid residue between position A3 and F4.

[0150] Particularly preferred positions are P10 and K32, i.e. in a particular interesting embodiment of the invention substitutions are made in positions P10 and K32, preferably P10Q+K32E.

[0151] Preferably, the substitution to be made in position 32 is K32E, the substitution to be made in position 10 is P10Q, the substitution to be made in position 33 is D33F, and the substitution to be made in position 34 is A34E. The amino acid residue to be inserted between position A3 and F4 is preferably a hydrophobic amino acid residue, in particular the insertion is A3AY. In an interesting embodiment of the invention the variant comprises at least one of the following further modifications: A3AY, P10Q, K32E, D33F, A34E or combinations thereof. Most preferably, the variant comprises one of the following further modifications: K32E, P10Q+K32E, A3AY+P10Q+K32E, A3AY+P10Q+K32E+D33F, A3AY+P10Q+K32E+A34E or A3AY+P10Q+K32E+D33F+A34E.

Modifications Outside the Gla Domain

[0152] A circulating rhFVIIa half-life of 2.3 hours was reported in "Summary Basis for Approval for NovoSeven®", FDA reference number 96-0597. Relatively high doses and frequent administration are necessary to reach and sustain the desired therapeutic or prophylactic effect. As a consequence adequate dose regulation is difficult to obtain and the need of frequent intravenous administrations imposes restrictions on the patient's way of living.

[0153] A molecule with a longer circulation half-life and/or increased bioavailability (such as an increased Area Under the Curve as compared to rhFVIIa when administered intravenously) would decrease the number of necessary administrations. Given the association of the current rhFVIIa product with frequent injections, and the potential for obtaining more optimal therapeutic FVIIa levels with concomitant enhanced therapeutic effect, there is a clear need for improved FVII- or FVIIa-like molecules.

[0154] Accordingly, a further object of the present invention is to provide improved FVII or FVII molecules (FVII or FVIIa variants) with an increased half-life and/or an increased bioavailability (such as an increased Area Under the Curve as compared to rhFVIIa, when administered intravenously) and which has an increased clotting activity.

[0155] Accordingly, in an interesting embodiment of the invention, the variant of the invention further comprises at least one introduced attachment group for a non-polypeptide moiety, where said attachment group has been introduced in a position located outside the Gla domain.

[0156] Thus, an interesting variant of the invention is a variant which, in its activated form and when compared to rhFVIIa, generates in increased Area Under the Curve when administered intravenously (AUC_{iv}), in particular when administered intravenously in rats.

[0157] More particularly, interesting variants of the present invention are such variants where the ratio between the AUC_{iv} of said variant, in its actvated form, and the AUC_{iv} of rhFVIIa is at least 1.25, such as at least 1.5, e.g. at least 1.75, more preferably at least 2, such as at least 3, even more preferably at least 4, such as at least 5, in particular when administered (intravenously) in rats.

[0158] This effect may correspond to an increased functional in vivo half-life and/or an increased serum half-life as compared to rhFVIIa. Accordingly, in another interesting embodiment of the invention, the ratio between the functional in vivo half-life or the serum half-life for the variant, in its activated form, and the functional in vivo half-life or the serum half-life for rhFVIIa is at least 1.25. More preferably, the ratio between the relevant half-life for the variant, in its activated form, and the relevant half-life for rhFVIIa is at least 1.5, such as at least 1.75, e.g. at least 2, even more preferably at least 3, such as at least 4, e.g. at least 5.

[0159] One way to increase the circulation half-life of a protein is to ensure that renal clearance of the protein is reduced. This may be achieved by conjugating the protein to a chemical moiety, which is capable of conferring reduced renal clearance to the protein.

[0160] Furthermore, attachment of a chemical moiety to the protein or substitution of amino acids exposed to proteolysis may effectively block a proteolytic enzyme from contact leading to proteolytic degradation of the protein. Polyethylene glycol (PEG) is one such chemical moiety that has been used in the preparation of therapeutic protein products. WO 98/32466 suggests that FVII, among many other proteins, may be PEGylated but does not contain any further information in this respect. WO 01/58935 discloses a new strategy for developing FVII or FVIIa molecules having inter alia an increased half-life.

[0161] A number of suitable modifications leading to an increase in AUC_{iv}, functional in vivo half-life and/or serum half-life are disclosed in WO 01/58935. The variants disclosed in WO 01/58935 are the result of a generally new strategy for developing improved FVII or FVIIa molecules. The specific modifications described in WO 01/58935 may advantageously be combined with the modifications described previously herein.

[0162] The polypeptide variant may also be attached to a serine proteinase inhibitor to inhibit the catalytic site of the polypeptide variant. Alternatively, one or more of the amino acid residues present in the catalytic site (S344, D242 and H193) may be mutated in order to render the resulting variant inactive. One example of such a mutation includes S344A.

[0163] The introduced amino acid residue comprising an attachment group for a non-polypeptide moiety is selected on the basis of the nature of the non-polypeptide moiety of choice and, in most instances, on the basis of the method in which conjugation between the polypeptide variant and the non-polypeptide moiety is to be achieved. For instance, when the non-polypeptide moiety is a polymer molecule such as a polyethylene glycol or polyalkylene oxide derived molecule, amino acid residues comprising an attachment group may be selected from the group consisting of lysine, cysteine, aspartic acid, glutamic acid, histidine, and tyrosine, preferably lysine, cysteine, aspartic acid and glutamic acid, more preferably lysine and cysteine, in particular cysteine.

[0164] Whenever an attachment group for a non-polypeptide moiety is to be introduced into the parent polypeptide, the position of the amino acid residue to be modified is preferably located at the surface of the parent FVII or FVIIa polypeptide, and more preferably occupied by an amino acid residue which has at least 25% of its side chain exposed to the surface (as defined in Example 1 herein), preferably at least 50% of its side chain exposed to the surface (as defined in Example 1 herein). Such positions have been identified on the basis of an analysis of a 3D structure of the hFVII or hFVIIa molecule as described in the Materials and Methods section herein.

[0165] Furthermore, the position to be modified according to this aspect of the invention is preferably selected from a part of the FVII or FVIIa molecule that is located outside the tissue factor binding site, and/or outside the active site region, and/or outside the ridge of the active site binding cleft. These sites/regions are identified in Example 1 herein. It should be emphasized, however, that in certain situations, e.g. in case an inactivated polypeptide variant is desired, it may be advantageous to perform modifications in or close to such regions. For example, it is contemplated that one or more attachment groups for the non-polypeptide moieties, such as attachment groups for N-glycosylation sites, may advantageously be introduced in the active site region or at the ridge of the active site binding cleft of the FVII or FVIIa molecule. The active site region, the tissue factor binding site and the ridge of the active site binding cleft are defined in Example 1 herein and are constituted by the following residues:

[0166] I153, Q167, V168, L169, L170, L171, Q176, L177, C178, G179, G180, T181, V188, V189, S190, A191, A192, H193, C194, F195, D196, K197, I198, W201, V228, I229, I230, P231, S232, T233, Y234, V235, P236, G237, T238, T239, N240, H241, D242, I243, A244, L245, L246, V281, S282, G283, W284, G285, Q286, T293, T324, E325, Y326, M327, F328, D338, S339, C340, K341, G342, D343, S344, G345, G346, P347, H348, L358, T359, G360, I361, V362, S363, W364, G365, C368, V376, Y377, T378, R379, V380, Q382, Y383, W386, L387, L400 and F405 (active site region);

[0167] L13, K18, F31, E35, R36, L39, F40, I42, S43, S60, K62, D63, Q64, L65, I69, C70, F71, C72, L73, P74, F76, E77, G78, R79, E82, K85, Q88, I90, V92, N93, E94, R271, A274, F275, V276, R277, F278, R304, L305, M306, T307, Q308, D309, Q312, Q313, E325 and R379 (tissue factor binding site); and

[0168] N173, A175, K199, N200, N203, D289, R290, G291, A292, P321 and T370 (the ridge of the active site binding cleft).

[0169] In order to determine an optimal distribution of attachment groups, the distance between amino acid residues located at the surface of the FVII or FVIIa polypeptide is calculated on the basis of a 3D structure of the hFVII or hFVIIa polypeptide. More specifically, the distance between the CB's of the amino acid residues comprising such attachment groups, or the distance between the functional group (NZ for lysine, CG for aspartic acid, CD for glutamic acid, SG for cysteine) of one and the CB of another amino acid residue comprising an attachment group are determined. In case of glycine, CA is used instead of CB. In the FVII or FVIIa part of the polypeptide variant of the invention, any of said distances is preferably more than 8 Å, in particular more than 10 Å in order to avoid or reduce heterogeneous conjugation.

[0170] In case of introduction of an attachment group, an amino acid residue comprising such group is introduced into

the position, preferably by substitution of the amino acid residue occupying such position.

[0171] The exact number of attachment groups present and available for conjugation in the FVII or FVIIa polypeptide is dependent on the effect desired to be achieved by the conjugation. The effect to be obtained is, e.g., dependent on the nature and degree of conjugation (e.g. the identity of the non-polypeptide moiety, the number of non-polypeptide moieties desirable or possible to conjugate to the polypeptide variant, where they should be conjugated or where conjugation should be avoided, etc.).

[0172] Functional in vivo half-life is inter alia dependent on the molecular weight of the protein, and the number of attachment groups needed for providing increased half-life thus depends on the molecular weight of the non-polypeptide moiety in question. In one embodiment, the polypeptide variant of the invention has a molecular weight of at least 67 IcDa, in particular at least 70 kDa, e.g., as measured by SDS-PAGE according to Laemmli, U.K., *Nature Vol* 227 (1970), p 680-85. FVII itself has a molecular weight of about 53 kDa, and therefore additional 10-20 kDa is required to obtain the desired effect. This may, e.g., be provided by conjugating 2-4 10 kDa PEG molecules or as otherwise described herein.

[0173] The total number of amino acid residues to be modified outside the Gla domain in the parent FVII or FVIIa polypeptide (as compared to the amino acid sequence shown in SEQ ID NO:1) will typically not exceed 10. Preferably, the FVII or FVIIa variant comprises an amino acid sequence which differs in 1-10 amino acid residues from amino acid residues 46-406 shown in SEQ ID NO:1, typically in 1-8 or in 2-8 amino acid residues, e.g. in 1-5 or in 2-5 amino acid residues, such as in 1-4 or in 1-3 amino acid residues, e.g. in 1, 2 or 3 amino acid residues from amino acid residues 46-406 shown in SEQ ID NO:1.

[0174] Analogously, the polypeptide variant of the invention may contain 1-10 (additional) non-polypeptide moieties, typically 1-8 or 2-8 (additional) non-polypeptide moieties, preferably 1-5 or 2-5 (additional) non-polypeptide moieties, such as 1-4 or 1-3 (additional) non-polypeptide moieties, e.g. 1, 2 or 3 (additional) non-polypeptide moieties. It will be understood that such additional non-polypeptide moieties are covalently attached to an attachment group located outside the Gla domain.

Introduction of Additional Sugar Moieties

[0175] In a preferred embodiment of the invention, an attachment group for a sugar moiety, such as a glycosylation site, in particular an in vivo glycosylation site, such as an N-glycosylation site, has been introduced in a position located outside the Gla domain.

[0176] When used in the present context, the term "naturally occurring glycosylation site" covers the glycosylation sites at postions N145, N322, S52 and S60. In a similar way, the term "naturally occurring O-glycosylation site" includes the positions S52 and S60, whereas the term "naturally occurring N-glycosylation site" includes positions N145 and N322.

[0177] Thus, in a very interesting embodiment of the invention, the non-polypeptide moiety is a sugar moiety and the introduced attachment group is a glycosylation site, preferably an in vivo glycosylation site, such as an O-glycosylation site or an N-glycosylation site, in particular an N-glycosylation site. Typically, 1-10 glycosylation sites, in particular N-glycosylation sites, have been introduced, preferably 1-8, 1-6, 14 or 1-3 glycosylation sites, in particular N-glycosylation sites, have been introduced in (a) positions(s) located outside the Gla domain. For example 1, 2 or 3 glycosylation sites, in particular N-glycosylation sites, may have been introduced outside the Gla domain, preferably by substitution. Analogously, the variant may comprise 1-10 introduced sugar moieties, preferably 1-8, 1-6, 1-4 or 1-3 introduced sugar moieties. For example, the variant may contain 1, 2 or 3 introduced sugar moieties.

[0178] It will be understood that in order to prepare a polypeptide variant, wherein the polypeptide variant comprises one or more glycosylation sites, the polypeptide variant must be expressed in a host cell capable of attaching sugar (oligosaccharide) moieties at the glycosylation site(s) or alternatively subjected to in vitro glycosylation. Examples of glycosylating host cells are given in the section further below entitled "Coupling to a sugar moiety".

[0179] Examples of positions, wherein the glycosylation sites, in particular N-glycosylation sites, may be introduced include, but is not limited to, positions comprising an amino acid residue having an amino acid residue having at least 25% of its side chain exposed to the surface (as defined in Example 1 herein), such as in a position comprising an amino acid residue having at least 50% of its side chain exposed to the surface (as defined in Example 1 herein). The position is preferably selected from a part of the molecule that is located outside the tissue factor binding site and/or the active site region and/or outside the ridge of the active site cleft. These sites/regions are identified in Example 1 herein. It should be understood that when the term "at least 25% (or at least 50%) of its side chain exposed to the surface" is used in connection with introduction of an N-glycosylation site this term refers to the surface accessibility of the amino acid side chain in the position where the sugar moiety is actually attached. In many cases it will be necessary to introduce a serine or a threonine residue in position +2 relative to the asparagine residue to which the sugar moiety is actually attached (unless, of course, this position is already occupied by a serine or a threonine residue) and these positions, where the serine or threonine residues are introduced, are allowed to be buried, i.e. to have less than 25% of their side chains exposed to the surface.

[0180] Specific and preferred examples of such substitutions creating an N-glycosylation site include a substitution selected from the group consisting of A51N, G58N, G58N+S60T, T106N, K109N, G124N, K143N+N145T, A175T, I205S, I205T, V253N, T267N, T267N+S269T, S314N+K316S, S314N+K316T, R315N+V317S, R315N+V317T, K316N+G318S, K316N+G318T, G318N, D334N and combinations thereof. More preferably, the N-glycosylation site is introduced by a substitution selected from the group consisting of A51N, G58N+S60T, T106N, K109N, G124N, K143N+N145T, A175T, I205T, V253N, T267N+S269T, S314N+K316T, R315N+V317T, K316N+G318T, G318N, D334N and combinations thereof. Even more preferably, the N-glycosylation site is introduced by a substitution selected from the group consisting of T106N, A175T, I205T, V253N, T267N+S269T and combinations thereof. Most preferably, the N-glycosylation site is introduced by a substitution

selected from the group consisting of T106N, I205T, V253N, T267N+S269T and combinations thereof.

[0181] In one embodiment, only one N-glycosylation site has been introduced by substitution. In another embodiment, two or more (such as two) N-glycosylation sites have been introduced by substitution. Examples of preferred substitutions creating two N-glycosylation sites include substitutions selected from the group consisting of A51N+G58N, A51N+G58N+S60T, A51N+T106N, A51N+K109N, A51N+G124N, A51N+K143N+N145T, A51N+A175T, A51N+I205T, A51N+V253N, A51N+T267N+S269T, A51N+S314N+K316T, A51N+R315N+V317T, A51N+K316N+G318T, A51N+G318N, A51N+D334N, G58N+T106N, G58N+K109N, G58N+G124N, G58N+K143N+N145T, G58N+A175T, G58N+I205T, G58N+V253N, G58N+T267N+S269T, G58N+S314N+K316T, G58N+R315N+V317T, G58N+K316N+G318T, G58N+G318N, G58N+D334N, G58N+S60T+T106N, G58N+S60T+K109N, G58N+S60T+G124N, G58N+S60T+K143N+N145T, G58N+S60T+A175T, G58N+S60T+I205T, G58N+S60T+V253N, G58N+S60T+T267N+S269T, G58N+S60T+S314N+K316T, G58N+S60T+R315N+V317T, G58N+S60T+K316N+G318T, G58N+S60T+G318N, G58N+S60T+D334N, T106N+K109N, T106N+G124N, T106N+K143N+N145T, T106N+A175T, T106N+I205T, T106N+V253N, T106N+T267N+S269T, T106N+S314N+K316T, T106N+R315N+V317T, T106N+K316N+G318T, T106N+G318N, T106N+D334N, K109N+G124N, K109N+K143N+N145T, K109N+A175T, K109N+I205T, K109N+V253N, K109N+T267N+S269T, K109N+S314N+K316T, K109N+R315N+V317T, K109N+K316N+G318T, K109N+G318N, K109N+D334N, G124N+K143N+N145T, G124N+A175T, G124N+I205T, G124N+V253N, G124N+T267N+S269T, G124N+S314N+K316T, G124N+R315N+V317T, G124N+K316N+G318T, G124N+G318N, G124N+D334N, K143N+N145T+A175T, K143N+N145T+I205T, K143N+N145T+V253N, K143N+N145T+T267N+S269T, K143N+N145T+S314N+K316T, K143N+N145T+R315N+V317T, K143N+N145T+K316N+G318T, K143N+N145T+G318N, K143N+N145T+D334N, A175T+I205T, A175T+V253N, A175T+T267N+S269T, A175T+S314N+K316T, A175T+R315N+V317T, A175T+K316N+G318T, A175T+G318N, A175T+D334N, I205T+V253N, I205T+T267N+S269T, I205T+S314N+K316T, I205T+R315N+V317T, I205T+K316N+G318T, I205T+G318N, I205T+D334N, V253N+T267N+S269T, V253N+S314N+K316T, V253N+R315N+V317T, V253N+K316N+G318T, V253N+G318N, V253N+D334N, T267N+S269T+S314N+K316T, T267N+S269T+R315N+V317T, T267N+S269T+K316N+G318T, T267N+S269T+G318N, T267N+S269T+D334N, S314N+K316T+R315N+V317T, S314N+K316T+G318N, S314N+K316T+D334N, R315N+V317T+K316N+G318T, R315N+V317T+G318N, R315N+V317T+D334N and G318N+D334N. More preferably, the substitutions are selected from the group consisiting of T106N+A175T, T106N+I205T, T106N+V253N, T106N+T267N+S269T, A175T+I205T, A175T+V253N, A175T+T267N+S269T, I205T+V253N, I205T+T267N+S269T and V253N+T267N+S269T, even more preferably from the group consisiting of T106N+I205T, T106N+V253N, T106N+T267N+S269T, I205T+V253N, I205T+T267N+S269T and V253N+T267N+S269T.

[0182] In an even further embodiment, three or more (such as three) N-glycosylation sites have been introduced by substitution. Examples of preferred substitutions creating three N-glycosylation sites include substitutions selected from the group consisiting of T106N+A175T+I205T, T106N+A175T+V253N, T106N+A175T+T267N+S269T, T106N+I205T+V253N, T106N+I205T+T267N+S269T, T106N+V253N+T267N+S269T, A175T+I205T+V253N, A175T+I205T+T267N+S269T, A175T+V253N+T267N+S269T and I205T+V253N+T267N+S269T. More preferably, the substitutions are selected from the group consisting of T106N+I205T+V253N, T106N+I205T+T267N+S269T, T106N+V253N+T267N+S269T and I205T+V253N+T267N+S269T.

[0183] As discussed above, it is preferred that the N-glycosylation site is introduced in a position which does neither form part of the tissue factor binding site nor form part of the active site region and the ridge of the active site binding cleft as defined herein. It is envisaged that such glycosylation variants will primarily belong to the class of active polypeptide variants as defined hereinbefore.

[0184] It will be understood that any of the modifications mentioned in the above sections may be combined.

Other Modifications

[0185] In a further embodiment of the present invention, the FVII or FVIIa variant may, in addition to the modifications described in the sections above, also contain mutations, which are known to increase the intrinsic activity of the polypeptide, e.g. such as those described in WO 02/22776

[0186] Examples of preferred substitutions include substitutions selected from the group consisting of V158D, E296D, M298Q, L305V and K337A. More preferably, said substitutions are selected from the group consisting of V158D+E296D+M298Q+L305V+K337A, V158D+E296D+M298Q+K337A, V158D+E296D+M298Q+L305V, V158D+E296D+M298Q, M298Q, L305V+K337A, L305V and K337A.

[0187] In a further embodiment of the present invention, the FVII or FVIIa variant may, in addition to the modifications described in the sections above, also contain mutations, which cause a decreased inhibition by TFPI. One example includes the substitution K341Q disclosed by Neuenschwander et al., *Biochemistry* 1995; 34:8701-8707. Other examples include D196K, D196N, G237L, G237GAA and combinations thereof.

[0188] As already indicated above, the variant may also contain conservative amino acid substitutions.

Specific Examples of Most the Preferred Variants of the Invention

[0189] Specifc examples of the most preferred variants of FVII or FVIIa are given below: S43Q, K62E, L65Q, S43Q+K62E, S43Q+L65Q, K62E+L65Q, S43Q+K62E+L65Q, P10Q+K32E+S43Q, P10Q+K32E+K62E, P10Q+K32E+L65Q, P10Q+K32E+S43Q+K62E, P10Q+K32E+S43Q+L65Q, P10Q+K32E+K62E+L65Q, P10Q+K32E+S43Q+K62E+L65Q

[0190] It will be understood that any of the above-mentioned preferred variants may be combined with at least one further modification performed outside the Gla domain. In particular any of the above-mentioned preferred variants may be combined with (a) substitution(s) selected from the group consisting of T106N, I205T, V253N, T267N+S269T,

T106N+I205T, T106N+V253N, T106N+T267N+S269T, I205T+V253N, I205T+T267N+S269T, V253N+T267N+S269T, T106N+I205T+V253N, T106N+I205T+T267N+S269T, T106N+V253N+T267N+S269T and I205T+V253N+T267N+S269T.

The Non-Polypeptide Moiety

[0191] Based on the present disclosure the skilled person will be aware that amino acid residues comprising other attachment groups may be introduced by substitution into the parent polypeptide, using the same approach as that illustrated above with N-glycosylation sites. For instance, one or more amino acid residues comprising an acid group (glutamic acid or aspartic acid), tyrosine or lysine may be introduced into the positions discussed above. In particular, one or more cysteine residues may be introduced in the positions discussed above.

[0192] As indicated further above the non-polypeptide moiety of the conjugated variant is preferably selected from the group consisting of a polymer molecule, a lipophilic compound, a sugar moiety (by way of in vivo glycosylation) and an organic derivatizing agent. All of these agents may confer desirable properties to the variant polypeptide, in particular increased $AUCI_{iv}$, increased functional in vivo half-life and/or increased plasma half-life. The variant polypeptide is normally conjugated to only one type of non-polypeptide moiety, but may also be conjugated to two or more different types of non-polypeptide moieties, e.g. to a polymer molecule and a sugar moiety, to a lipophilic group and a sugar moiety, to an organic derivatizing agent and a sugar moiety, to a lipophilic group and a polymer molecule, etc. The conjugation to two or more different non-polypeptide moieties may be done simultaneous or sequentially.

Methods of Preparing a Conjugated Variant of the Invention

[0193] In the following sections "Conjugation to a polymer molecule", "Conjugation to a sugar moiety", "Conjugation to an organic derivatizing agent" and "Conjugation to a lipophilic compound", conjugation to specific types of non-polypeptide moieties is described. In general, a conjugated variant according to the invention may be produced by culturing an appropriate host cell under conditions conducive for the expression of the variant polypeptide, and recovering the variant polypeptide, wherein a) the variant polypeptide comprises at least one N- or O-glycosylation site and the host cell is an eukaryotic host cell capable of in vivo glycosylation, and/or b) the variant polypeptide is subjected to conjugation to a non-polypeptide moiety in vitro.

[0194] It will be understood that the conjugation should be designed so as to produce the optimal molecule with respect to the number of non-polypeptide moieties attached, the size and form of such molecules (e.g. whether they are linear or branched), and the attachment site(s) in the polypeptide. The molecular weight of the non-polypeptide moiety to be used may, e.g., be chosen on the basis of the desired effect to be achieved. For instance, if the primary purpose of the conjugation is to achieve a conjugated variant having a high molecular weight (e.g. to reduce renal clearance) it is usually desirable to conjugate as few high molecular weight non-polypeptide moieties as possible to obtain the desired molecular weight. When a high degree of shielding is desirable this may be obtained by use of a sufficiently high number of low molecular weight non-polypeptide moieties (e.g. with a molecular weight of from about 300 Da to about 5 kDa, such as a molecular weight of from 300 Da to 2 kDa).

Conjugation to a Polymer Molecule

[0195] The polymer molecule to be coupled to the variant polypeptide may be any suitable polymer molecule, such as a natural or synthetic homo-polymer or hetero-polymer, typically with a molecular weight in the range of about 300-100,000 Da, such as about 500-20,000 Da, more preferably in the range of about 500-15,000 Da, even more preferably in the range of about 2-12 kDa, such as in the range of about 3-10 kDa. When the term "about" is used herein in connection with a certain molecular weight, the word "about" indicates an approximate average molecular weight and reflects the fact that there will normally be a certain molecular weight distribution in a given polymer preparation.

[0196] Examples of homo-polymers include a polyol (i.e. poly-OH), a polyamine (i.e. poly-NH_2) and a polycarboxylic acid (i.e. poly-COOH). A hetero-polymer is a polymer comprising different coupling groups, such as a hydroxyl group and an amine group.

[0197] Examples of suitable polymer molecules include polymer molecules selected from the group consisting of polyalkylene oxide (PAO), including polyalkylene glycol (PAG), such as polyethylene glycol (PEG) and polypropylene glycol (PPG), branched PEGs, poly-vinyl alcohol (PVA), poly-carboxylate, poly-(vinylpyrolidone), polyethylene-co-maleic acid anhydride, polystyrene-co-maleic acid anhydride, dextran, including carboxymethyl-dextran, or any other biopolymer suitable for reducing immunogenicity and/or increasing functional in vivo half-life and/or serum half-life. Another example of a polymer molecule is human albumin or another abundant plasma protein. Generally, polyalkylene glycol-derived polymers are biocompatible, non-toxic, non-antigenic, non-immunogenic, have various water solubility properties, and are easily excreted from living organisms.

[0198] PEG is the preferred polymer molecule, since it has only few reactive groups capable of cross-linking compared to, e.g., polysaccharides such as dextran. In particular, mono-functional PEG, e.g. methoxypolyethylene glycol (mPEG), is of interest since its coupling chemistry is relatively simple (only one reactive group is available for conjugating with attachment groups on the polypeptide). Consequently, as the risk of cross-linking is eliminated, the resulting conjugated variants are more homogeneous and the reaction of the polymer molecules with the variant polypeptide is easier to control.

[0199] To effect covalent attachment of the polymer molecule(s) to the variant polypeptide, the hydroxyl end groups of the polymer molecule must be provided in activated form, i.e. with reactive functional groups (examples of which include primary amino groups, hydrazide (HZ), thiol, succinate (SUC), succinimidyl succinate (SS), succinimidyl succinamide (SSA), succinimidyl propionate (SPA), succinimidyl butyrate (SBA), succinimidy carboxymethylate (SCM), benzotriazole carbonate (BTC), N-hydroxysuccinimide (NHS), aldehyde, nitrophenylcarbonate (NPC), and tresylate (TRES)). Suitable activated polymer molecules are commercially available, e.g. from Shearwater Polymers, Inc., Huntsville, Ala., USA, or from PolyMASC Pharmaceuticals plc, UK.

[0200] Alternatively, the polymer molecules can be activated by conventional methods known in the art, e.g. as disclosed in WO 90/13540. Specific examples of activated linear or branched polymer molecules for use in the present invention are described in the Shearwater Polymers, Inc. 1997 and 2000 Catalogs (Functionalized Biocompatible Polymers for Research and pharmaceuticals, Polyethylene Glycol and Derivatives, incorporated herein by reference).

[0201] Specific examples of activated PEG polymers include the following linear PEGs: NHS-PEG (e.g. SPA-PEG, SSPA-PEG, SBA-PEG, SS-PEG, SSA-PEG, SC-PEG, SG-PEG, and SCM-PEG), and NOR-PEG, BTC-PEG, EPOX-PEG, NCO-PEG, NPC-PEG, CDI-PEG, ALD-PEG, TRES-PEG, VS-PEG, IODO-PEG, and MAL-PEG, and branched PEGS such as PEG2-NHS and those disclosed in U.S. Pat. No. 5,932,462 and U.S. Pat. No. 5,643,575, both of which are incorporated herein by reference. Furthermore, the following publications, incorporated herein by reference, disclose useful polymer molecules and/or PEGylation chemistries: U.S. Pat. No. 5,824,778, U.S. Pat. No. 5,476,653, WO 97/32607, EP 0 229 108, EP 0 402 378, U.S. Pat. No. 4,902,502, U.S. Pat. No. 5,281,698, U.S. Pat. No. 5,122,614, U.S. Pat. No. 5,219,564, WO 92/16555, WO 94/04193, WO 94/14758, WO 94/17039, WO 94/18247, WO 94/28024, WO 95/00162, WO 95/11924, WO95/13090, WO 95/33490, WO 96/00080, WO 97/18832, WO 98/41562, WO 98/48837, WO 99/32134, WO 99/32139, WO 99/32140, WO 96/40791, WO 98/32466, WO 95/06058, EP 0 439 508, WO 97/03106, WO 96/21469, WO 95/13312, EP 0 921 131, U.S. Pat. No. 5,736,625, WO 98/05363, EP 0 809 996, U.S. Pat. No. 5,629,384, WO 96/41813, WO 96/07670, U.S. Pat. No. 5,473,034, U.S. Pat. No. 5,516,673, EP 0 605 963, U.S. Pat. No. 5,382,657, EP 0 510 356, EP 0 400 472, EP 0 183 503 and EP 0 154 316.

[0202] Specific examples of activated PEG polymers particularly preferred for coupling to cysteine residues, include the following linear PEGs: vinylsulfone-PEG (VS-PEG), preferably vinylsulfone-mPEG (VS-mPEG); maleimide-PEG (MAL-PEG), preferably maleimide-mPEG (MAL-mPEG) and orthopyridyl-disulfide-PEG (OPSS-PEG), preferably orthopyridyl-disulfide-mPEG (OPSS-mPEG). Typically, such PEG or mPEG polymers will have a size of about 5 kDa, about 10 kD, about 12 kDa or about 20 kDa.

[0203] The conjugation of the polypeptide variant and the activated polymer molecules is conducted by use of any conventional method, e.g. as described in the following references (which also describe suitable methods for activation of polymer molecules): Harris and Zalipsky, eds., Poly(ethylene glycol) Chemistry and Biological Applications, AZC, Washington; R. F. Taylor, (1991), "Protein immobilisation. Fundamental and applications", Marcel Dekker, N.Y.; S. S. Wong, (1992), "Chemistry of Protein Conjugation and Crosslinking", CRC Press, Boca Raton; G. T. Hermanson et al., (1993), "Immobilized Affinity Ligand Techniques", Academic Press, N.Y.).

[0204] The skilled person will be aware that the activation method and/or conjugation chemistry to be used depends on the attachment group(s) of the variant polypeptide (examples of which are given further above), as well as the functional groups of the polymer (e.g. being amine, hydroxyl, carboxyl, aldehyde, sulfydryl, succinimidyl, maleimide, vinysulfone or haloacetate). The PEGylation may be directed towards conjugation to all available attachment groups on the variant polypeptide (i.e. such attachment groups that are exposed at the surface of the polypeptide) or may be directed towards one or more specific attachment groups, e.g. the N-terminal amino group as described in U.S. Pat. No. 5,985,265 or to cysteine residues. Furthermore, the conjugation may be achieved in one step or in a stepwise manner (e.g. as described in WO 99/55377).

[0205] For PEGylation to cysteine residues (see above) the FVII or FVIIa variant is usually treated with a reducing agent, such as dithiothreitol (DDT) prior to PEGylation. The reducing agent is subsequently removed by any conventional method, such as by desalting. Conjugation of PEG to a cysteine residue typically takes place in a suitable buffer at pH 6-9 at temperatures varying from 4° C. to 25° C. for periods up to 16 hours.

[0206] It will be understood that the PEGylation is designed so as to produce the optimal molecule with respect to the number of PEG molecules attached, the size and form of such molecules (e.g. whether they are linear or branched), and the attachment site(s) in the variant polypeptide. The molecular weight of the polymer to be used may e.g. be chosen on the basis of the desired effect to be achieved.

[0207] In connection with conjugation to only a single attachment group on the protein (e.g. the N-terminal amino group), it may be advantageous that the polymer molecule, which may be linear or branched, has a high molecular weight, preferably about 10-25 kDa, such as about 15-25 kDa, e.g. about 20 kDa.

[0208] Normally, the polymer conjugation is performed under conditions aimed at reacting as many of the available polymer attachment groups with polymer molecules. This is achieved by means of a suitable molar excess of the polymer relative to the polypeptide. Typically, the molar ratios of activated polymer molecules to polypeptide are up to about 1000-1, such as up to about 200-1, or up to about 100-1. In some cases the ration may be somewhat lower, however, such as up to about 50-1, 10-1, 5-1, 2-1 or 1-1 in order to obtain optimal reaction.

[0209] It is also contemplated according to the invention to couple the polymer molecules to the polypeptide through a linker. Suitable linkers are well known to the skilled person. A preferred example is cyanuric chloride (Abuchowski et al., *J Biol Chem* 1977; 252; 3578-3581; U.S. Pat. No. 4,179,337; Shafer et al., *J Polym Sci Polym Chem Ed* 1986; 24; 375-378).

[0210] Subsequent to the conjugation, residual activated polymer molecules are blocked according to methods known in the art, e.g. by addition of primary amine to the reaction mixture, and the resulting inactivated polymer molecules are removed by a suitable method.

[0211] It will be understood that depending on the circumstances, e.g. the amino acid sequence of the variant polypeptide, the nature of the activated PEG compound being used and the specific PEGylation conditions, including the molar ratio of PEG to polypeptide, varying degrees of PEGylation may be obtained, with a higher degree of PEGylation generally being obtained with a higher ratio of PEG to variant polypeptide. The PEGylated variant polypeptides resulting from any given PEGylation process will, however,

normally comprise a stochastic distribution of conjugated polypeptide variants having slightly different degrees of PEGylation.

Conjugation to a Sugar Moiety

[0212] In order to achieve in vivo glycosylation of a FVII molecule comprising one or more glycosylation sites the nucleotide sequence encoding the variant polypeptide must be inserted in a glycosylating, eucaryotic expression host. The expression host cell may be selected from fungal (filamentous fungal or yeast), insect or animal cells or from transgenic plant cells. In one embodiment the host cell is a mammalian cell, such as a CHO cell, BHK or HEK, e.g. HEK 293, cell, or an insect cell, such as an SF9 cell, or a yeast cell, e.g. *S. cerevisiae* or *Pichia pastoris*, or any of the host cells mentioned hereinafter.

[0213] Covalent in vitro coupling of sugar moieties (such as dextran) to amino acid residues of the variant polypeptide may also be used, e.g. as described, for example in WO 87/05330 and in Aplin etl al., *CRC Crit Rev. Biochem* 1981; 259-306. The in vitro coupling of sugar moieties or PEG to protein- and peptide-bound Gln-residues can be carried out by transglutaminases (TGases). Transglutaminases catalyse the transfer of donor amine-groups to protein- and peptide-bound Gin-residues in a so-called cross-linking reaction. The donor-amine groups can be protein- or peptide-bound, such as the ϵ-amino-group in Lys-residues or it can be part of a small or large organic molecule. An example of a small organic molecule functioning as amino-donor in TGase-catalysed cross-linking is putrescine (1,4-diaminobutane). An example of a larger organic molecule functioning as amino-donor in TGase-catalysed cross-linking is an amine-containing PEG (Sato et al., *Biochemistry* 1996; 35; 13072-13080).

[0214] TGases, in general, are highly specific enzymes, and not every Gln-residues exposed on the surface of a protein is accessible to TGase-catalysed cross-linking to amino-containing substances. On the contrary, only few Gln-residues are naturally functioning as TGase substrates but the exact parameters governing which Gln-residues are good TGase substrates remain unknown. Thus, in order to render a protein susceptible to TGase-catalysed cross-linking reactions it is often a prerequisite at convenient positions to add stretches of amino acid sequence known to function very well as TGase substrates. Several amino acid sequences are known to be or to contain excellent natural TGase substrates e.g. substance P, elafin, fibrinogen, fibronectin, α_2-plasmin inhibitor, α-caseins, and β-caseins.

Conjugation to an Organic Derivatizing Agent

[0215] Covalent modification of the variant polypeptide may be performed by reacting one or more attachment groups of the variant polypeptide with an organic derivatizing agent. Suitable derivatizing agents and methods are well known in the art. For example, cysteinyl residues most commonly are reacted with α-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, α-bromo-β-(4-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole. Histidyl residues are derivatized by reaction with diethylpyrocarbonateat pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful. The reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0. Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing α-amino-containing residues include imidoesters such as methyl picolinimidate, pyridoxal phosphate, pyridoxal, chloroborohydride, trinitrobenzenesulfonic acid, O-methylisourea, 2,4-pentanedione and transaminase-catalyzed reaction with glyoxylate. Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pKa of the guanidine functional group.

[0216] Furthermore, these reagents may react with the groups of lysine as well as the arginine guanidino group. Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R—N═C═N—R'), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl)carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Conjugation to a Lipophilic Compound

[0217] The variant polypeptide and the lipophilic compound may be conjugated to each other, either directly or by use of a linker. The lipophilic compound may be a natural compound such as a saturated or unsaturated fatty acid, a fatty acid diketone, a terpene, a prostaglandin, a vitamine, a carotenoide or steroide, or a synthetic compound such as a carbon acid, an alcohol, an amine and sulphonic acid with one or more alkyl-, aryl-, alkenyl- or other multiple unsaturated compounds. The conjugation between the variant polypeptide and the lipophilic compound, optionally through a linker may be done according to methods known in the art, e.g. as described by Bodanszky in Peptide Synthesis, John Wiley, New York, 1976 and in WO 96/12505.

Attachment of Serine Protease Inhibitor

[0218] Attachment of a serine protease inhibitor can be performed in accordance with the method described in WO 96/12800.

Conjugation of a Tagged Polypeptide

[0219] In an alternative embodiment the polypeptide variant is expressed as a fusion protein with a tag, i.e. an amino acid sequence or peptide stretch made up of typically 1-30, such as 1-20 amino acid residues. Besides allowing for fast and easy purification, the tag is a convenient tool for achieving conjugation between the tagged polypeptide variant and the non-polypeptide moiety. In particular, the tag may be used for achieving conjugation in microtiter plates or other carriers, such as paramagnetic beads, to which the tagged polypeptide variant can be immobilised via the tag. The conjugation to the tagged polypeptide variant in, e.g.,

microtiter plates has the advantage that the tagged polypeptide variant can be immobilised in the microtiter plates directly from the culture broth (in principle without any purification) and subjected to conjugation. Thereby, the total number of process steps (from expression to conjugation) can be reduced. Furthermore, the tag may function as a spacer molecule, ensuring an improved accessibility to the immobilised polypeptide variant to be conjugated. The conjugation using a tagged polypeptide variant may be to any of the non-polypeptide moieties disclosed herein, e.g. to a polymer molecule such as PEG.

[0220] The identity of the specific tag to be used is not critical as long as the tag is capable of being expressed with the polypeptide variant and is capable of being immobilised on a suitable surface or carrier material. A number of suitable tags are commercially available, e.g. from Unizyme Laboratories, Denmark. The subsequent cleavage of the tag from the polypeptide variant may be achieved by use of commercially available enzymes.

Inactivation of the FVII/FVIIa Variants of the Invention

[0221] In another interesting embodiment of the invention, the variants disclosed herein may be inactivated. The inactivated form is capable of competing with wild-type FVII or FVIIa for binding to TF and inhibiting clotting activity, and it is envisaged that such inactivated variants will be very potent tissue factor antagonists. Thus, in another aspect the present invention relates to the FVIIa/FVIIa variants described herein in their inactivated forms as well as to such inactivated FVIIa/FVIIa variants for use as medicaments. More particularly, the inactivated variant of the invention may be used for the manufacture of a medicament for the treatment or prophylaxis of a FVIIa/TF-related disease or disorder in a mammal. For example, the inactivated variant of the invention may be used for the manufacture of a medicament for the treatment or prophylaxis of diseases where anticoagulant activity is desirable, such as prophylaxis or treatment of patients being in hypercoagulable states, such as patients with sepsis, deep-vein thrombosis, patients in risk of myocardial infections or thrombotic stroke, pulmonary embolism, patients with acute coronary syndromes (myocardial infarction and unstable angina pectoris), patients undergoing coronary cardiac, prevention of cardiac events and restonosis for patients receiving angioplasty, patients with peripheral vascular diseases. The inactivated variant of the invention may also be used for the manufacture of a medicament for the treatment of respiratory diseases, tumor growth and metastasis. Analogously, the inactivated variant of the invention may be used in a method for treating a mammal having a FVIIa/TF-related disease or disorder (such as one or more of the diseases or disorders mentioned above), comprising administering to a mammal in need thereof an effective amount of such an inactivated conjugate or composition.

[0222] As used herein, the term "inactivated", when used in connection with the variants of the invention, is intended to mean a variant having less than 5% of the clotting activity of hFVIIa or rhFVIIa when measured in the "Whole Blood Assay" described herein.

[0223] The variants described herein may be inactivated by methods well-known in the art.

[0224] For example, an active FVII or FVIIa polypeptide may be rendered inactive by carbamylating the α-amino acid group 1153 or by complexing the polypeptide to a serine proteinase inhibitor, in accordance with the method described in WO 96/12800. A suitable serine inhibitor protein is, e.g., selected from the group consisting of an organophosphor compound, a sulfanylfluoride, a peptide halomethylketone, preferably a Dansyl-Phe-Pro-Arg chloromethylketone, Dansyl-Glu-Glu-Arg chlormethylketone, Dansyl-Phe-Phe-Arg chlormethylketone or a Phe-Phe-Arg chlormethylketone, or an azapeptide.

[0225] Alternatively, the variants of the invention may be rendered inactive by removing at least one amino acid residue occupying a position selected from the group consisting of R152, I153, S344, D242 and H193. The removal may be effected by substitution or deletion of one or more of the above-identified amino acid residues. Preferably, the removal is effected by substitution, in particular by conservative substitution. Accordingly, the inactivated FVII or FVIIa polypeptide used herein may comprise one or more of the following substitutions: R152X, I153×, S344X, D242X or H193X, wherein X is any amino acid residue, preferably one leading to a conservative substitution. For instance, the inactivated FVII or FVIIa polypeptide comprises the mutations R152X, wherein X is any amino acid residue other than lysine (since lysine forms part of a protease cleavage site). Other examples of specific substitutions include I153A/V/L; S344T/A/G/Y, preferably S344A; D242E/A and/or H193R/A.

[0226] Another approach includes performing modifications in or close to the active site region. For example, it is contemplated that one or more attachment groups for the non-polypeptide moieties, such as attachment groups for N-glycosylation sites, may advantageously be introduced in the active site region or at the ridge of the active site binding cleft of the FVII or FVIIa variant. The active site region, the tissue factor binding site and the ridge of the active site binding cleft are defined in Example 1 herein.

[0227] Thus, specific examples of substitutions creating such an N-glycosylation site include substitutions selected from the group consisting of I153N+G155S/T, Q167N+L169S/T, V168N+L170S/T, L169N+L171S/T, L170N+V172S/T, L171N+N173S/T, A175S/T, A175N+L177S/T, L177N+G179S/T, G179N, G180N+L182S/T, T181N+I183S/T, V188N, V189N+A191S/T, S190N+A192S/T, A191N+H193S/T, H193N+F195S/T, F195N+K197S/T, D196N+I198S/T, K197N+K199S/T, I198N+N200S/T, K199N+W201S/T, W201N+N203S/T, R202S/T, I205S/T, V228N+I230S/T, I229N+P231S/T, I230N, P231N, S232N+Y234S/T, T233N+V235S/T, Y234N+P236S/T, V235N+G237S/T, P236N, G237N, T238N+N240S/T, T239N+H241S/T, H241N+I243S/T, D242S/T, I243N+L245S/T, A244N+L246S/T, L245N+R247S/T, L246N+L246S/T, V281N+G283S/T, S282N+W284S/T, G283N+G285S/T, W284N+Q286S/T, G285N+L287S/T, Q286N+L288S/T, D289N+G291S/T, R290N+A292S/T, G291N, A292N+A294S/T, T293N+L295S/T, P321N+I323S/T, T324N+Y326S/T, Y326N+F327S/T, F328N+A330S/T, S339N+K341 S/T, K341N+D343S/T, G342N+S344S/T, D343N+G345S/T, S344N+G346S/T, G345N+P347S/T, P347N+A349S/T, H348N, L358N+G360S/T, T359N+I361S/T, G360N+V362S/T, I361N, V362N+W364S/T, S363N+G365S/T, W364N+Q366S/T, G365N+G367S/T, T370N+G372S/T, V376N, Y377N+R379S/T, T378N+V380S/T, V380N+Q382S/T, Q382N+I384S/T, Y383N+E385S/T,

W386N+Q388S/T, L387N+K389S/T, L400N+R402S/T and combinations thereof. Preferably, the substitution is selected from the group consisting of D289N+G291S/T, R290N+A292S/T, G291N, A292N+A294S/T, T293N+L295S/T, S339N+K341S/T, K341N+D343S/T, G342N+S344S/T, D343N+G345S/T, and combinations thereof. More preferably, the substitution is selected from the group consisting of D289N+G291T, R290N+A292T, G291N, A292N+A294T, T293N+L295T, S339N+K341T, K341N+D343T, G342N+S344T, D343N+G345T, and combinations thereof, in particular G291N.

[0228] Typically, an inactivated variant has significantly reduced clotting activity as compared to wild-type hFVIIa or rhFVIIa. Preferably, the inactivated variant has less than 4% of the clotting activity of hFVIIa or rhFVIIa when assayed in Whole Blood Assay described herein. More preferably the inactivated variant has less than 3% of the clotting activity, such as less than 2% of the clotting activity, e.g. less than 1% of the clotting activity of hFVIIa or rhFVIIa when assayed in the Whole Blood Assay described herein.

[0229] As will be understood, details and particulars concerning the inactivated variants of the invention (e.g. preferred substitutions, formulation of the variants, etc.) will be the same or analogous to the (active) variant aspect of the invention, whenever appropriate. Thus, statements and details concerning the inactivated variants of the invention will apply mutatis mutandis to the active variants disclosed herein, whenever appropriate.

Methods of Preparing a Polypeptide Variant of the Invention

[0230] The polypeptide variants of the present invention, optionally in glycosylated form, may be produced by any suitable method known in the art. Such methods include constructing a nucleotide sequence encoding the polypeptide variant and expressing the sequence in a suitable transformed or transfected host. Preferably, the host cell is a gammacarboxylating host cell such as a mammalian cell. However, polypeptide variants of the invention may be produced, albeit less efficiently, by chemical synthesis or a combination of chemical synthesis or a combination of chemical synthesis and recombinant DNA technology.

[0231] A nucleotide sequence encoding a polypeptide variant of the invention may be constructed by isolating or synthesizing a nucleotide sequence encoding human wild-type FVII and then changing the nucleotide sequence so as to effect introduction (i.e. insertion or substitution) or removal (i.e. deletion or substitution) of the relevant amino acid residue(s).

[0232] The nucleotide sequence is conveniently modified by site-directed mutagenesis in accordance with conventional methods. Alternatively, the nucleotide sequence is prepared by chemical synthesis, e.g. by using an oligonucleotide synthesizer, wherein oligonucleotides are designed based on the amino acid sequence of the desired polypeptide variant, and preferably selecting those codons that are favored in the host cell in which the recombinant polypeptide variant will be produced. For example, several small oligonucleotides coding for portions of the desired polypeptide variant may be synthesized and assembled by PCR, ligation or ligation chain reaction (LCR) (Barany, PNAS 88:189-193, 1991). The individual oligonucleotides typically contain 5' or 3' overhangs for complementary assembly.

[0233] Alternative nucleotide sequence modification methods are available for producing polypeptide variants for high throughput screening, for instance methods which involve homologous cross-over such as disclosed in U.S. Pat. No. 5,093,257, and methods which involve gene shuffling, i.e. recombination between two or more homologous nucleotide sequences resulting in new nucleotide sequences having a number of nucleotide alterations when compared to the starting nucleotide sequences. Gene shuffling (also known as DNA shuffling) involves one or more cycles of random fragmentation and reassembly of the nucleotide sequences, followed by screening to select nucleotide sequences encoding polypeptides with desired properties. In order for homology-based nucleic acid shuffling to take place, the relevant parts of the nucleotide sequences are preferably at least 50% identical, such as at least 60% identical, more preferably at least 70% identical, such as at least 80% identical. The recombination can be performed in vitro or in vivo.

[0234] Examples of suitable in vitro gene shuffling methods are disclosed by Stemmer et al. (1994), Proc. Natl. Acad. Sci. USA; vol. 91, pp. 10747-10751; Stemmer (1994), Nature, vol. 370, pp. 389-391; Smith (1994), Nature vol. 370, pp. 324-325; Zhao et al., Nat. Biotechnol. 1998, March; 16(3): 258-61; Zhao H. and Arnold, F B, Nucleic Acids Research, 1997, Vol. 25. No. 6 pp. 1307-1308; Shao et al., Nucleic Acids Research 1998, Jan. 15; 26(2): pp. 681-83; and WO 95/17413.

[0235] An example of a suitable in vivo shuffling method is disclosed in WO 97/07205. Other techniques for mutagenesis of nucleic acid sequences by in vitro or in vivo recombination are disclosed e.g. in WO 97/20078 and U.S. Pat. No. 5,837,458. Examples of specific shuffling techniques include "family shuffling", "synthetic shuffling" and "in silico shuffling".

[0236] Family shuffling involves subjecting a family of homologous genes from different species to one or more cycles of shuffling and subsequent screening or selection. Family shuffling techniques are disclosed e.g. by Crameri et al. (1998), Nature, vol. 391, pp. 288-291; Christians et al. (1999), Nature Biotechnology, vol. 17, pp. 259-264; Chang et al. (1999), Nature Biotechnology, vol. 17, pp. 793-797; and Ness et al. (1999), Nature Biotechnology, vol. 17, 893-896.

[0237] Synthetic shuffling involves providing libraries of overlapping synthetic oligonucleotides based e.g. on a sequence alignment of homologous genes of interest. The synthetically generated oligonucleotides are recombined, and the resulting recombinant nucleic acid sequences are screened and if desired used for further shuffling cycles. Synthetic shuffling techniques are disclosed in WO 00/42561.

[0238] In silico shuffling refers to a DNA shuffling procedure, which is performed or modelled using a computer system, thereby partly or entirely avoiding the need for physically manipulating nucleic acids. Techniques for in silico shuffling are disclosed in WO 00/42560.

[0239] Once assembled (by synthesis, site-directed mutagenesis or another method), the nucleotide sequence encoding the polypeptide is inserted into a recombinant vector and operably linked to control sequences necessary for expression of the FVII in the desired transformed host cell.

[0240] It should of course be understood that not all vectors and expression control sequences function equally well to express the nucleotide sequence encoding the polypeptide variants described herein. Neither will all hosts function equally well with the same expression system. However, one of skill in the art may make a selection among these vectors, expression control sequences and hosts without undue experimentation. For example, in selecting a vector, the host must be considered because the vector must replicate in it or be able to integrate into the chromosome. The vector's copy number, the ability to control that copy number, and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered. In selecting an expression control sequence, a variety of factors should also be considered. These include, for example, the relative strength of the sequence, its controllability, and its compatibility with the nucleotide sequence encoding the polypeptide, particularly as regards potential secondary structures. Hosts should be selected by consideration of their compatibility with the chosen vector, the toxicity of the product coded for by the nucleotide sequence, their secretion characteristics, their ability to fold the polypeptide variant correctly, their fermentation or culture requirements, and the ease of purification of the products coded for by the nucleotide sequence.

[0241] The recombinant vector may be an autonomously replicating vector, i.e. a vector, which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector is one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

[0242] The vector is preferably an expression vector, in which the nucleotide sequence encoding the polypeptide variant of the invention is operably linked to additional segments required for transcription of the nucleotide sequence. The vector is typically derived from plasmid or viral DNA. A number of suitable expression vectors for expression in the host cells mentioned herein are commercially available or described in the literature. Useful expression vectors for eukaryotic hosts, include, for example, vectors comprising expression control sequences from SV40, bovine papilloma virus, adenovirus and cytomegalovirus. Specific vectors are, e.g., pcDNA3.1(+)\Hyg (Invitrogen, Carlsbad, Calif., USA) and pCI-neo (Stratagene, La Jola, Calif., USA). Useful expression vectors for yeast cells include the 2μ plasmid and derivatives thereof, the POT1 vector (U.S. Pat. No. 4,931,373), the pJSO37 vector described in Okkels, Ann. New York Acad. Sci. 782, 202-207, 1996, and pPICZ A, B or C (Invitrogen). Useful vectors for insect cells include pVL941, pBG311 (Cate et al., "Isolation of the Bovine and Human Genes for Mullerian Inhibiting Substance And Expression of the Human Gene In Animal Cells", Cell, 45, pp. 685-98 (1986), pBluebac 4.5 and pMelbac (both available from Invitrogen). Useful expression vectors for bacterial hosts include known bacterial plasmids, such as plasmids from *E. coli*, including pBR322, pET3a and pET12a (both from Novagen Inc., WI, USA), wider host range plasmids, such as RP4, phage DNAs, e.g., the numerous derivatives of phage lambda, e.g., NM989, and other DNA phages, such as M13 and filamentous single stranded DNA phages.

[0243] Other vectors for use in this invention include those that allow the nucleotide sequence encoding the polypeptide variant to be amplified in copy number. Such amplifiable vectors are well known in the art. They include, for example, vectors able to be amplified by DHFR amplification (see, e.g., Kaufman, U.S. Pat. No. 4,470,461, Kaufman and Sharp, "Construction Of A Modular Dihydrafolate Reductase cDNA Gene: Analysis Of Signals Utilized For Efficient Expression", Mol. Cell. Biol., 2, pp. 1304-19 (1982)) and glutamine synthetase ("GS") amplification (see, e.g., U.S. Pat. No. 5,122,464 and EP 338,841).

[0244] The recombinant vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. An example of such a sequence (when the host cell is a mammalian cell) is the SV40 origin of replication. When the host cell is a yeast cell, suitable sequences enabling the vector to replicate are the yeast plasmid 2μ replication genes REP 1-3 and origin of replication.

[0245] The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or the *Schizosaccharomyces pombe* TPI gene (described by P. R. Russell, Gene 40, 1985, pp. 125-130), or one which confers resistance to a drug, e.g. ampicillin, kanamycin, tetracyclin, chloramphenicol, neomycin, hygromycin or methotrexate. For *Saccharomyces cerevisiae*, selectable markers include ura3 and leu2. For filamentous fungi, selectable markers include amdS, pyrG, arcB, niaD and sC.

[0246] The term "control sequences" is defined herein to include all components, which are necessary or advantageous for the expression of the polypeptide variant of the invention. Each control sequence may be native or foreign to the nucleic acid sequence encoding the polypeptide variant. Such control sequences include, but are not limited to, a leader sequence, polyadenylation sequence, propeptide sequence, promoter, enhancer or upstream activating sequence, signal peptide sequence, and transcription terminator. At a minimum, the control sequences include a promoter.

[0247] A wide variety of expression control sequences may be used in the present invention. Such useful expression control sequences include the expression control sequences associated with structural genes of the foregoing expression vectors as well as any sequence known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof.

[0248] Examples of suitable control sequences for directing transcription in mammalian cells include the early and late promoters of SV40 and adenovirus, e.g. the adenovirus 2 major late promoter, the MT-1 (metallothionein gene) promoter, the human cytomegalovirus immediate-early gene promoter (CMV), the human elongation factor 1α (EF-1a) promoter, the *Drosophila* minimal heat shock protein 70 promoter, the Rous Sarcoma Virus (RSV) promoter, the human ubiquitin C (UbC) promoter, the human growth hormone terminator, SV40 or adenovirus E1b region polyadenylation signals and the Kozak consensus sequence (Kozak, M. *J Mol Biol* 1987 Aug. 20; 196(4):947-50).

[0249] In order to improve expression in mammalian cells a synthetic intron may be inserted in the 5' untranslated

region of the nucleotide sequence encoding the polypeptide. An example of a synthetic intron is the synthetic intron from the plasmid pCI-Neo (available from Promega Corporation, Wis., USA).

[0250] Examples of suitable control sequences for directing transcription in insect cells include the polyhedrin promoter, the P10 promoter, the *Autographa californica* polyhedrosis virus basic protein promoter, the baculovirus immediate early gene 1 promoter and the baculovirus 39K delayed-early gene promoter, and the SV40 polyadenylation sequence. Examples of suitable control sequences for use in yeast host cells include the promoters of the yeast α-mating system, the yeast triose phosphate isomerase (TPI) promoter, promoters from yeast glycolytic genes or alcohol dehydrogenase genes, the ADH2-4c promoter, and the inducible GAL promoter. Examples of suitable control sequences for use in filamentous fungal host cells include the ADH3 promoter and terminator, a promoter derived from the genes encoding *Aspergillus oryzae* TAKA amylase triose phosphate isomerase or alkaline protease, an *A. niger* α-amylase, *A. niger* or *A. nidulans* glucoamylase, *A. nidulans* acetamidase, *Rhizomucor miehei* aspartic proteinase or lipase, the TPI1 terminator and the ADH3 terminator. Examples of suitable control sequences for use in bacterial host cells include promoters of the lac system, the trp system, the TAC or TRC system, and the major promoter regions of phage lambda.

[0251] The presence or absence of a signal peptide will, e.g., depend on the expression host cell used for the production of the polypeptide variant to be expressed (whether it is an intracellular or extracellular polypeptide) and whether it is desirable to obtain secretion. For use in filamentous fungi, the signal peptide may conveniently be derived from a gene encoding an *Aspergillus* sp. amylase or glucoamylase, a gene encoding a *Rhizomucor miehei* lipase or protease or a *Humicola lanuginosa* lipase. The signal peptide is preferably derived from a gene encoding *A. oryzae* TAKA amylase, *A. niger* neutral α-amylase, *A. niger* acid-stable amylase, or *A. niger* glucoamylase. For use in insect cells, the signal peptide may conveniently be derived from an insect gene (cf. WO 90/05783), such as the Lepidopteran *manduca sexta* adipokinetic hormone precursor, (cf. U.S. Pat. No. 5,023,328), the honeybee melittin (Invitrogen), ecdysteroid UDPglucosyltransferase (egt) (Murphy et al., Protein Expression and Purification 4, 349-357 (1993) or human pancreatic lipase (hpl) (Methods in Enzymology 284, pp. 262-272, 1997). A preferred signal peptide for use in mammalian cells is that of hFVII or the murine Ig kappa light chain signal peptide (Coloma, M (1992) J. Imm. Methods 152:89-104). For use in yeast cells suitable signal peptides have been found to be the α-factor signal peptide from *S. cereviciae* (cf. U.S. Pat. No. 4,870,008), a modified carboxypeptidase signal peptide (cf. L. A. Valls et al., Cell 48, 1987, pp. 887-897), the yeast BAR1 signal peptide (cf. WO 87/02670), the yeast aspartic protease 3 (YAP3) signal peptide (cf. M. Egel-Mitani et al., Yeast 6, 1990, pp. 127-137), and the synthetic leader sequence TA57 (WO98/32867). For use in *E. coli* cells a suitable signal peptide have been found to be the signal peptide ompA (EP581821).

[0252] The nucleotide sequence of the invention encoding a polypeptide variant, whether prepared by site-directed mutagenesis, synthesis, PCR or other methods, may optionally include a nucleotide sequence that encode a signal peptide. The signal peptide is present when the polypeptide variant is to be secreted from the cells in which it is expressed. Such signal peptide, if present, should be one recognized by the cell chosen for expression of the polypeptide variant. The signal peptide will typically be the one normally associated with human wild-type FVII.

[0253] Any suitable host may be used to produce the polypeptide variant, including bacteria (although not particularly preferred), fungi (including yeasts), plant, insect, mammal, or other appropriate animal cells or cell lines, as well as transgenic animals or plants. Examples of bacterial host cells include grampositive bacteria such as strains of *Bacillus*, e.g. *B. brevis* or *B. subtilis*, or *Streptomyces*, or gramnegative bacteria, such as strains of *E. coli*. The introduction of a vector into a bacterial host cell may, for instance, be effected by protoplast transformation (see, e.g., Chang and Cohen, 1979, *Molecular General Genetics* 168: 111-115), using competent cells (see, e.g., Young and Spizizin, 1961, *Journal of Bacteriology* 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, *Journal of Molecular Biology* 56: 209-221), electroporation (see, e.g., Shigekawa and Dower, 1988, *Biotechniques* 6: 742-751), or conjugation (see, e.g., Koehler and Thorne, 1987, *Journal of Bacteriology* 169: 5771-5278). Examples of suitable filamentous fungal host cells include strains of *Aspergillus*, e.g. *A. oryzae, A. niger*, or *A. nidulans, Fusarium* or *Trichoderma*. Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known per se. Suitable procedures for transformation of *Aspergillus* host cells are described in EP 238 023 and U.S. Pat. No. 5,679,543. Suitable methods for transforming *Fusarium* species are described by Malardier et al., 1989, *Gene* 78: 147-156 and WO 96/00787. Examples of suitable yeast host cells include strains of *Saccharomyces*, e.g. *S. cerevisiae, Schizosaccharomyces, Kluyveromyces, Pichia*, such as *P. pastoris* or *P. methanolica, Hansenula*, such as *H. Polymorpha* or *Yarrowia*. Yeast may be transformed using the procedures described by Becker and Guarente, In Abelson, J. N. and Simon, M. I., editors, *Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology*, Volume 194, pp 182-187, Academic Press, Inc., New York; Ito et al., 1983, *Journal of Bacteriology* 153: 163; Hinnen et al., 1978, *Proceedings of the National Academy of Sciences USA* 75: 1920: and as disclosed by Clontech Laboratories, Inc, Palo Alto, Calif., USA (in the product protocol for the Yeastmaker™ Yeast Transformation System Kit). Examples of suitable insect host cells include a *Lepidoptora* cell line, such as *Spodoptera frugiperda* (Sf9 or Sf21) or *Trichoplusioa ni* cells (High Five) (U.S. Pat. No. 5,077,214). Transformation of insect cells and production of heterologous polypeptides therein may be performed as described by Invitrogen. Examples of suitable mammalian host cells include Chinese hamster ovary (CHO) cell lines, (e.g. CHO-K1; ATCC CCL61), Green Monkey cell lines (COS) (e.g. COS 1 (ATCC CRL-1650), COS 7 (ATCC CRL-1651)); mouse cells (e.g. NS/O), Baby Hamster Kidney (BHK) cell lines (e.g. ATCC CRL-1632 or ATCC CCL-10), and human cells (e.g. HEK 293 (ATCC CRL-1573)), as well as plant cells in tissue culture. Additional suitable cell lines are known in the art and available from public depositories such as the American Type Culture Collection, Rockville, Md. Also, the mammalian cell, such as a CHO cell, may be modified to express sialyltransferase, e.g. 1,6-sialyltrans-

ferase, e.g. as described in U.S. Pat. No. 5,047,335, in order to provide improved glycosylation of the polypeptide variant.

[0254] In order to increase secretion it may be of particular interest to produce the polypeptide variant of the invention together with an endoprotease, in particular a PACE (paired basic amino acid converting enzyme) (e.g. as described in U.S. Pat. No. 5,986,079), such as a Kex2 endoprotease (e.g. as described in WO 00/28065).

[0255] Methods for introducing exogeneous DNA into mammalian host cells include calcium phosphate-mediated transfection, electroporation, DEAE-dextran mediated transfection, liposome-mediated transfection, viral vectors and the transfection method described by Life Technologies Ltd, Paisley, UK using Lipofectamin 2000. These methods are well known in the art and e.g. described by Ausbel et al. (eds.), 1996, Current Protocols in Molecular Biology, John Wiley & Sons, New York, USA. The cultivation of mammalian cells are conducted according to established methods, e.g. as disclosed in Animal Cell Biotechnology, Methods and Protocols, Edited by Nigel Jenkins, 1999, Human Press Inc, Totowa, N.J., USA and Harrison M A and Rae I F, General Techniques of Cell Culture, Cambridge University Press 1997.

[0256] In the production methods of the present invention, the cells are cultivated in a nutrient medium suitable for production of the polypeptide variant using methods known in the art. For example, the cell may be cultivated by shake flask cultivation, small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermenters performed in a suitable medium and under conditions allowing the polypeptide to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or may be prepared according to published compositions (e.g., in catalogues of the American Type Culture Collection). If the polypeptide variant is secreted into the nutrient medium, the polypeptide can be recovered directly from the medium. If the polypeptide variant is not secreted, it can be recovered from cell lysates.

[0257] The resulting polypeptide variant may be recovered by methods known in the art. For example, the polypeptide variant may be recovered from the nutrient medium by conventional procedures including, but not limited to, centrifugation, filtration, extraction, spray drying, evaporation, or precipitation.

[0258] The polypeptides may be purified by a variety of procedures known in the art including, but not limited to, chromatography (e.g., ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (e.g., preparative isoelectric focusing), differential solubility (e.g., ammonium sulfate precipitation), HPLC, or extraction (see, e.g., *Protein Purification*, J.-C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989).

[0259] Single chain polypeptide variants of the invention can be purified and activated to two-chain polypeptide variants by a number of methods as described in the literature (Broze and Majerus, 1980, J. Biol. Chem. 255:1242-47 and Hedner and Kisiel, 1983, J. Clin. Invest. 71:1836-41). Another method whereby single chain polypeptide variants can be purified is by incorporation of Zn ions during purification as described in U.S. Pat. No. 5,700,914.

[0260] In a preferred embodiment the polypeptide variant is purified as a single chain polypeptide variant, which further is optionally PEGylated. The optionally PEGylated single chain polypeptide variant is activated by either use of an immobilized enzyme (e.g. factors IIa, IXa, Xa and XIIa) or by autoactivation using a positively charged ion exchange matrix or the like.

[0261] It is advantageous to first purify the polypeptide variant in its single chain form, then PEGylate (if desired) and lastly activate by one of the methods described above or by autoactivation as described by Pedersen et al, 1989, Biochemistry 28: 9331-36. The advantage of carrying out PEGylation before activation is that PEGylation of the new amino-terminus formed by cleavage of R152-1153 is avoided. PEGylation of this new amino-terminus would render the molecule inactive since the formation of a hydrogen bond between D242 and the amino group of 1153 is necessary for activity.

Pharmaceutical Composition of the Invention and its Use

[0262] In a further aspect, the present invention relates to a composition, in particular to a pharmaceutical composition, comprising a polypeptide variant of the invention and a pharmaceutically acceptable carrier or excipient.

[0263] The polypeptide variant or the pharmaceutical composition according to the invention may be used as a medicament.

[0264] Due to the high clotting efficiency, the polypeptide variant of the invention, or the pharmaceutical composition of the invention, is particular useful for the treatment of haemorrage, including uncontrollable bleeding events, such as uncontrollable bleding events in connection with trauma, thrombocytopenia, patients in anticoagulant treatment, and cirrhosis patients, such as cirrhosis patients with variceal bleeds, or other upper gastrointestinal bleedings, and in patients undergoing orthotopic liver transplantation, or liver resection (allowing for transfusion free surgery).

[0265] Trauma is defined as an injury to living tissue caused by an extrinsic agent. It is the 4th leading cause of death in the US and places a large financial burden on the economy.

[0266] Trauma is classified as either blunt or penetrative. Blunt trauma results in internal compression, organ damage and internal haemorrhage whereas penetrative trauma (as the consequence of an agent penetrating the body and destroying tissue, vessels and organs) results in external haemorrhage.

[0267] Haemorrhage, as a result of trauma, can start a cascade of problems. For example physiological compensation mechanisms are initiated with initial peripheral and mesenteric vasoconstriction to shunt blood to the central circulation. If circulation is not restored, hypovolemia shock (multiple organ failure due to inadequate perfusion) ensues. Since tissues throughout the body become starved for oxygen, anaerobic metabolism begins. However, the concomitant lactic acid leads the blood pH to drop and metabolic acidosis develops. If acidosis is severe and uncorrected, the patient may develop multisystem failure and die.

[0268] Although the majority of trauma patients are hypothermic on arrival in the emergency room due to the environmental conditions at the scene, inadequate protection, intravenous fluid administration and ongoing blood loss worsen the hypothermic state. Deficiencies in coagulation factors can result from blood loss or transfusions. Meanwhile, acidosis and hypothermia interfere with blood clotting mechanisms. Thus coagulopathy develops, which in turn, may mask surgical bleeding sites and hamper the control of mechanical bleeding. Hypothermia, coagulopathy and acidosis are often characterised as the "trauma triad of death"

[0269] Trauma may be caused by several events. For example, road traffic accidents result in many different types of trauma. Whilst some road traffic accidents are likely to result in penetrative trauma, many road traffic accidents are likely to inflict blunt trauma to both head and body. However, these various types of trauma can all result in coagulopathy in the patient. Road traffic accidents are the leading cause of accidental death in the US. There are over 42,000 deaths from them in the US each year. Many trauma patients die at the location of the accident either whilst being treated by the paramedics, before they arrive or in transit to the ER.

[0270] Another example includes gunshot wounds. Gunshot wounds are traumas that can result in massive bleeding. They are penetrative and destroy tissue as the bullet passes through the body, whether it be in the torso or a limb. In the US about 40,000 people a year die from gunshot wounds

[0271] A further example includes falls. Falls result in a similar profile of trauma type to road traffic accidents. By falling onto a solid object or the ground from height can cause both penetrative and decelerative blunt trauma. In the US, falls are a common cause of accidental death, numbering about 13,000.

[0272] A still further example includes machinery accidents. A smaller number of people die in the US from machinery accident related deaths, whether struck by, or entangled in machinery. The figures are small but significant—around 2,000.

[0273] A still further example includes stab wounds. Stab wounds are penetrative injuries that can also cause massive bleeding. The organs most likely to be damaged in a stab wound are the liver, small intestine and the colon.

[0274] Cirrhosis of the liver is the terminal sequel of prolonged repeated injury to the hepatic parenchyma. The end result is the formation of broad bands of fibrous tissue separating regenerative nodules that do not maintain the normal organization of liver lobules and thus cause deteriorated liver function. Patients have prolonged prothrombin times as a result of the depletion of vitamin K-dependent coagulation factors. Pathogenetically, liver cirrhosis should be regarded as the final common pathway of chronic liver injury, which can result from any form of intense repeated prolonged liver cell injury. Cirrhosis of the liver may be caused by direct liver injury, including chronic alcoholism, chronic viral hepatitis (types B, C, and D), and auto immune hepatitis as well as by indirect injury by way of bile duct damage, including primary biliary cirrhosis, primary sclerosing cholangitis and biliary atresia. Less common causes of cirrhosis include direct liver injury from inherited disease such as cystic fibrosis, alpha-1-antitrypsin deficiency, hemochromatosis, Wilson's disease, galactosemia, and glycogen storage disease.

[0275] Transplantation is primarily reserved for late stage cirrhotic patients, where it is the key intervention for treating the disease. To be eligible for transplantation, a patient must be classified as Child's B or C, as well as meet additional criteria for selection. Last year, in the US alone, 4,954 transplants were performed.

[0276] It has been estimated that there are 6,000 bleeding episodes associated with patients undergoing resection each year. This correlates with the reserved position of this procedure although seems slightly high in comparison with transplantation numbers. Accurate data on the incidence of variceal bleeding is hard to obtain. The key facts known are that at the time of diagnosis, varices are present in about 60% of decompensated and 30% of compensated patients and that about 30% of these patients with varices will experience a bleed and that each episode of variceal bleeding is associated with a 30% risk of mortality.

[0277] Thrombocytopenia is caused by one of three mechanisms-decreased bone marrow production, increased splenic sequestration, or accelerated destruction of platelets. Thrombocytopenia is a risk factor for hemorrhage, and platelet transfusion reduces the incidence of bleeding. The threshold for prophylactic platelet transfusion is 10,000/μl. In patients without fever or infections, a threshold of 5000/μl may be sufficient to prevent spontaneous hemorrhage. For invasive procedures, 50,000/μl platelets is the usual target level. In patients who develop antibodies to platelets following repeated transfusions, bleeding can be extremely difficult to control.

[0278] Thus, in a further aspect the present invention relates to the use of a polypeptide variant of the invention for the manufacture of a medicament for the treatment of diseases or disorders wherein clot formation is desirable. A still further aspect of the present invention relates to a method for treating a mammal having a disease or disorder wherein clot formation is desirable, comprising administering to a mammal in need thereof an effective amount of a polypeptide variant or the pharmaceutical composition of the invention.

[0279] Examples of diseases/disorders wherein clot formation is desirable include, but is not limited to, hemorrhages, including brain hemorrhages, as well as uncontrolled bleedings, such as trauma. Further examples include patients undergoing living transplantations, patients undergoing resection, thrombocytopenic patients, cirrhotic patients, patients with variceal bleedings, haemophilia A, haemophilia B and von Willebrands disease.

[0280] The polypeptide variant of the invention is administered to patients in a therapeutically effective dose, normally one approximately paralleling that employed in therapy with rFVII such as NovoSeven®, or at lower dosage. By "therapeutically effective dose" herein is meant a dose that is sufficient to produce the desired effects in relation to the condition for which it is administered. The exact dose will depend on the circumstances, and will be ascertainable by one skilled in the art using known techniques. Normally, the dose should be capable of preventing or lessening the severity or spread of the condition or indication being treated. It will be apparent to those of skill in the art that an effective amount of a polypeptide variant or composition of the invention depends, inter alia, upon the disease, the dose, the administration schedule, whether the

polypeptide variant or composition is administered alone or in conjunction with other therapeutic agents, the plasma half-life of the compositions, and the general health of the patient. Preferably, the polypeptide variant or composition of the invention is administered in an effective dose, in particular a dose which is sufficient to normalize the coagulation disorder.

[0281] The polypeptide variant of the invention is preferably administered in a composition including a pharmaceutically acceptable carrier or excipient. "Pharmaceutically acceptable" means a carrier or excipient that does not cause any untoward effects in patients to whom it is administered. Such pharmaceutically acceptable carriers and excipients are well known in the art (see, for example, Remington's Pharmaceutical Sciences, 18th edition, A. R. Gennaro, Ed., Mack Publishing Company [1990]; Pharmaceutical Formulation Development of Peptides and Proteins, S. Frokjaer and L. Hovgaard, Eds., Taylor & Francis [2000]; and Handbook of Pharmaceutical Excipients, 3rd edition, A. Kibbe, Ed., Pharmaceutical Press [2000]).

[0282] The polypeptide variant of the invention can be formulated into pharmaceutical compositions by well-known methods. Suitable formulations are described by Remington's Pharmaceutical Sciences by E. W. Martin (Mark Pubi. Co., 16th Ed., 1980).

[0283] The polypeptide variants of the invention can be used "as is" and/or in a salt form thereof. Suitable salts include, but are not limited to, salts with alkali metals or alkaline earth metals, such as sodium, potassium, calcium and magnesium, as well as e.g. zinc salts. These salts or complexes may by present as a crystalline and/or amorphous structure.

[0284] The pharmaceutical composition of the invention may be administered alone or in conjunction with other therapeutic agents. These agents may be incorporated as part of the same pharmaceutical composition or may be administered separately from the polypeptide variant of the invention, either concurrently or in accordance with another treatment schedule. In addition, the polypeptide variant or pharmaceutical composition of the invention may be used as an adjuvant to other therapies.

[0285] A "patient" for the purposes of the present invention includes both humans and other mammals. Thus, the methods are applicable to both human therapy and veterinary applications. The pharmaceutical composition comprising the polypeptide variant of the invention may be formulated in a variety of forms, e.g. as a liquid, gel, lyophilized, or as a compressed solid. The preferred form will depend upon the particular indication being treated and will be apparent to one skilled in the art.

[0286] In particular, the pharmaceutical composition comprising the polypeptide variant of the invention may be formulated in lyophilised or stable soluble form. The polypeptide variant may be lyophilised by a variety of procedures known in the art. The polypeptide variant may be in a stable soluble form by the removal or shielding of proteolytic degradation sites as described herein. The advantage of obtaining a stable soluble preparation lies in easier handling for the patient and, in the case of emergencies, quicker action, which potentially can become life saving. The preferred form will depend upon the particular indication being treated and will be apparent to one of skill in the art.

[0287] The administration of the formulations of the present invention can be performed in a variety of ways, including, but not limited to, orally, subcutaneously, intravenously, intracerebrally, intranasally, transdermally, intraperitoneally, intramuscularly, intrapulmonary, vaginally, rectally, intraocularly, or in any other acceptable manner. The formulations can be administered continuously by infusion, although bolus injection is acceptable, using techniques well known in the art, such as pumps or implantation. In some instances the formulations may be directly applied as a solution or spray.

Parenterals

[0288] A preferred example of a pharmaceutical composition is a solution, in particular an aqueous solution, designed for parenteral administration. Although in many cases pharmaceutical solution formulations are provided in liquid form, appropriate for immediate use, such parenteral formulations may also be provided in frozen or in lyophilized form. In the former case, the composition must be thawed prior to use. The latter form is often used to enhance the stability of the active compound contained in the composition under a wider variety of storage conditions, as it is recognized by those skilled in the art that lyophilized preparations are generally more stable than their liquid counterparts. Such lyophilized preparations are reconstituted prior to use by the addition of one or more suitable pharmaceutically acceptable diluents such as sterile water for injection or sterile physiological saline solution.

[0289] In case of parenterals, they are prepared for storage as lyophilized formulations or aqueous solutions by mixing, as appropriate, the polypeptide variant having the desired degree of purity with one or more pharmaceutically acceptable carriers, excipients or stabilizers typically employed in the art (all of which are termed "excipients"), for example buffering agents, stabilizing agents, preservatives, isotonifiers, non-ionic surfactants or detergents, antioxidants and/or other miscellaneous additives.

[0290] Buffering agents help to maintain the pH in the range which approximates physiological conditions. They are typically present at a concentration ranging from about 2 mM to about 50 mM. Suitable buffering agents for use in the present invention include both organic and inorganic acids and salts thereof such as citrate buffers (e.g., monosodium citrate-disodium citrate mixture, citric acid-trisodium citrate mixture, citric acid-monosodium citrate mixture, etc.), succinate buffers (e.g., succinic acid-monosodium succinate mixture, succinic acid-sodium hydroxide mixture, succinic acid-disodium succinate mixture, etc.), tartrate buffers (e.g., tartaric acid-sodium tartrate mixture, tartaric acid-potassium tartrate mixture, tartaric acid-sodium hydroxide mixture, etc.), fumarate buffers (e.g., fumaric acid-monosodium fumarate mixture, fumaric acid-disodium fumarate mixture, monosodium fumarate-disodium fumarate mixture, etc.), gluconate buffers (e.g., gluconic acid-sodium glyconate mixture, gluconic acid-sodium hydroxide mixture, gluconic acid-potassium glyuconate mixture, etc.), oxalate buffer (e.g., oxalic acid-sodium oxalate mixture, oxalic acid-sodium hydroxide mixture, oxalic acid-potassium oxalate mixture, etc.), lactate buffers (e.g., lactic acid-sodium lactate mixture, lactic acid-sodium hydroxide mixture, lactic acid-potassium lactate mixture, etc.) and acetate buffers (e.g., acetic acid-sodium acetate

mixture, acetic acid-sodium hydroxide mixture, etc.). Additional possibilities are phosphate buffers, histidine buffers and trimethylamine salts such as Tris.

[0291] Stabilizers refer to a broad category of excipients, which can range in function from a bulking agent to an additive which solubilizes the therapeutic agent or helps to prevent denaturation or adherence to the container wall. Typical stabilizers can be polyhydric sugar alcohols (enumerated above); amino acids such as arginine, lysine, glycine, glutamine, asparagine, histidine, alanine, ornithine, L-leucine, 2-phenylalanine, glutamic acid, threonine, etc., organic sugars or sugar alcohols, such as lactose, trehalose, stachyose, mannitol, sorbitol, xylitol, ribitol, myoinisitol, galactitol, glycerol and the like, including cyclitols such as inositol; polyethylene glycol; amino acid polymers; sulfur-containing reducing agents, such as urea, glutathione, thioctic acid, sodium thioglycolate, thioglycerol, α-monothioglycerol and sodium thiosulfate; low molecular weight polypeptides (i.e. <10 residues); proteins such as human serum albumin, bovine serum albumin, gelatin or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; monosaccharides such as xylose, mannose, fructose and glucose; disaccharides such as lactose, maltose and sucrose; trisaccharides such as raffinose, and polysaccharides such as dextran. Stabilizers are typically present in the range of from 0.1 to 10,000 parts by weight based on the active protein weight.

[0292] Preservatives are added to retard microbial growth, and are typically added in amounts of about 0.2%-1% (w/v). Suitable preservatives for use with the present invention include phenol, benzyl alcohol, meta-cresol, methyl paraben, propyl paraben, octadecyldimethylbenzyl ammonium chloride, benzalkonium halides (e.g. benzalkonium chloride, bromide or iodide), hexamethonium chloride, alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol and 3-pentanol.

[0293] Isotonicifiers are added to ensure isotonicity of liquid compositions and include polyhydric sugar alcohols, preferably trihydric or higher sugar alcohols, such as glycerin, erythritol, arabitol, xylitol, sorbitol and mannitol. Polyhydric alcohols can be present in an amount between 0.1% and 25% by weight, typically 1% to 5%, taking into account the relative amounts of the other ingredients.

[0294] Non-ionic surfactants or detergents (also known as "wetting agents") may be present to help solubilizing the therapeutic agent as well as to protect the therapeutic polypeptide against agitation-induced aggregation, which also permits the formulation to be exposed to shear surface stress without causing denaturation of the polypeptide. Suitable non-ionic surfactants include polysorbates (20, 80, etc.), polyoxamers (184, 188 etc.), Pluronic® polyols, polyoxyethylene sorbitan monoethers (Tween®-20, Tween®-80, etc.).

[0295] Additional miscellaneous excipients include bulking agents or fillers (e.g. starch), chelating agents (e.g. EDTA), antioxidants (e.g., ascorbic acid, methionine, vitamin E) and cosolvents.

[0296] The active ingredient may also be entrapped in microcapsules prepared, for example, by coascervation techniques or by interfacial polymerization, for example hydroxymethylcellulose, gelatin or poly-(methylmethacylate) microcapsules, in colloidal drug delivery systems (for example liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, supra.

[0297] Parenteral formulations to be used for in vivo administration must be sterile. This is readily accomplished, for example, by filtration through sterile filtration membranes.

Sustained Release Preparations

[0298] Examples of sustained-release preparations include semi-permeable matrices of solid hydrophobic polymers containing the polypeptide variant, the matrices having a suitable form such as a film or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate) or poly(vinylalcohol)), polylactides, copolymers of L-glutamic acid and ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the ProLease® technology or Lupron Depot® (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(−)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for long periods such as up to or over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated polypeptides remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37° C., resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S—S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

[0299] The invention is further described in the following non-limiting examples.

Materials and Methods

Accessible Surface Area (ASA)

[0300] The computer program Access (B. Lee and F. M. Richards, J. Mol. Biol. 55: 379-400 (1971)) version 2 (© 1983 Yale University) is used to compute the accessible surface area (ASA) of the individual atoms in the structure. This method typically uses a probe-size of 1.4A and defines the Accessible Surface Area (ASA) as the area formed by the center of the probe. Prior to this calculation all water molecules and all hydrogen atoms should be removed from the coordinate set, as should other atoms not directly related to the protein.

Fractional ASA of Side Chain

[0301] The fractional ASA of the side chain atoms is computed by division of the sum of the ASA of the atoms in the side chain with a value representing the ASA of the side chain atoms of that residue type in an extended Ala-x-Ala tripeptide (See Hubbard, Campbell & Thornton (1991) J. Mol. Biol. 220,507-530). For this example the CA atom is regarded as a part of the side chain of Glycine residues but

not for the remaining residues. The following table is used as standard 100% ASA for the side chain:

Ala	69.23 Å^2
Arg	200.35 Å^2
Asn	106.25 Å^2
Asp	102.06 Å^2
Cys	96.69 Å^2
Gln	140.58 Å^2
Glu	134.61 Å^2
Gly	32.28 Å^2
His	147.00 Å^2
Ile	137.91 Å^2
Leu	140.76 Å^2
Lys	162.50 Å^2
Met	156.08 Å^2
Phe	163.90 Å^2
Pro	119.65 Å^2
Ser	78.16 Å^2
Thr	101.67 Å^2
Trp	210.89 Å^2
Tyr	176.61 Å^2
Val	114.14 Å^2

[0302] Residues not detected in the structure are defined as having 100% exposure as they are thought to reside in flexible regions. The gamma-carboxy glutamic acids at positions 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35 are all defined as being 100% exposed.

Determining Distances Between Atoms

[0303] The distance between atoms is most easily determined using molecular graphics software e.g. InsightII® v. 98.0, MSI INC.

Active Site Region

[0304] The active site region is defined as any residues having at least one atom within 10 Å of any atom in the catalytic triad (residues H193, D242, S344).

Determination of Tissue Factor Binding Site

[0305] The TF binding site is defined as comprising all residues having their accessible surface area changed upon TF binding. This is determined by at least two ASA calculations; one on the isolated ligand(s) in the ligand(s)/receptor(s) complex and one on the complete ligand(s)/receptor(s) complex.

Measurement of Reduced Sensitivity to Proteolytic Degradation

[0306] Proteolytic degradation can be measured using the assay described in U.S. Pat. No. 5,580,560, Example 5, where proteolysis is autoproteolysis.

[0307] Furthermore, reduced proteolysis can be tested in an in vivo model using radiolabelled samples and comparing proteolysis of rhFVIIa and the polypeptide variant of the invention by withdrawing blood samples and subjecting these to SDS-PAGE and autoradiography.

[0308] Irrespectively of the assay used for determining proteolytic degradation, "reduced proteolytic degradation" is intended to mean a measurable reduction in cleavage compared to that obtained by rhFVIIa as measured by gel scanning of Coomassie stained SDS-PAGE gels, HPLC or as measured by conserved catalytic activity in comparison to wild type using the tissue factor independent activity assay decribed below.

Determination of the Molecular Weight of Polypeptide Variants

[0309] The molecular weight of polypeptide variants is determined by either SDS-PAGE, gel filtration, Western Blots, matrix assisted laser desorption mass spectrometry or equilibrium centrifugation, e.g. SDS-PAGE according to Laemmli, U.K., Nature Vol 227 (1970), pp. 680-85.

Determination of Tissue Factor Binding Affinity

[0310] The capacity of variants to bind to tissue factor may be evaluated using one or more of the three BIAcore® assays described in Dickinson et al. Proc. Natl. Acad. Sci. USA 1996, 93: 14379-14384; Roberge et al. Biochemistry 2001, 40: 9522-9531; and Ruf et al. Biochemistry 1999, 38(7): 1957-1966.

Determination of TFPI Inhibition

[0311] FVII inhibition by TFPI can be monitored in the amidolytic assay described in Chang et al. Biochemistry 1999, 38: 10940-10948.

Determination of TFPI Affinity

[0312] The capacity of variants to bind to TFPI is evaluated using one or more of the three BLAcore® assays described in Dickinson et al. Proc. Natl. Acad. Sci. USA 1996, 93: 14379-14384; Roberge et al. Biochemistry 2001, 40: 9522-9531; and Ruf et al. Biochemistry 1999, 38(7): 1957-1966.

Determination of Phospholipid Membrane Binding Affinity

[0313] Phospholipid membrane binding affinity may be determined as described in Nelsestuen et al., Biochemistry, 1977; 30; 10819-10824 or as described in Example 1 in U.S. Pat. No. 6,017,882.

TF-Independent Factor X Activation Assay

[0314] This assay has been described in detail on page 39826 in Nelsestuen et al., J Biol Chem, 2001; 276:39825-39831.

[0315] Briefly, the molecule to be assayed (either hFVIIa, rhFVIIa or the polypeptide variant of the invention in its activated form) is mixed with a source of phospholipid (preferably phosphatidylcholine and phosphatidylserine in a ratio of 8:2) and relipidated Factor X in Tris buffer containing BSA. After a specified incubation time the reaction is stopped by addition of excess EDTA. The concentration of factor Xa is then measured from absorbance change at 405 nm after addition of a chromogenic substrate (S-2222, Chromogenix). After correction from background the tissue factor independent activity of rhFVIIa (a_{wt}) is determined as the absorbance change after 10 minutes and the tissue factor independent activity of the polypeptide variant of the invention (a_{vaiant}) is also determined as the absorbance change after 10 minutes. The ratio between the activity of the polypeptide variant, in its activated form, and the activity of rhFVIIa is defined as $a_{variant}/a_{wt}$.

Clotting Assay

[0316] The clotting activity of the FVIIa and variants thereof were measured in one-stage assays and the clotting

times were recorded on a Thrombotrack IV coagulometer (Medinor). Factor VII-depleted human plasma (American Diagnostica) was reconstituted and equilibrated at room temperature for 15-20 minutes. 50 microliters of plasma was then transferred to the coagulometer cups.

[0317] FVIIa and variants thereof were diluted in Glyoxaline Buffer (5.7 mM barbiturate, 4.3 mM sodium citrate, 117 mM NaCl, 1 mg/ml BSA, pH 7.35). The samples were added to the cup in 50 ul and incubated at 37° C. for 2 minutes.

[0318] Thromboplastin (Medinor) was reconstituted with water and $CaCl_2$ was added to a final concentration of 4.5 mM. The reaction was initiated by adding 100 μl thromboplastin.

[0319] To measure the clotting activity in the absence of TF the same assay was used without addition of thromboplastin. Data was analysed using PRISM software.

Whole Blood Assay

[0320] The clotting activity of FVIIa and variants thereof were measured in one-stage assays and the clotting times were recorded on a Thrombotrack IV coagulometer (Medinor). 100 μl of FVIIa or variants thereof were diluted in a buffer containing 10 mM glycylglycine, 50 mM NaCl, 37.5 mM $CaCl_2$, pH 7.35 and transferred to the reaction cup. The clotting reaction was initiated by addition of 50 μl blood containing 10% 0.13 M tri-sodium citrate as anticoagulant. Data was analysed using Excel or PRISM software.

Amidolytic Assay

[0321] The ability of the variants to cleave small peptide substrates can be measured using the chromogenic substrate S-2288 (D-Ile-Pro-Arg-p-nitroanilide). FVIIa is diluted to about 10-90 nM in assay buffer (50 mM Na-Hepes pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 0.1% BSA, IU/ml Heparin). Furthermore, soluble TF (sTF) is diluted to 50-450 nM in assay buffer. 120 μl of assay buffer is mixed with 20 μl of the FVIIa sample and 20 μl sTF. After 5 min incubation at room temperature with gentle shaking, followed by 10 min incubation at 37° C., the reaction is started by addition of the S-2288 substrate to 1 mM and the absorption at 405 nm is determined at several time points.

ELISA Assay

[0322] FVII/FVIIa (or variant) concentrations are determined by ELISA. Wells of a microtiter plate are coated with an antibody directed against the protease domain using a solution of 2 μg/ml in PBS (100 μl per well). After overnight coating at R.T., the wells are washed 4 times with THT buffer (100 mM NaCl, 50 mM Tris-HCl pH 7.2 0.05% Tween-20). Subsequently, 200 μl of 1% Casein (diluted from 2.5% stock using 100 mM NaCl, 50 mM Tris-HCl pH 7.2) is added per well for blocking. After 1 hr incubation at R.T., the wells are emptied, and 100 μl of sample (optionally diluted in dilution buffer (THT+0.1% Casein)) is added. After another incubation of 1 hr at room temperature, the wells are washed 4 times with THT buffer, and 100 μl of a biotin-labelled antibody directed against the EGF-like domain (1 μg/ml) is added. After another 1 hr incubation at R.T., followed by 4 more washes with THT buffer, 100 μl of streptavidin-horse radish peroxidase (DAKO A/S, Glostrup, Denmark, 1/10000 diluted) is added. After another 1 hr incubation at R.T., followed by 4 more washes with THT buffer, 100 μl of TMB (3,3',5,5'-tetramethylbenzidine, Kem-en-Tech A/S, Denmark) is added. After 30 min incubation at R.T. in the dark, 100 μl of 1 M H_2SO_4 is added and OD_{450nm} is determined. A standard curve is prepared using rhFVIIa (NovoSeven®).

[0323] Alternatively, FVII/FVIIa or variants may be quantified through the Gla domain rather than through the protease domain. In this ELISA set-up, wells are coated overnight with an antibody directed against the EGF-like domain and for detection, a calcium-dependent biotin-labelled monoclonal anti-Gla domain antibody is used (2 μg/ml, 100 μl per well). In this set-up, 5 mM $CaCl_2$ is added to the THT and dilution buffers.

EXAMPLES

Example 1

[0324] The X-ray structure of hFVIIa in complex with soluble tissue factor by Banner et al., J Mol Biol, 1996; 285:2089 is used for this example. It is noted that the numbering of residues in the reference does not follow the sequence. Here we have used the sequential numbering according to SEQ ID NO:1. The gamma-carboxy glutamic acids at positions 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35 are all here named Glu (three letter abbreviation) or E (one letter abbreviation). Residues 143-152 are not present in the structure.

Surface Exposure

[0325] Performing fractional ASA calculations on FVII fragments alone combined with the definition of accessibilities of non standard and/or missing residues described in the methods resulted in the following residues having more than 25% of their side chain exposed to the surface: A1, N2, A3, F4, L5, E6, E7, L8, R9, P10, S12, L13, E14, E16, K18, E19, E20, Q21, S23, F24, E25, E26, R28, E29, F31, K32, D33, A34, E35, R36, K38, L39, W41, I42, S43, S45, G47, D48, Q49, A51, S52, S53, Q56, G58, S60, K62, D63, Q64, L65, Q66, S67, I69, F71, L73, P74, A75, E77, G78, R79, E82, T83, H84, K85, D86, D87, Q88, L89, I90, V92, N93, E94, G97, E99, S103, D104, H105, T106, G107, T108, K109, S111, R113, E116, G117, S119, L120, L121, A122, D123, G124, V125, S126, T128, P129, T130, V131, E132, I140, L141, E142, K143, R144, N145, A146, S147, K148, P149, Q150, G151, R152, G155, K157, V158, P160, K161, E163, L171, N173, G174, A175, N184, T185, I186, H193, K197, K199, N200, R202, N203, I205, S214, E215, H216, D217, G218, D219, S222, R224, S232, T233, V235, P236, G237, T238, T239, N240, H249, Q250, P251, V253, T255, D256, E265, R266, T267, E270, R271, F275, V276, R277, F278, L280, L287, L288, D289, R290, G291, A292, T293, L295, E296, N301, M306, T307, Q308, D309, L311, Q312, Q313, R315, K316, V317, G318, D319, S320, P321, N322, T324, E325, Y326, Y332, S333, D334, S336, K337, K341, G342, H351, R353, G354, Q366, G367, T370, V371, G372, R379, E385, Q388, K389, R392, S393, E394, P395, R396, P397, G398, V399, L400, L401, R402, P404 and P406.

[0326] The following residues had more than 50% of their side chain exposed to the surface: A1, A3, F4, L5, E6, E7, L8, R9, P10, E14, E16, K18, E19, E20, Q21, S23, E25, E26, E29, K32, A34, E35, R36, K38, L39, I42, S43, G47, D48, A51, S52, S53, Q56, G58, S60, K62, L65, Q66, S67, I69, F71, L73, P74, A75, E77, G78, R79, E82, H84, K85, D86, D87, Q88, L89, I90, V92, N93, E94, G97, T106, G107,

T108, K109, S111, E116, S119, L121, A122, D123, G124, V131, E132, L141, E142, K143, R144, N145, A146, S147, K148, P149, Q150, G151, R152, G155, K157, P160, N173, G174, A175, K197, K199, N200, R202, S214, E215, H216, G218, R224, V235, P236, G237, T238, H249, Q250, V253, D256, T267, F275, R277, F278, L288, D289, R290, G291, A292, T293, L295, N301, M306, Q308, D309, L311, Q312, Q313, R315, K316, G318, D319, N322, E325, D334, K341, G354, G367, V371, E385, K389, R392, E394, R396, P397, G398, R402, P404 and P406.

Tissue Factor Binding Site

[0327] Performing ASA calculations the following residues in human FVII change their ASA in the complex. These residues were defined as constituting the tissue factor binding site: L13, K18, F31, E35, R36, L39, F40, I42, S43, S60, K62, D63, Q64, L65, I69, C70, F71, C72, L73, P74, F76, E77, G78, R79, E82, K85, Q88, I90, V92, N93, E94, R271, A274, F275, V276, R277, F278, R304, L305, M306, T307, Q308, D309, Q312, Q313, E325 and R379.

Active Site Region

[0328] The active site region is defined as any residue having at least one atom within a distance of 10 Å from any atom in the catalytic triad (residues H193, D242, S344): I153, Q167, V168, L169, L170, L171, Q176, L177, C178, G179, G180, T181, V188, V189, S190, A191, A192, H193, C194, F195, D196, K197, I198, W201, V228, I229, I230, P231, S232, T233, Y234, V235, P236, G237, T238, T239, N240, H241, D242, I243, A244, L245, L246, V281, S282, G283, W284, G285, Q286, T293, T324, E325, Y326, M327, F328, D338, S339, C340, K341, G342, D343, S344, G345, G346, P347, H348, L358, T359, G360, 1361, V362, S363, W364, G365, C368, V376, Y377, T378, R379, V380, Q382, Y383, W386, L387, L400 and F405.

The Ridge of the Active Site Binding Cleft

[0329] The ridge of the active site binding cleft region was defined by visual inspection of the FVIIa structure 1FAK-.pdb as: N173, A175, K199, N200, N203, D289, R290, G291, A292, P321 and T370.

Example 2

Design of an Expression Cassette for Expression of rhFVII in Mammalian Cells

[0330] The DNA sequence shown in SEQ ID NO:2, encompassing the short form of the full length cDNA encoding human blood coagulation factor VII with its native short signal peptide (Hagen et al., 1986. PNAS 83:2412), was synthesized in order to facilitate high expression in mammalian cells. First the ATG start codon context was modified according to the Kozak consensus sequence (Kozak, M. *J Mol Biol* 1987 Aug. 20; 196(4):947-50), so that there is a perfect match to the consensus sequence upstream of the ATG start codon. Secondly the open reading frame of the native human blood coagulation factor cDNA was modified by making a bias in the codon usage towards the codons frequently used in highly expressed human genes. Further, two translational stop codons were inserted at the end of the open reading frame in order to facilitate efficient translational stop. The fully synthetic and expression optimized human FVII gene was assembled from 70-mer DNA oligonucleotides and finally amplified using end primers inserting BamHI and HindIII sites at the 5' and 3' ends respectively using standard PCR techniques.

[0331] A vector for the cloning of the generated PCR product encompassing the expression cassette for factor VII was prepared by cloning the intron from pCINeo (Promega). The synthetic intron from pCI-Neo was amplified using standard PCR conditions and the primers:

```
                                                    (SEQ ID NO:3)
CBProFpr174: AGCTGGCTAGCCACTGGGCAGGTAAGTATCA
and

                                                    (SEQ ID NO:4)
CBProFpr175: TGGCGGGATCCTTAAGAGCTGTAATTGAACT
```

resulting in a 332 bp PCR fragment. The fragment was cut with NheI and BamHI before cloning into pcDNA3.1I/HygR (obtained from Invitrogen) resulting in PF#34.

[0332] The expression cassette for human factor VII was cloned between the BamHI and HindIII sites of PF#34, resulting in plasmid PF#226.

[0333] In order to allow for cloning of FVII-variant genes between the NheI and PmeI sites of the UCOE based expression-plasmid CET720 a derivative of PF#226 lacking the NheI site was created. Using PF#226 as template a DNA fragment was made by PCR using the primers CBProFpr219 (SEQ ID NO:5) and CBProFpr499 (SEQ ID NO:6). This fragment was cut with XbaI and XhoI and cloned between the XbaI and XhoI sites of PF#226 resulting in the plasmid PF#444. PF#444-derivative plasmids encoding variants of the invention were constructed by standard PCR-based site-directed mutagenesis using PF#444 as template and custom-synthesized primers. Specific CET720 based expression plasmids were generated by excision of the variant gene from the corresponding PF444-derivative by NheI and PmeI and then cloned into NheI and PmeI cut CET720. For example the CET720-derivative pB0088 encoding the FVII S43Q variant was made by excision of the variant gene by NheI and PmeI from pB0075 and cloned into NheI and PmeI cut CET720.

Example 3

Construction of Expression Vectors Encoding Polypeptide Variants of the Invention

[0334] The following primers were used pair vice for the primary PCRs:

```
[L39H]rhFVII
CBProFpr526:        GCTGAGCGGACCAAACACTTTTGGATTAGC
                    (SEQ ID NO:7 - direct primer)
CBProFpr527:        GCTAATCCAAAAGTGTTTGGTCCGCTCAGC
                    (SEQ ID NO:8 - reverse primer)
```

-continued

[I42R]rhFVII

CBProFpr530: CCAAACTGTTTTGGCGCAGCTATAGCGATG
(SEQ ID NO:9 - direct primer)

CBProFpr531: CATCGCTATAGCTGCGCCAAAACAGTTTGG
(SEQ ID NO:10 - reverse primer)

[S43Q]rhFVII

CBProFpr534: AACTGTTTTGGATTCAGTATAGCGATGGCG
(SEQ ID NO:11 - direct primer)

CBProFpr535: CGCCATCGCTATACTGAATCCAAAACAGTT
(SEQ ID NO:12 - reverse primer)

[K62E]rhFVII

CBProFpr536: AACGGGGGCTCCTGCGAGGACCAGCTGCAG
(SEQ ID NO:13 - direct primer)

CBProFpr537: CTGCAGCTGGTCCTCGCAGGAGCCCCCGTT
(SEQ ID NO:14 - reverse primer)

[K62R]rhFVII

CBProFpr538: GGGGGCTCCTGCCGCGACCAGCTGCAGAGC
(SEQ ID NO:15 - direct primer)

CBProFpr539: GCTCTGCAGCTGGTCGCGGCAGGAGCCCCC
(SEQ ID NO:16 - reverse primer)

[F71E]rhFVII

CBProFpr540: GAGCTATATCTGCGAGTGCCTGCCTGCCTT
(SEQ ID NO:17 - direct primer)

CBProFpr541: AAGGCAGGCAGGCACTCGCAGATATAGCTC
(SEQ ID NO:18 - reverse primer)

[E82Q]rhFVII

CBProFpr542: GGGGCGCAATTGCCAGACCCATAAGGATGA
(SEQ ID NO:19 - direct primer)

CBProFpr543: TCATCCTTATGGGTCTGGCAATTGCGCCCC
(SEQ ID NO:20 - reverse primer)

[L39E]rhFVII

LoB069: GAGCGGACCAAAGAGTTTTGGATTAGC
(SEQ ID NO:21 - direct primer)

LoB070: GCTAATCCAAAACTCTTTGGTCCGCTG
(SEQ ID NO:22 - reverse primer)

[L39Q]rhFVII

LoB071: GAGCGGACCAAACAGTTTTGGATTAGC
(SEQ ID NO 23 - direct primer)

LoB072: GCTAATCCAAAACTGTTTGGTCCGCTC
(SEQ ID NO:24 - reverse primer)

[L65Q]rhFVII

LoB075: CTGCAAAGACCAGCAGCAGAGCTATATCTGC
(SEQ ID NO:25 - direct primer)

LoB076: GCAGATATAGCTCTGCTGCTGGTCTTTGCAG
(SEQ ID NO:26 - reverse primer)

[L65S]rhFVII

LoB077: CTGCAAAGACCAGTCCCAGAGCTATATCTGC
(SEQ ID NO:27 - direct primer)

LoB078: GCAGATATAGCTCTGGGACTGGTCTTTGCAG
(SEQ ID NO:28 - reverse primer)

[F71D]rhFVII

LoB079: CAGAGCTATATCTGCGACTGCCTGCCTGCC
(SEQ ID NO:29 - direct primer)

LoB080: GGCAGGCAGGCAGTCGCAGATATAGCTCTG
(SEQ ID NO:30 - reverse primer)

[F71Y]rhFVII

LoB081: CAGAGCTATATCTGCTACTGCCTGCCTGC
(SEQ ID NO:31 - direct primer)

LoB082: GCAGGCAGGCAGTAGCAGATATAGCTCTG
(SEQ ID NO: 32 - reverse primer)

[K62E + L65Q]rhFVII

CBProFpr703: GAACGGGGGCTCCTGCGAGGACCAGCAGCAGAGCTATATCTGC
(SEQ ID NO:33 - direct primer)

CBProFpr704: GCAGATATAGCTCTGCTGCTGGTCCTCGCAGGAGCCCCCGTTC
(SEQ ID NO:34 - reverse primer)

Example 4

Expression of FVII or FVII Variants in CHO K1 Cells

[0335] The cell line CHO K1 (ATCC # CCL-61) was seeded at 50% confluence in T-25 flasks using MEMα, 10% FCS (Gibco/BRL Cat # 10091), P/S and 5 μg/ml phylloquinone and allowed to grow until confluent. The confluent mono cell layer was transfected with 5 μg of the relevant plasmid described above using the Lipofectamine 2000 transfection agent (Life technologies) according to the manufacturer's instructions. Twenty four hours post transfection a sample was drawn and quantified using e.g. an ELISA recognizing the EGF1 domain of human factor VII. At this time point relevant selection (e.g. Hygromycin B) may be applied to the cells with the purpose of generating a pool of stable transfectants. When using CHO K1 cells and the Hygromycin B resistance gene as selectable marker on the plasmid, this is usually achieved within one week.

Example 5

Generation of CHO-K1 Cells Stably Expressing Polypeptide Variants

[0336] A vial of CHO-K1 transfectant pool was thawed and the cells seeded in a 175 cm^2 tissue flask containing 25 ml of MEMα, 10% FCS, phylloquinone (5 μg/ml), 100 U/l penicillin, 100 μg/l streptomycin and grown for 24 hours. The cells were harvested, diluted and plated in 96 well microtiter plates at a cell density of ½-1 cell/well. After a week of growth, colonies of 20-100 cells were present in the wells and those wells containing only one colony were labelled. After a further two weeks, the media in all wells containing only one colony was substituted with 200 μl fresh medium. After 24 hours, a medium sample was withdrawn and analysed by e.g. ELISA. High producing clones were selected and used to produce FVII or variants thereof.

Example 6

Small-Scale Purification of Polypeptide Variants and Subsequent Activation with Prothrombin Activator

[0337] FVII and variants thereof were purified and activated as follows. The procedure was performed at 4° C. 100 mM NaCl and 10 mM $CaCl_2$ wa added to 1200 ml harvested culture media followed by pH adjustment to 7.5 and sterile filtering. An affinity column was prepared by coupling a monoclonal calcium-dependent antiGla-domain antibody to CNBr-activated Sepharose FF using about 5.5 mg antibody coupled per ml resin. The prepared culture media was applied overnight to a 2 ml monoclonal antibody affinity column pre-equilibrated with 10 mM Tris, 100 mM NaCl, 35 mM $CaCl_2$, pH 7.5. The monoclonal antibody affinity matrix with bound FVII was subsequently unpacked by transferring the column material from the column to and empty tube.

[0338] FVII or variants thereof were activated to FVIIa by incubation with prothrombin activator from *Oxyuranus scutellatus* (OSII) while still being bound to the affinity matrix. FVIIa was recovered by repacking the column material followed by elution using 10 mM Tris, 25 mM NaCl, 5 mM EDTA, pH 8.6.

[0339] The eluate from the first chromatographic step was loaded directly onto a second and final chromatographic column, which consisted of a POROS HQ50 column pre-equilibrated with 10 mM Tris, 25 mM NaCl, 5 mM EDTA, pH 8.6. FVIIa was eluted from the POROS HQ50 column using 10 mM Tris, 25 mM NaCl, 35 mM $CaCl_2$, pH 7.5 after washing the column with 10 mM Tris, 25 mM NaCl, pH 8.6. FVIIa eluted from the POROS HQ50 column was stored at −80° C. without further modification.

Example 7

Large-Scale Purification of Polypeptide Variants and Subsequent Activation

[0340] FVII and FVII variants are purified as follows. The procedure is performed at 4° C. The harvested culture media from large-scale production is ultrafiltered using a Millipore TFF system with 30 KDa cut-off Pellicon membranes. After concentration of the medium, citrate is added to 5 mM and the pH is adjusted to 8.6. If necessary, the conductivity is lowered to below 10 mS/cm. Subsequently, the sample is applied to a Q-sepharose FF column, equilibrated with 50 mM NaCl, 10 mM Tris pH 8.6. After washing the column with 100 mM NaCl, 10 mM Tris pH 8.6, followed by 150 mM NaCl, 10 mM Tris pH 8.6, FVII is eluted using 10 mM Tris, 25 mM NaCl, 35 mM $CaCl_2$, pH 8.6.

[0341] For the second chromatographic step, an affinity column is prepared by coupling of a monoclonal Calcium-dependent antiGla-domain antibody to CNBr-activated Sepharose FF. About 5.5 mg antibody is coupled per ml resin. The column is equilibrated with 10 mM Tris, 100 mM NaCl, 35 mM $CaCl_2$, pH 7.5. NaCl is added to the sample to a concentration of 100 mM NaCl and the pH is adjusted to 7.4-7.6. After O/N application of the sample, the column is washed with 100 mM NaCl, 35 mM $CaCl_2$, 10 mM Tris pH 7.5, and the FVII protein is eluted with 100 mM NaCl, 50 mM citrate, 75 mM Tris pH 7.5.

[0342] For the third chromatographic, the conductivity of the sample is lowered to below 10 mS/cm, if necessary, and the pH is adjusted to 8.6. The sample is then applied to a Q-sepharose column (equilibrated with 50 mM NaCl, 10 mM Tris pH 8.6) at a density around 3-5 mg protein per ml gel to obtain efficient activation. After application, the column is washed with 50 mM NaCl, 10 mM Tris pH 8.6 for about 4 hours with a flow of 3-4 column volumes (cv) per hour. The FVII protein is eluted using a gradient of 0-100% of 500 mM NaCl, 10 mM Tris pH 8.6 over 40 cv. FVII containing fractions are pooled.

[0343] For the final chromatographic step, the conductivity is lowered to below 10 mS/cm. Subsequently, the sample is applied to a Q-sepharose column (equilibrated with 140 mM NaCl, 10 mM glycylglycine pH 8.6) at a concentration of 3-5 mg protein per ml gel. The column is then washed with 140 mM NaCl, 10 mM glycylglycine pH 8.6 and FVII is eluted with 140 mM NaCl, 15 mM $CaCl_2$, 10 mM glycylglycine pH 8.6. The eluate is diluted to 10 mM $CaCl_2$ and the pH is adjusted 6.8-7.2. Finally, Tween-80 is added to 0.01% and the pH is adjusted to 5.5 for storage at −80° C.

Example 8

Experimental Results

[0344] Subjecting the variants of the invention (purified as described in Example 6 above) to the "Whole Blood Assay" revealed that the variants exhibit a significantly increased clotting activity (or reduced clotting time) as compared to rhFVIIa. The experimental results are shown in **FIGS. 1-3**.

SEQUENCE LISTING

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             20                  25                  30

Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp
         35                  40                  45

Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln
     50                  55                  60

Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn
 65                  70                  75                  80

Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly
                 85                  90                  95

Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys
            100                 105                 110

Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr
        115                 120                 125

Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg
    130                 135                 140

Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro
145                 150                 155                 160

Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln
                165                 170                 175

Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala
            180                 185                 190

His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu
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Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg
    210                 215                 220

Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn
225                 230                 235                 240

His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp
                245                 250                 255

His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr
            260                 265                 270

Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu
        275                 280                 285

Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg
    290                 295                 300

Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser
305                 310                 315                 320

Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser
                325                 330                 335
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Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr
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Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys
        355                 360                 365

Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile
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Leu Arg Ala Pro Phe Pro
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                                                          Ala
                                                           1

aat gcc ttt ctg gaa gag ctc cgc cct ggc tcc ctg gaa cgc gaa tgc      165
Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu Cys
              5                  10                  15

aaa gag gaa cag tgc agc ttt gag gaa gcc cgg gag att ttc aaa gac      213
Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys Asp
         20                  25                  30

gct gag cgg acc aaa ctg ttt tgg att agc tat agc gat ggc gat cag      261
Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp Gln
     35                  40                  45

tgc gcc tcc agc cct tgc cag aac ggg ggc tcc tgc aaa gac cag ctg      309
Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln Leu
 50                  55                  60                  65

cag agc tat atc tgc ttc tgc ctg cct gcc ttt gag ggg cgc aat tgc      357
Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn Cys
                 70                  75                  80

gaa acc cat aag gat gac cag ctg att tgc gtc aac gaa aac ggg ggc      405
Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly Gly
             85                  90                  95

tgc gag cag tac tgc agc gat cac acg ggc acg aag cgg agc tgc cgc      453
Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys Arg
        100                 105                 110

tgc cac gaa ggc tat agc ctc ctg gct gac ggg gtg tcc tgc acg ccc      501
Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr Pro
    115                 120                 125

acg gtg gaa tac cct tgc ggg aag att ccc att cta gaa aag cgg aac      549
Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg Asn
130                 135                 140                 145

gct agc aaa ccc cag ggc cgg atc gtc ggc ggg aag gtc tgc cct aag      597
Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro Lys
                150                 155                 160

ggg gag tgc ccc tgg cag gtc ctg ctc ctg gtc aac ggg gcc cag ctg      645
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Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu Gly
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gaa cac gat ctg tcc gag cat gac ggg gac gaa cag tcc cgc cgg gtg      789
Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg Val
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gct cag gtc atc att ccc tcc acc tat gtg cct ggc acg acc aat cac      837
Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn His
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Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr Leu
        260                 265                 270

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Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu Asp
    275                 280                 285

cgg ggc gct acc gct ctc gag ctg atg gtg ctc aac gtc ccc cgg ctg     1029
Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg Leu
290                 295                 300                 305

atg acc cag gac tgc ctg cag cag tcc cgc aaa gtg ggg gac tcc ccc     1077
Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser Pro
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Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser Lys
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Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr Arg
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ggg acc tgg tac ctc acc ggg atc gtc agc tgg ggc cag ggc tgc gcc     1221
Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys Ala
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acg gtg ggg cac ttt ggc gtc tac acg cgc gtc agc cag tac att gag     1269
Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile Glu
370                 375                 380                 385

tgg ctg cag aag ctc atg cgg agc gaa ccc cgg ccc ggg gtg ctc ctg     1317
Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu Leu
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gcagatatag ctctgctgct ggtcctcgca ggagcccccg ttc 43

1. A Factor VII (FVII) or Factor VIIa (FVIIa) polypeptide variant having an amino acid sequence comprising 1-15 amino acid modifications relative to human Factor VII (hFVII) or human Factor VIIa (hFVIIa) having the amino acid sequence shown in SEQ ID NO:1, wherein said variant sequence comprises a substitution in at least one position selected from the group consisting of L39, I42, S43, K62, L65, F71, E82 and F275,

with the proviso that said variant is not

[K32E+D33N+A34T+K38T+L39E]hFVII/hFVIIa or

[A1Y+K32E+D33N+A34T+K38T+L39E]hFVII/hFVIIa or

[A1Y+A3S+F4GK+K32E+D33N+A34T+K38T+L39E] hFVII/hFVIIa or

[A1Y+L8F+R9V+P10Q+K32E+D33N+A34T+K38T+ L39E]hFVII/hFVIIa or

[A1Y+A3S+F4GK+L8F+R9V+P10Q+K32E+D33N+ A34T+K38T+L39E]hFVII/hFVIIa

or [A3S+F4GK+K32E+D33N+A34T+K38T+L39E] hFVII/hFVIIa or

[A3S+F4GK+L8F+R9V+P10Q+K32E+D33N+A34T+ K38T+L39E]hFVII/hFVIIa or

[L8F+R9V+P10Q+K32E+D33N+A34T+K38T+L39E] hFVII/hFVIIa or

[I42N]hFVII/hFVIIa or [I42S]hFVII/hFVIIa or [I42A] hFVII/hFVIIa or

[I42Q]hFVII/hFVIIa.

2. The variant according to claim 1, wherein said variant sequence comprises at least one substitution selected from the group consisting of L39E, L39Q, L39H, I42R, S43H, S43Q, K62E, K62R, L65Q, L65S, F71D, F71Y, F71E, F71Q, F71N, E82Q, E82N, E82K and F275H,

with the proviso that said variant is not

[K32E+D33N+A34T+K38T+L39E]hFVII/hFVIIa or

[A1Y+K32E+D33N+A34T+K38T+L39E]hFVII/hFVIIa or

[A1Y+A3S+F4GK+K32E+D33N+A34T+K38T+L39E] hFVII/hFVIIa or

[A1Y+L8F+R9V+P10Q+K32E+D33N+A34T+K38T+ L39E]hFVII/hFVIIa or

[A1Y+A3S+F4GK+L8F+R9V+P10Q+K32E+D33N+ A34T+K38T+L39E]hFVII/hFVIIa

or [A3S+F4GK+K32E+D33N+A34T+K38T+L39E] hFVII/hFVIIa or

[A3S+F4GK+L8F+R9V+P10Q+K32E+D33N+A34T+ K38T+L39E]hFVII/hFVIIa or

[L8F+R9V+P10Q+K32E+D33N+A34T+K38T+L39E] hFVII/hFVIIa.

3.-6. (canceled)

7. The variant according to claim 1, wherein said variant comprises at least two substitutions selected from the group consisting of L65Q, F71Y, K62E and S43Q.

8.-12. (canceled)

13. The variant according to claim 1, wherein said variant comprises at least one amino acid modification in the Gla domain.

14. The variant according to claim 13, wherein said at least one modification in the Gla domain comprises a substitution in at least one position selected from the group consisting of P10, K32, D33 and A34.

15.-24. (canceled)

25. The variant according to claim 1, wherein at least one amino acid residue comprising an attachment group for a non-polypeptide moiety has been introduced in a position located outside the Gla domain.

26. The variant according to claim 25, wherein at least one non-polypeptide moiety is covalently attached to at least one of said attachment groups.

27. The variant according to claim 26, wherein said non-polypeptide moiety is a sugar moiety.

28. The variant according to claim 25, wherein said attachment group is a glycosylation site.

29. (canceled)

30. The variant according to claim 28, wherein said glycosylation site is introduced by substitution and said introduced glycosylation site is an in vivo glycosylation site.

31. (canceled)

32. The variant according to claim 30, wherein said introduced in vivo glycosylation site is an N-glycosylation site.

33.-34. (canceled)

35. The variant according to claim 32, wherein said N-glycosylation site is introduced by a substitution selected from the group consisting of A51N, G58N, G48N+S60T, T106N, K109N, G124N, K143N+N145T, A175T, I205S, I205T, V253N, T267N, T267N+S269T, S314N+K316S, S314N+K316T, R315N+V317S, R315N+V317T, K316N+ G318S, K316N+G318T, G318N, and D334N.

36.-46. (canceled)

47. The variant according to claim 1, wherein said variant further comprises at least one modification in a position selected from the group of 157, 158, 296, 298, 305, 334, 336, 337 and 374.

48.-49. (canceled)

50. The variant according to claim 1, wherein said variant is in its activated form.

51. A nucleotide sequence encoding the variant according to claim 1.

52. (canceled)

53. A host cell comprising the nucleotide sequence according to claim 51.

54. The host cell according to claim 53, wherein said host cell is a gammacarboxylating cell capable of in vivo glycosylation.

55. A pharmaceutical composition comprising the variant according to claim 1, and a pharmaceutical acceptable carrier or excipient.

56.-61. (canceled)

62. A method for treating a mammal having a disease or a disorder wherein clot formation is desirable, comprising administering to a mammal in need thereof an effective amount of the pharmaceutical composition according to claim 55.

63. The method according to claim 62, wherein said disease or disorder is selcted from the group consisting of hemorrhage; uncontrolled bleedings, such as trauma; cirrhosis; thrombocytopenia; haemophilia A and haemophilia B.

64.-66. (canceled)

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Ostergaard et al. (43) Pub. Date: Sep. 14, 2006

(54) **COAGULATION FACTOR VII POLYPEPTIDES**

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C07K 14/745	(2006.01)

(52) **U.S. Cl.** **435/69.6**; 435/320.1; 435/325; 530/383; 514/12; 536/23.5

(57) **ABSTRACT**

The present invention relates to novel coagulation Factor VII polypeptides, polynucleotide constructs encoding such polypeptides, as well as vectors and host cells comprising and expressing the polynucleotide, pharmaceutical compositions, uses and methods of treatment.

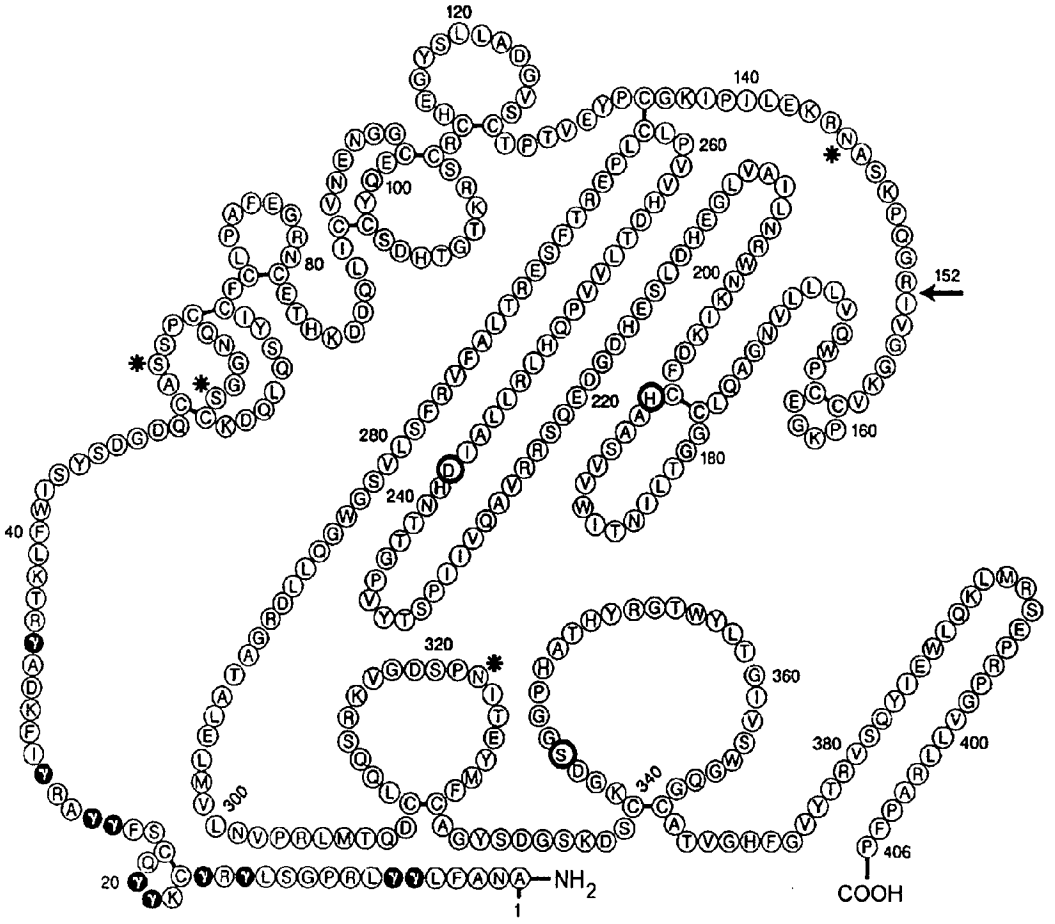


Fig. 1

SEQ ID NO: 1 (The amino acid sequence of native human coagulation Factor VII):

```
Ala-Asn-Ala-Phe-Leu-GLA-GLA-Leu-Arg-Pro-Gly-Ser-Leu-GLA-Arg-GLA-Cys-
Lys-
                    5                  10                  15

GLA-GLA-Gln-Cys-Ser-Phe-GLA-GLA-Ala-Arg-GLA-Ile-Phe-Lys-Asp-Ala-GLA-
Arg-
     20                  25                  30                  35

Thr-Lys-Leu-Phe-Trp-Ile-Ser-Tyr-Ser-Asp-Gly-Asp-Gln-Cys-Ala-Ser-Ser-
Pro-
                40                  45                  50

Cys-Gln-Asn-Gly-Gly-Ser-Cys-Lys-Asp-Gln-Leu-Gln-Ser-Tyr-Ile-Cys-Phe-
Cys-
 55                  60                  65                  70

Leu-Pro-Ala-Phe-Glu-Gly-Arg-Asn-Cys-Glu-Thr-His-Lys-Asp-Asp-Gln-Leu-
Ile-
          75                  80                  85
90

Cys-Val-Asn-Glu-Asn-Gly-Gly-Cys-Glu-Gln-Tyr-Cys-Ser-Asp-His-Thr-Gly-
Thr-
                    95                 100                 105

Lys-Arg-Ser-Cys-Arg-Cys-His-Glu-Gly-Tyr-Ser-Leu-Leu-Ala-Asp-Gly-Val-
Ser-
    110                 115                 120                 125

Cys-Thr-Pro-Thr-Val-Glu-Tyr-Pro-Cys-Gly-Lys-Ile-Pro-Ile-Leu-Glu-Lys-
Arg-
                130                 135                 140
```

```
        Asn-Ala-Ser-Lys-Pro-Gln-Gly-Arg-Ile-Val-Gly-Gly-Lys-Val-Cys-Pro-Lys-
Gly-
        145                 150                 155                 160

        Glu-Cys-Pro-Trp-Gln-Val-Leu-Leu-Leu-Val-Asn-Gly-Ala-Gln-Leu-Cys-Gly-
Gly-
                165                 170                 175
180

        Thr-Leu-Ile-Asn-Thr-Ile-Trp-Val-Val-Ser-Ala-Ala-His-Cys-Phe-Asp-Lys-
Ile-
                        185                 190                 195

        Lys-Asn-Trp-Arg-Asn-Leu-Ile-Ala-Val-Leu-Gly-Glu-His-Asp-Leu-Ser-Glu-
His-
            200                 205                 210                 215

        Asp-Gly-Asp-Glu-Gln-Ser-Arg-Arg-Val-Ala-Gln-Val-Ile-Ile-Pro-Ser-Thr-
Tyr-
                    220                 225                 230

        Val-Pro-Gly-Thr-Thr-Asn-His-Asp-Ile-Ala-Leu-Leu-Arg-Leu-His-Gln-Pro-
Val-
        235                 240                 245                 250

        Val-Leu-Thr-Asp-His-Val-Val-Pro-Leu-Cys-Leu-Pro-Glu-Arg-Thr-Phe-Ser-
Glu-
                255                 260                 265
270

        Arg-Thr-Leu-Ala-Phe-Val-Arg-Phe-Ser-Leu-Val-Ser-Gly-Trp-Gly-Gln-Leu-
Leu-
                        275                 280                 285

        Asp-Arg-Gly-Ala-Thr-Ala-Leu-Glu-Leu-Met-Val-Leu-Asn-Val-Pro-Arg-Leu-
Met-
            290                 295                 300                 305
```

```
Thr-Gln-Asp-Cys-Leu-Gln-Gln-Ser-Arg-Lys-Val-Gly-Asp-Ser-Pro-Asn-Ile-
Thr-
                310                 315                 320

Glu-Tyr-Met-Phe-Cys-Ala-Gly-Tyr-Ser-Asp-Gly-Ser-Lys-Asp-Ser-Cys-Lys-
Gly-
325                 330                 335                 340

Asp-Ser-Gly-Gly-Pro-His-Ala-Thr-His-Tyr-Arg-Gly-Thr-Trp-Tyr-Leu-Thr-
Gly-
          345                 350                 355
360

Ile-Val-Ser-Trp-Gly-Gln-Gly-Cys-Ala-Thr-Val-Gly-His-Phe-Gly-Val-Tyr-
Thr-
                  365                 370                 375

Arg-Val-Ser-Gln-Tyr-Ile-Glu-Trp-Leu-Gln-Lys-Leu-Met-Arg-Ser-Glu-Pro-
Arg-
     380                 385                 390                 395

Pro-Gly-Val-Leu-Leu-Arg-Ala-Pro-Phe-Pro
              400                 405 406
```

Fig. 2

COAGULATION FACTOR VII POLYPEPTIDES

FIELD OF THE INVENTION

[0001] The present invention relates to novel human coagulation Factor VII polypeptides, as well as polynucleotide constructs encoding such polypeptides, vectors and host cells comprising and expressing the polynucleotide, pharmaceutical compositions comprising Factor VII polypeptides, uses and methods of treatment.

BACKGROUND OF THE INVENTION

[0002] Blood coagulation is a process consisting of a complex interaction of various blood components (or factors) that eventually gives rise to a fibrin clot. Generally, the blood components, which participate in what has been referred to as the coagulation "cascade", are enzymatically inactive proteins (proenzymes or zymogens) that are converted to proteolytic enzymes by the action of an activator (which itself is an activated clotting factor). Coagulation factors that have undergone such a conversion are generally referred to as "active factors", and are designated by the addition of the letter "a" to the name of the coagulation factor (e.g. Factor VIIa).

[0003] Initiation of the haemostatic process is mediated by the formation of a complex between tissue factor, exposed as a result of injury to the vessel wall, and Factor VIIa. This complex then converts Factors IX and X to their active forms. Factor Xa converts limited amounts of prothrombin to thrombin on the tissue factor-bearing cell. Thrombin activates platelets and Factors V and VIII into Factors Va and VIIIa, both cofactors in the further process leading to the full thrombin burst. This process includes generation of Factor Xa by Factor IXa (in complex with factor VIIIa) and occurs on the surface of activated platelets. Thrombin finally converts fibrinogen to fibrin resulting in formation of a fibrin clot.

[0004] Factor VII is a trace plasma glycoprotein that circulates in blood as a single-chain zymogen. The zymogen is catalytically inactive. Single-chain Factor VII may be converted to two-chain Factor VIIa by Factor Xa, Factor XIIa, Factor IXa, Factor VIIa or thrombin in vitro. Factor Xa is believed to be the major physiological activator of Factor VII. The conversion of zymogen Factor VII into the activated two-chain molecule occurs by cleavage of an internal Arg_{152}-Ile_{153} peptide bond.

[0005] It is often desirable to stimulate the coagulation cascade in a subject. Factor VIIa has been used to control bleeding disorders that have several causes such as clotting factor deficiencies (e.g. haemophilia A and B or deficiency of coagulation Factors XI or VII) or clotting factor inhibitors. Factor VIIa has also been used to control excessive bleeding occurring in subjects with a normally functioning blood clotting cascade (no clotting factor deficiencies or inhibitors against any of the coagulation factors). Such bleeding may, for example, be caused by a defective platelet function, thrombocytopenia or von Willebrand's disease. Bleeding is also a major problem in connection with surgery and other forms of tissue damage.

[0006] European Patent No. 200,421 (ZymoGenetics) relates to the nucleotide sequence encoding human Factor VII and the recombinant expression of Factor VII in mammalian cells.

[0007] Dickinson et al. (*Proc. Nat. Acad. Sci. USA* 93, 14379-14384, 1996) discloses Factor VII polypeptides wherein certain amino acids have been individually replaced by Ala. Iwanaga et al. (*Thromb. Haemost.* (supplement august 1999), 466, abstract 1474) relates to Factor VIIa variants wherein residues 316-320 are deleted or residues 311-322 are replaced with the corresponding residues from trypsin.

[0008] There is still a need in the art for improved Factor VII polypeptides having procoagulant activity. In particular, there is a need for Factor VII polypeptides with increased TF independent activity.

SUMMARY OF THE INVENTION

[0009] The present invention relates to Factor VII polypeptides with increased TF independent activity, such as novel coagulation Factor VII polypeptides with the same or increased activity compared to wild type Factor VIIa and with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa.

[0010] In a first aspect, the present invention relates to a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa with the proviso that the Factor VII polypeptide is not I69A-FVII.

[0011] In a second aspect, the invention relates to a composition comprising a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa with the proviso that the Factor VII polypeptide is not I69A-FVII.

[0012] In a third aspect, the invention relates to a pharmaceutical composition comprising a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa; and optionally, a pharmaceutically acceptable carrier.

[0013] In a further aspect, the invention relates to a polynucleotide construct encoding a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa with the proviso that the Factor VII polypeptide is not I69A-FVII.

[0014] In a further aspect, the invention relates to a eukaryotic host cell comprising a polynucleotide construct encoding a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa with the proviso that the Factor VII polypeptide is not I69A-FVII.

[0015] In a further aspect, the invention relates to a method for producing a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type

human Factor VIIa with the proviso that the Factor VII polypeptide is not I69A-FVII, the method comprising cultivating a eukaryotic host cell comprising a polynucleotide construct encoding the Factor VII polypeptide in an appropriate growth medium under conditions allowing protein synthesis from said polynucleotide construct and recovering said Factor VII polypeptide from the culture medium.

[0016] In a further aspect, the invention relates to a use of a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa; for the preparation of a medicament for the treatment of bleeding episodes or for the enhancement of the normal haemostatic system.

[0017] In a further aspect, the invention relates to a method for the treatment of bleeding episodes or bleeding disorders in a subject or for the enhancement of the normal haemostatic system, the method comprising administering to a subject in need thereof a therapeutically or prophylactically effective amount of a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa.

[0018] In a further aspect, the invention relates to a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa, wherein said Factor VII polypeptide is selected from:

[0019] i) a Factor VII polypeptide comprising one or more amino acid substitution selected from $K18X_1$, $R36X_2$, $S43X_3$, $K62X_4$, $Q64X_5$, $L65X_6$, $I69X_7$, $F71X_8$, $L73X_9$, $P74X_{10}$, $E77X_{11}$, $G78X_{12}$, $R79X_{13}$, $K85X_{14}$, $Q88X_{15}$, $N93X_{16}$, $F275X_{17}$, $R277X_{18}$, $M306X_{19}$, $T307X_{20}$, $Q308X_{21}$, $D309X_{22}$, or $R379X_{23}$ corresponding to amino acids at positions of SEQ ID NO: 1, which different amino acid decreases the Tissue Factor binding affinity, wherein

[0020] X_1 is E, D, A, or F;

[0021] X_2 is E, D, K, A, F, or N;

[0022] X_3 is E, D, K, R, A, or F;

[0023] X_4 is D, A, or F;

[0024] X_5 is E, D, K, R, A, F, or N;

[0025] X_6 is E, D, K, R, A, or F;

[0026] X_7 is C, D, E, F, G, H, K, L, M, N, P, Q, R, S, T, V, W, or Y;

[0027] X_8 is A, G, H, I, K, L, M, P, R, S, T, V, or W;

[0028] X_9 is A, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y;

[0029] X_{10} is A, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, or Y;

[0030] X_{11} is A, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

[0031] X_{12} is A, D, E, F, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

[0032] X_{13} is A, D, E, F, G, H, I, K, L, M, N, P, Q, S, T, V, W, or Y;

[0033] X_{14} is A, D, E, F, G, I, L, M, P, S, T, V, W, or Y;

[0034] X_{15} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;

[0035] X_{16} is A, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y;

[0036] X_{17} is A, D, E, G, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

[0037] X_{18} is A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y;

[0038] X_{19} is A, E, F, G, H, I, K, L, P, Q, R, S, T, V, W, or Y;

[0039] X_{20} is A, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, V, W, or Y;

[0040] X_{21} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;

[0041] X_{22} is A, F, G, H, I, K, L, M, N, P, Q, R, V, W, or Y; and

[0042] X_{23} is A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y;

[0043] and/or

[0044] ii) a Factor VII polypeptide, wherein one or more amino acid corresponding to amino acids at positions selected from K18, R36, K62, Q64, L65, I69, F71, L73, P74, E77, G78, R79, K85, Q88, N93, F275, R277, M306, Q308, D309, or R379 of SEQ ID NO: 1 have been substituted with a cysteine amino acid residue, which cysteine amino acid residue decreases the Tissue Factor binding affinity, and which cysteine amino acid residue is optionally conjugated with a chemical group, which chemical group increases the molecular weight of said Factor VII polypeptide;

[0045] and/or

[0046] iii) a Factor VII polypeptide comprising one or more N-glycosylation site N-Xaa-S/T introduced by amino acid substitutions corresponding to amino acids starting at positions selected from R36, Q64, I69, F71, P74, E77, G78, Q88, N93, F275, M306, T307, or D309 of SEQ ID NO: 1, wherein Xaa is any amino acid except P, which introduced N-glycosylation site decreases the Tissue Factor binding affinity.

[0047] In a further aspect, the invention relates to a composition comprising a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa, wherein said Factor VII polypeptide is selected from:

[0048] i) a Factor VII polypeptide comprising one or more amino acid substitution selected from $K18X_1$, $R36X_2$, $S43X_3$, $K62X_4$, $Q64X_5$, $L65X_6$, $I69X_7$, $F71X_8$, $L73X_9$, $P74X_{10}$, $E77X_{11}$, $G78X_{12}$, $R79X_{13}$, $K85X_{14}$, $Q88X_{15}$, $N93X_{16}$, $F275X_{17}$, $R277X_{18}$, $M306X_{19}$,

$T307X_{20}$, $Q308X_{21}$, $D309X_{22}$, or $R379X_{23}$ corresponding to amino acids at positions of SEQ ID NO: 1, which different amino acid decreases the Tissue Factor binding affinity, wherein

[0049] X_1 is E, D, A, or F;

[0050] X_2 is E, D, K, A, F, or N;

[0051] X_3 is E, D, K, R, A, or F;

[0052] X_4 is D, A, or F;

[0053] X_5 is E, D, K, R, A, F, or N;

[0054] X_6 is E, D, K, R, A, or F;

[0055] X_7 is C, D, E, F, G, H, K, L, M, N, P, Q, R, S, T, V, W, or Y;

[0056] X_8 is A, G, H, I, K, L, M, P, R, S, T, V, or W;

[0057] X_9 is A, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y;

[0058] X_{10} is A, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, or Y;

[0059] X_{11} is A, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

[0060] X_{12} is A, D, E, F, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

[0061] X_{13} is A, D, E, F, G, H, I, K, L, M, N, P, Q, S, T, V, W, or Y;

[0062] X_{14} is A, D, E, F, G, I, L, M, P, S, T, V, W, or Y;

[0063] X_{15} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;

[0064] X_{16} is A, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y;

[0065] X_{17} is A, D, E, G, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

[0066] X_{18} is A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y;

[0067] X_{19} is A, E, F, G, H, I, K, L, P, Q, R, S, T, V, W, or Y;

[0068] X_{20} is A, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, V, W, or Y;

[0069] X_{21} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;

[0070] X_{22} is A, F, G, H, I, K, L, M, N, P, Q, R, V, W, or Y; and

[0071] X_{23} is A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y;

[0072] and/or

[0073] ii) a Factor VII polypeptide, wherein one or more amino acid corresponding to amino acids at positions selected from K18, R36, K62, Q64, L65, I69, F71, L73, P74, E77, G78, R79, K85, Q88, N93, F275, R277, M306, Q308, D309, or R379 of SEQ ID NO: 1 have been substituted with a cysteine amino acid residue, which cysteine amino acid residue decreases the Tissue Factor binding affinity, and which cysteine amino acid residue is optionally conjugated with a chemical group, which chemical group increases the molecular weight of said Factor VII polypeptide;

[0074] and/or

[0075] iii) a Factor VII polypeptide comprising one or more N-glycosylation site N-Xaa-S/T introduced by amino acid substitutions corresponding to amino acids starting at positions selected from R36, Q64, I69, F71, P74, E77, G78, Q88, N93, F275, M306, T307, or D309 of SEQ ID NO: 1, wherein Xaa is any amino acid except P, which introduced N-glycosylation site decreases the Tissue Factor binding affinity.

[0076] In a further aspect, the invention relates to a pharmaceutical composition comprising a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa, wherein said Factor VII polypeptide is selected from:

[0077] i) a Factor VII polypeptide comprising one or more amino acid substitution selected from $K18X_1$, $R36X_2$, $S43X_3$, $K62X_4$, $Q64X_5$, $L65X_6$, $I69X_7$, $F71X_8$, $L73X_9$, $P74X_{10}$, $E77X_{11}$, $G78X_{12}$, $R79X_{13}$, $K85X_{14}$, $Q88X_{15}$, $N93X_{16}$, $F275X_{17}$, $R277X_{18}$, $M306X_{19}$, $T307X_{20}$, $Q308X_{21}$, $D309X_{22}$, or $R379X_{23}$ corresponding to amino acids at positions of SEQ ID NO: 1, which different amino acid decreases the Tissue Factor binding affinity, wherein

[0078] X_1 is E, D, A, or F;

[0079] X_2 is E, D, K, A, F, or N;

[0080] X_3 is E, D, K, R, A, or F;

[0081] X_4 is D, A, or F;

[0082] X_5 is E, D, K, R, A, F, or N;

[0083] X_6 is E, D, K, R, A, or F;

[0084] X_7 is C, D, E, F, G, H, K, L, M, N, P, Q, R, S, T, V, W, or Y;

[0085] X_8 is A, G, H, I, K, L, M, P, R, S, T, V, or W;

[0086] X_9 is A, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y;

[0087] X_{10} is A, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, or Y;

[0088] X_{11} is A, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

[0089] X_{12} is A, D, E, F, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

[0090] X_{13} is A, D, E, F, G, H, I, K, L, M, N, P, Q, S, T, V, W, or Y;

[0091] X_{14} is A, D, E, F, G, I, L, M, P, S, T, V, W, or Y;

[0092] X_{15} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;

[0093] X_{16} is A, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y;

[0094] X_{17} is A, D, E, G, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

[0095] X_{18} is A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y;

[0096] X_{19} is A, E, F, G, H, I, K, L, P, Q, R, S, T, V, W, or Y;

[0097] X_{20} is A, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, V, W, or Y;

[0098] X_{21} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;

[0099] X_{22} is A, F, G, H, I, K, L, M, N, P, Q, R, V, W, or Y; and

[0100] X_{23} is A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y;

[0101] and/or

[0102] ii) a Factor VII polypeptide, wherein one or more amino acid corresponding to amino acids at positions selected from K18, R36, K62, Q64, L65, I69, F71, L73, P74, E77, G78, R79, K85, Q88, N93, F275, R277, M306, Q308, D309, or R379 of SEQ ID NO: 1 have been substituted with a cysteine amino acid residue, which cysteine amino acid residue decreases the Tissue Factor binding affinity, and which cysteine amino acid residue is optionally conjugated with a chemical group, which chemical group increases the molecular weight of said Factor VII polypeptide;

[0103] and/or

[0104] iii) a Factor VII polypeptide comprising one or more N-glycosylation site N-Xaa-S/T introduced by amino acid substitutions corresponding to amino acids starting at positions selected from R36, Q64, I69, F71, P74, E77, G78, Q88, N93, F275, M306, T307, or D309 of SEQ ID NO: 1, wherein Xaa is any amino acid except P, which introduced N-glycosylation site decreases the Tissue Factor binding affinity;

and optionally, a pharmaceutically acceptable carrier.

[0105] In a further aspect, the invention relates to a use of a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa, wherein said Factor VII polypeptide is selected from:

[0106] i) a Factor VII polypeptide comprising one or more amino acid substitution selected from $K18X_1$, $R36X_2$, $S43X_3$, $K62X_4$, $Q64X_5$, $L65X_6$, $I69X_7$, $F71X_8$, $L73X_9$, $P74X_{10}$, $E77X_{11}$, $G78X_{12}$, $R79X_{13}$, $K85X_{14}$, $Q88X_{15}$, $N93X_{16}$, $F275X_{17}$, $R277X_{18}$, $M306X_{19}$, $T307X_{20}$, $Q308X_{21}$, $D309X_{22}$, or $R379X_{23}$ corresponding to amino acids at positions of SEQ ID NO: 1, which different amino acid decreases the Tissue Factor binding affinity, wherein

[0107] X_1 is E, D, A, or F;

[0108] X_2 is E, D, K, A, F, or N;

[0109] X_3 is E, D, K, R, A, or F;

[0110] X_4 is D, A, or F;

[0111] X_5 is E, D, K, R, A, F, or N;

[0112] X_6 is E, D, K, R, A, or F;

[0113] X_7 is C, D, E, F, G, H, K, L, M, N, P, Q, R, S, T, V, W, or Y;

[0114] X_8 is A, G, H, I, K, L, M, P, R, S, T, V, or W;

[0115] X_9 is A, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y;

[0116] X_{10} is A, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, or Y;

[0117] X_{11} is A, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

[0118] X_{12} is A, D, E, F, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

[0119] X_{13} is A, D, E, F, G, H, I, K, L, M, N, P, Q, S, T, V, W, or Y;

[0120] X_{14} is A, D, E, F, G, I, L, M, P, S, T, V, W, or Y;

[0121] X_{15} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;

[0122] X_{16} is A, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y;

[0123] X_{17} is A, D, E, G, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

[0124] X_{18} is A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y;

[0125] X_{19} is A, E, F, G, H, I, K, L, P, Q, R, S, T, V, W, or Y;

[0126] X_{20} is A, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, V, W, or Y;

[0127] X_{21} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;

[0128] X_{22} is A, F, G, H, I, K, L, M, N, P, Q, R, V, W, or Y; and

[0129] X_{23} is A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y;

[0130] and/or

[0131] ii) a Factor VII polypeptide, wherein one or more amino acid corresponding to amino acids at positions selected from K18, R36, K62, Q64, L65, I69, F71, L73, P74, E77, G78, R79, K85, Q88, N93, F275, R277, M306, Q308, D309, or R379 of SEQ ID NO: 1 have been substituted with a cysteine amino acid residue, which cysteine amino acid residue decreases the Tissue Factor binding affinity, and which cysteine amino acid residue is optionally conjugated with a chemical group, which chemical group increases the molecular weight of said Factor VII polypeptide;

[0132] and/or

[0133] iii) a Factor VII polypeptide comprising one or more N-glycosylation site N-Xaa-S/T introduced by amino acid substitutions corresponding to amino acids starting at positions selected from R36, Q64, I69, F71, P74, E77, G78, Q88, N93, F275, M306, T307, or D309 of SEQ ID NO: 1, wherein Xaa is any amino acid except P, which introduced N-glycosylation site decreases the Tissue Factor binding affinity;

for the preparation of a medicament for the treatment of bleeding episodes or for the enhancement of the normal haemostatic system.

[0134] In a further aspect, the invention relates to a method for the treatment of bleeding episodes or bleeding disorders in a subject or for the enhancement of the normal haemostatic system, the method comprising administering to a subject in need thereof a therapeutically or prophylactically effective amount of a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa, wherein said Factor VII polypeptide is selected from:

[0135] i) a Factor VII polypeptide comprising one or more amino acid substitution selected from $K18X_1$, $R36X_2$, $S43X_3$, $K62X_4$, $Q64X_5$, $L65X_6$, $I69X_7$, $F71X_8$, $L73X_9$, $P74X_{10}$, $E77X_{11}$, $G78X_{12}$, $R79X_{13}$, $K85X_{14}$, $Q88X_{15}$, $N93X_{16}$, $F275X_{17}$, $R277X_{18}$, $M306X_{19}$, $T307X_{20}$, $Q308X_{21}$, $D309X_{22}$, or $R379X_{23}$ corresponding to amino acids at positions of SEQ ID NO: 1, which different amino acid decreases the Tissue Factor binding affinity, wherein

[0136] X_1 is E, D, A, or F;

[0137] X_2 is E, D, K, A, F, or N;

[0138] X_3 is E, D, K, R, A, or F;

[0139] X_4 is D, A, or F;

[0140] X_5 is E, D, K, R, A, F, or N;

[0141] X_6 is E, D, K, R, A, or F;

[0142] X_7 is C, D, E, F, G, H, K, L, M, N, P, Q, R, S, T, V, W, or Y;

[0143] X_8 is A, G, H, I, K, L, M, P, R, S, T, V, or W;

[0144] X_9 is A, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y;

[0145] X_{10} is A, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, or Y;

[0146] X_{11} is A, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

[0147] X_{12} is A, D, E, F, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

[0148] X_{13} is A, D, E, F, G, H, I, K, L, M, N, P, Q, S, T, V, W, or Y;

[0149] X_{14} is A, D, E, F, G, I, L, M, P, S, T, V, W, or Y;

[0150] X_{15} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;

[0151] X_{16} is A, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y;

[0152] X_{17} is A, D, E, G, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

[0153] X_{18} is A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y;

[0154] X_{19} is A, E, F, G, H, I, K, L, P, Q, R, S, T, V, W, or Y;

[0155] X_{20} is A, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, V, W, or Y;

[0156] X_{21} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;

[0157] X_{22} is A, F, G, H, I, K, L, M, N, P, Q, R, V, W, or Y; and

[0158] X_{23} is A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y;

[0159] and/or

[0160] ii) a Factor VII polypeptide, wherein one or more amino acid corresponding to amino acids at positions selected from K18, R36, K62, Q64, L65, I69, F71, L73, P74, E77, G78, R79, K85, Q88, N93, F275, R277, M306, Q308, D309, or R379 of SEQ ID NO: 1 have been substituted with a cysteine amino acid residue, which cysteine amino acid residue decreases the Tissue Factor binding affinity, and which cysteine amino acid residue is optionally conjugated with a chemical group, which chemical group increases the molecular weight of said Factor VII polypeptide;

[0161] and/or

[0162] iii) a Factor VII polypeptide comprising one or more N-glycosylation site N-Xaa-S/T introduced by amino acid substitutions corresponding to amino acids starting at positions selected from R36, Q64, I69, F71, P74, E77, G78, Q88, N93, F275, M306, T307, or D309 of SEQ ID NO: 1, wherein Xaa is any amino acid except P, which introduced N-glycosylation site decreases the Tissue Factor binding affinity.

[0163] In a further aspect the present invention relates to a composition comprising wild type human FVIIa and a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa.

[0164] In a further aspect the present invention relates to a method for the treatment of bleeding episodes in a subject or for the enhancement of the normal haemostatic system, the method comprising administering to a subject in need thereof a therapeutically or prophylactically effective amount of:

[0165] a) composition comprising wild type human FVIIa and a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa; or

[0166] b) a first composition comprising wild type human FVIIa and a second composition comprising a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa.

[0167] In a further aspect the present invention relates to a process for preparing a composition comprising wild type human FVIIa and a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type

human Factor VIIa, wherein the process comprises the step of: mixing wild type human FVIIa with a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa in an aqueous medium.

[0168] In a further aspect the present invention relates to a use of a composition comprising wild type human FVIIa and a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa, for the preparation of a medicament for the treatment of bleeding episodes in a subject or for the enhancement of the normal haemostatic system.

BRIEF DESCRIPTION OF THE DRAWINGS

[0169] **FIG. 1** The structure of correctly processed human coagulation Factor VII, amino acids 1 to 406, with gamma carboxylated Glu-residues (γ) and glycosylation (*). The arrow at amino acid residue 152 shows the site where single-chain Factor VII is cleaved to be converted to activated two-chain Factor VII (FVIIa).

[0170] **FIG. 2** The full amino acid sequence of native (wild type) human coagulation Factor VII (SEQ ID NO:1).

DETAILED DESCRIPTION OF THE INVENTION

[0171] The present invention relates to novel coagulation Factor VII polypeptides with substantially the same or increased activity compared to wild type Factor VIIa and with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa. Increasing evidence suggests that therapeutic treatment with recombinant wild type human coagulation Factor VII works by a TF independent mechanism involving binding of FVII directly to activated thrombocytes and a generation of activated Factor Xa and eventually fibrin locally at the place of tissue damage. The Factor VII polypeptides of the present invention provide an alternative to traditional TF binding Factor VII polypeptides. Factor VII polypeptides that do not bind to TF may be an advantage in the therapeutic treatment of bleedings, when the exposure of TF is high, e.g. plaque rupture or sepsis. As used herein, Factor VII activity refers to its ability to promote blood clotting, which may be assessed, e.g., by measuring hydrolytic or proteolytic activity or by measuring thrombin formation.

[0172] As used herein, the terms "Factor VII polypeptide" or "FVII polypeptide" mean wild-type human Factor VII/VIIa (i.e., a polypeptide having the amino acid sequence disclosed in U.S. Pat. No. 4,784,950), as well as variants of Factor VII exhibiting substantially the same or improved biological activity relative to wild-type Factor VII, Factor VII-related polypeptides as well as Factor VII derivatives and Factor VII conjugates. Unless otherwise indicated, the term "Factor VII" is intended to encompass Factor VII polypeptides in their uncleaved (zymogen) form, as well as those that have been proteolytically processed to yield their respective bioactive forms, which may be designated Factor VIIa. Typically, Factor VII is cleaved between residues 152 and 153 to yield Factor VIIa. Such variants of Factor VII may exhibit different properties relative to human Factor VII, including stability, phospholipid binding, altered specific activity, and the like.

[0173] As used herein, "Factor VII-related polypeptides" encompasses polypeptides, including variants, in which the Factor VIIa biological activity has been substantially modified or reduced relative to the activity of wild-type Factor VIIa. These polypeptides include, without limitation, Factor VII or Factor VIIa into which specific amino acid sequence alterations have been introduced that modify or disrupt the bioactivity of the polypeptide.

[0174] The term "Factor VII derivative" as used herein, is intended to designate wild-type Factor VII, variants of Factor VII exhibiting substantially the same or improved biological activity relative to wild-type Factor VII and Factor VII-related polypeptides, in which one or more of the amino acids of the parent peptide have been chemically and/or enzymatically modified, e.g. by alkylation, glycosylation, PEGylation, acylation, ester formation or amide formation or the like. This includes but is not limited to PEGylated human Factor VIIa, cysteine-PEGylated human Factor VIIa and variants thereof.

[0175] The optional conjugation of a cysteine amino acid residue with the chemical group includes but are not limited to covalent attachment of polyethylene glycol (PEG), monomethoxy-polyethylene glycol, dextran, poly-(N-vinyl pyrrolidone)polyethylene glycol, propylene glycol homopolymers, a polypropylene oxide/ethylene oxide co-polymer, polypropylene glycol, polyoxyethylated polyols (e.g., glycerol) and polyvinyl alcohol, colominic acids or other carbohydrate based polymers, polymers of amino acids, and biotin derivatives. In one embodiment of the present invention, the chemical group is a biocompatible, non-toxic, non-immunogenic and water-soluble polymer. Preferably the chemical group is water-soluble in all proportions.

[0176] Methods for attaching PEG groups to cysteine residues are described in Roberts, M. J. et al, Advanced Drug Delivery Reviews 54 (2002) 459-476.

[0177] An "N-glycosylation site" has the sequence N-Xaa-S/T, wherein Xaa is any amino acid residue except proline, N is asparagine and S/T is either serine or threonine, such as serine or threonine, such as threonine.

[0178] Specific examples of activated PEG polymers particularly preferred for coupling to cysteine residues, include the following linear PEGs: vinylsulfone-PEG (VS-PEG), such as vinylsulfone-mPEG (VS-mPEG); maleimide-PEG (MAL-PEG), such as maleimide-mPEG (MAL-mPEG) and orthopyridyl-disulfide-PEG (OPSS-PEG), such as orthopyridyl-disulfide-mPEG (OPSS-MPEG). Typically, such PEG or mPEG polymers will have a size of about 2 kDa, such as about 5 kDa, such as about 10 kD, such as about 12 kDa or such as about 20 kDa.

[0179] The skilled person will be aware that the activation method and/or conjugation chemistry to be used depends on the functional groups of the polymer (e.g. being amine, hydroxyl, carboxyl, aldehyde, sulfydryl, succinimidyl, maleimide, vinysulfone or haloacetate).

[0180] For conjugated with a chemical group to a cysteine residue (e.g. PEGylation) the FVII polypeptide is usually

treated with a reducing agent, such as dithiothreitol (DDT), β-mercaptoethanol, or glutathione (γ-glutamylcysteinylglycine) prior to PEGylation, such as described in Higashi, S. Matsumoto, N., Iwanaga, S. (1997) J. Biol. Chem., 272(41), 25724-25730. The reducing agent is subsequently removed by any conventional method, such as by desalting. Conjugation of PEG to a cysteine residue typically takes place in a suitable buffer at pH 6-9 at temperatures varying from 4° C. to 25° C. for periods up to 16 hours.

[0181] In one embodiment of the present invention, an introduced cysteine residue is conjugated by mixed disulfide bond-formation with a chemical group selected from the list consisting of glutathione (gamma-glutamylcysteinylglycine), gamma-glutamylcysteine and cysteine present in the host cell or in the culture medium during production of the FVII polypeptide.

[0182] In one embodiment of the invention the chemical group is selected from the group consisting of: dendrimer, polyalkylene oxide (PAO), polyalkylene glycol (PAG), polyethylene glycol (PEG), polypropylene glycol (PPG), branched PEGs, polyvinyl alcohol (PVA), poly-carboxylate, poly-vinylpyrolidone, polyethylene-co-maleic acid anhydride, polystyrene-co-maleic acid anhydride, dextran, carboxymethyl-dextran; serum protein binding-ligands, such as compounds which bind to albumin, such as fatty acids, C5-C24 fatty acid, aliphatic diacid (e.g. C5-C24), a structure (e.g. sialic acid derivatives or mimetics) which inhibits the glycans from binding to receptors (e.g. asialoglycoprotein receptor and mannose receptor), a small organic molecule containing moieties that under physiological conditions alters charge properties, such as carboxylic acids or amines, or neutral sub-stituents that prevent glycan specific recognition such as smaller alkyl substituents (e.g., C1-C5 alkyl), a low molecular organic charged radical (e.g. C1-C25), which may contain one or more carboxylic acids, amines sulfonic, phosphonic acids, or combination thereof; a low molecular neutral hydrophilic molecule (e.g. C1-C25), such as cyclodextrin, or a polyethylene chain which may optionally branched; polyethyleneglycol with a avarage molecular weight of 2-40 KDa; a well defined precission polymer such as a dendrimer with an excact molecular mass ranging from 700 to 20.000 Da, such as between 700-10.000 Da; and a substantially non-imunogenic polypeptide such as albumin or an antibody or part of an antibody optionally containing a Fc-domain.

[0183] The term "PEGylated human Factor VIIa" means human Factor VIIa, having a PEG molecule conjugated to a human Factor VIIa polypeptide. It is to be understood, that the PEG molecule may be attached to any part of the Factor VIIa polypeptide including any amino acid residue or carbohydrate moiety of the Factor VIIa polypeptide. The term "cysteine-PEGylated human Factor VIIa" means Factor VIIa having a PEG molecule conjugated to a sulfhydryl group of a cysteine introduced in human Factor VIIa.

[0184] The biological activity of Factor VIIa in blood clotting derives from its ability to (i) bind to tissue factor (TF) and (ii) catalyze the proteolytic cleavage of Factor IX or Factor X to produce activated Factor IX or X (Factor IXa or Xa, respectively). For purposes of the invention, Factor VIIa biological activity may be quantified by measuring the ability of a preparation to promote blood clotting using Factor VII-deficient plasma and thromboplastin, as described, e.g., in U.S. Pat. No. 5,997,864. In this assay, biological activity is expressed as the reduction in clotting time relative to a control sample and is converted to "Factor VII units" by comparison with a pooled human serum standard containing 1 unit/ml Factor VII activity. Alternatively, Factor VIIa biological activity may be quantified by (i) measuring the ability of Factor VIIa to produce of Factor Xa in a system comprising TF embedded in a lipid membrane and Factor X. (Persson et al., J. Biol. Chem. 272:19919-19924, 1997); (ii) measuring Factor X hydrolysis in an aqueous system; (iii) measuring its physical binding to TF using an instrument based on surface plasmon resonance (Persson, FEBS Letts. 413:359-363, 1997) and (iv) measuring hydrolysis of a synthetic substrate.

[0185] The terms "variant" or "variants", as used herein, is intended to designate Factor VII having the sequence of SEQ ID NO:1, wherein one or more amino acids of the parent protein have been substituted by another amino acid and/or wherein one or more amino acids of the parent protein have been deleted and/or wherein one or more amino acids have been inserted in protein and/or wherein one or more amino acids have been added to the parent protein. Such addition can take place either at the N-terminal end or at the C-terminal end of the parent protein or both. The "variant" or "variants" within this definition still have FVII activity in its activated form. In one embodiment a variant is 70% identical with the sequence of SEQ ID NO:1. In one embodiment a variant is 80% identical with the sequence of SEQ ID NO:1. In another embodiment a variant is 90% identical with the sequence of SEQ ID NO:1. In a further embodiment a variant is 95% identical with the sequence of SEQ ID NO:1.

[0186] Non-limiting examples of Factor VII variants having substantially the same biological activity as wild-type Factor VII include S52A-FVIIa, S60A-FVIIa (Lino et al., Arch. Biochem. Biophys. 352: 182-192, 1998); FVIIa variants exhibiting increased proteolytic stability as disclosed in U.S. Pat. No. 5,580,560; Factor VIIa that has been proteolytically cleaved between residues 290 and 291 or between residues 315 and 316 (Mollerup et al., Biotechnol. Bioeng. 48:501-505, 1995); oxidized forms of Factor VIIa (Kornfelt et al., Arch. Biochem. Biophys. 363:43-54, 1999); FVII variants as disclosed in PCT/DK02/00189; and FVII variants exhibiting increased proteolytic stability as disclosed in WO 02/38162 (Scripps Research Institute); FVII variants having a modified Gla-domain and exhibiting an enhanced membrane binding as disclosed in WO 99/20767 (University of Minnesota) and WO 00/66753 (University of Minnesota); and FVII variants as disclosed in WO 01/58935 (Maxygen ApS), WO 03/93465 (Maxygen ApS) and WO 04/029091 (Maxygen ApS).

[0187] Non-limiting examples of FVII variants having increased biological activity compared to wild-type FVIIa include FVII variants as disclosed in WO 01/83725, WO 02/22776, WO 02/077218, PCT/DK02/00635, Danish patent application PA 2002 01423, Danish patent application PA 2001 01627; WO 02/38162 (Scripps Research Institute); and FVIIa variants with enhanced activity as disclosed in JP 2001061479 (Chemo-Sero-Therapeutic Res Inst.). Non-limiting examples of Factor VII variants having substantially reduced or modified biological activity relative to wild-type Factor VII include R152E-FVIIa (Wildgoose et al., Biochem 29:3413-3420, 1990), S344A-FVIIa (Kazama et al., J. Biol.

Chem. 270:66-72, 1995), FFR-FVIIa (Holst et al., Eur. J. Vasc. Endovasc. Surg. 15:515-520, 1998), and Factor VIIa lacking the Gla domain, (Nicolaisen et al., FEBS Letts. 317:245-249, 1993). Examples of variants of factor VII, factor VII or factor VII-related polypeptides include, without limitation, wild-type Factor VII, L305V-FVII, L305V/M306D/D309S-FVII, L3051-FVII, L305T-FVII, F374P-FVII, V158T/M298Q-FVII, V158D/E296V/M298Q-FVII, K337A-FVII, M298Q-FVII, V158D/M298Q-FVII, L305V/K337A-FVII, V158D/E296V/M298Q/L305V-FVII, V158D/E296V/M298Q/K337A-FVII, V158D/E296V/M298Q/L305V/K337A-FVII, K157A-FVII, E296V-FVII, E296V/M298Q-FVII, V158D/E296V-FVII, V158D/M298K-FVII, and S336G-FVII, L305V/K337A-FVII, L305V/V158D-FVII, L305V/E296V-FVII, L305V/M298Q-FVII, L305V/V158T-FVII, L305V/K337A/V158T-FVII, L305V/K337A/M298Q-FVII, L305V/K337A/E296V-FVII, L305V/K337A/V158D-FVII, L305V/V158D/M298Q-FVII, L305V/V158D/E296V-FVII, L305V/V158T/M298Q-FVII, L305V/V158T/E296V-FVII, L305V/E296V/M298Q-FVII, L305V/V158D/E296V/M298Q-FVII, L305V/V158T/E296V/M298Q-FVII, L305V/V158T/K337A/M298Q-FVII, L305V/V158T/E296V/K337A-FVII, L305V/V158D/K337A/M298Q-FVII, L305V/V158D/E296V/K337A-FVII, 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[0188] The terminology for amino acid substitutions used is as follows. The first letter represents the amino acid naturally present at a position of human wild type FVII. The following number represents the position in human wild type FVII. The second letter represent the different amino acid substituting for (replacing) the natural amino acid. An example is M298Q, where a methionine at position 298 of human wild type FVII is replaced by a glutamine. In another example, V158T/M298Q, the valine in position 158 of human wild type FVII is replaced by a threonine and the methionine in position 298 of human wild type FVII is replaced by a Glutamine in the same Factor VII polypeptide.

[0189] In a further embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at least about 1.25. In one embodiment the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at least about 2.0. In a further embodiment the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at least about 4.0.

[0190] In a further embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at least about 1.25 when tested in a Factor VIIa activity assay. In one embodiment the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at least about 2.0 when tested in a Factor VIIa activity assay. In a further embodiment the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at least about 4.0 when tested in a Factor VIIa activity assay. The Factor VIIa activity may be measured by the assays described under "assays".

[0191] In a further embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at least about 1.25 when tested in the "In Vitro Hydrolysis Assay". In one embodiment the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at least about 2.0 when tested in the "In Vitro Hydrolysis Assay". In a further embodiment the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at least about 4.0 when tested in the "In Vitro Hydrolysis Assay".

[0192] In a further embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at least about 1.25 when tested in the "In Vitro Proteolysis Assay". In one embodiment the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at least about 2.0 when tested in the "In Vitro Proteolysis Assay". In a further embodiment the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at least about 4.0 when tested in the "In Vitro Proteolysis Assay". In a further embodiment the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at least about 8.0 when tested in the "In Vitro Proteolysis Assay".

[0193] The present invention is also suitable for Factor VII/VIIa variants with increased activity compared to wild type. Factor VII/VIIa variants with increased activity may be found by testing in suitable assays described in the following. These assays can be performed as a simple preliminary in vitro test. Thus, the section "assays" discloses a simple test (entitled "In Vitro Hydrolysis Assay") for the activity of Factor VIIa variants of the invention. Based thereon, Factor VIIa variants which are of particular interest are such variants where the ratio between the activity of the variant and the activity of wild type Factor VII is above 1.0, e.g. at least about 1.25, preferably at least about 2.0, such as at least about 3.0 or, even more preferred, at least about 4.0 when tested in the "In Vitro Hydrolysis Assay".

[0194] The activity of the variants can also be measured using a physiological substrate such as factor X ("In Vitro Proteolysis Assay") (see under "assays"), suitably at a concentration of 100-1000 nM, where the factor Xa generated is measured after the addition of a suitable chromogenic substrate (e.g. S-2765). In addition, the activity assay may be run at physiological temperature.

[0195] The ability of the Factor VIIa variants to generate thrombin can also be measured in an assay comprising all relevant coagulation factors and inhibitors at physiological concentrations (minus factor VIII when mimicking hemophilia A conditions) and activated platelets (as described on p. 543 in Monroe et al. (1997) Brit. J. Haematol. 99, 542-547 which is hereby incorporated as reference).

[0196] The term "identity" as known in the art, refers to a relationship between the sequences of two or more polypeptide molecules or two or more nucleic acid molecules, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between nucleic acid molecules or between polypeptides, as the case

may be, as determined by the number of matches between strings of two or more nucleotide residues or two or more amino acid residues. "Identity" measures the percent of identical matches between the smaller of two or more sequences with gap alignments (if any) addressed by a particular mathematical model or computer program (i.e., "algorithms").

[0197] The term "similarity" is a related concept, but in contrast to "identity", refers to a sequence relationship that includes both identical matches and conservative substitution matches. If two polypeptide sequences have, for example, {fraction (10/20)} identical amino acids, and the remainder are all non-conservative substitutions, then the percent identity and similarity would both be 50%. If, in the same example, there are 5 more positions where there are conservative substitutions, then the percent identity remains 50%, but the percent similarity would be 75% ({fraction (15/20)}). Therefore, in cases where there are conservative substitutions, the degree of similarity between two polypeptides will be higher than the percent identity between those two polypeptides.

[0198] The term "isolated polypeptide" refers to a polypeptide of the present invention that (1) has been separated from at least about 50 percent of polynucleotides, lipids, carbohydrates or other materials (i.e., contaminants) with which it is naturally associated, (2) is not covalently linked to all or a portion of a polypeptide to which the "isolated polypeptide" is linked in nature, (3) is operably linked covalently to a polypeptide to which it is not covalently linked in nature, or (4) does not occur in nature. Preferably, the isolated polypeptide is substantially free from any other contaminating polypeptides or other contaminants that are found in its natural environment which would interfere with its therapeutic, diagnostic, prophylactic or research use.

[0199] Conservative modifications to the amino acid sequence of SEQ ID NO:1 (and the corresponding modifications to the encoding nucleotides) outside the positions for substitutions claimed will produce FVII polypeptides having functional and chemical characteristics similar to those of naturally occurring FVII polypeptide. In contrast, substantial modifications in the functional and/or chemical characteristics of FVII polypeptides may be accomplished by selecting substitutions in the amino acid sequence of SEQ ID NO:1 that differ significantly in their effect on maintaining (a) the structure of the molecular backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain.

[0200] For example, a "conservative amino acid substitution" may involve a substitution of a native amino acid residue with a non-native residue such that there is little or no effect on the polarity or charge of the amino acid residue at that position. Furthermore, any native residue in the polypeptide may also be substituted with almandine, as has been previously described for "alanine scanning mutagenesis" (see, for example, MacLennan et al., 1998, Acta Physiol. Scand. Suppl. 643:55-67; Sasaki et al., 1998, Adv. Biophys. 35:1-24, which discuss alanine scanning mutagenesis).

[0201] Identity and similarity of related polypeptides can be readily calculated by known methods. Such methods include, but are not limited to, those described in Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part 1, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M. Stockton Press, New York, 1991; and Carillo et al., SIAM J. Applied Math., 48:1073 (1988).

[0202] Preferred methods to determine identity and/or similarity are designed to give the largest match between the sequences tested. Methods to determine identity and similarity are described in publicly available computer programs. Preferred computer program methods to determine identity and similarity between two sequences include, but are not limited to, the GCG program package, including GAP (Devereux et al., Nucl. Acid. Res., 12:387 (1984); Genetics Computer Group, University of Wisconsin, Madison, Wis.), BLASTP, BLASTN, and FASTA (Altschul et al., J. Mol. Biol., 215:403-410 (1990)). The BLASTX program is publicly available from the National Center for Biotechnology Information (NCBI) and other sources (BLAST Manual, Altschul et al. NCB/NLM/NIH Bethesda, Md. 20894; Altschul et al., supra). The well known Smith Waterman algorithm may also be used to determine identity.

[0203] Certain alignment schemes for aligning two amino acid sequences may result in the matching of only a short region of the two sequences, and this small aligned region may have very high sequence identity even though there is no significant relationship between the two full length sequences. Accordingly, in a preferred embodiment, the selected alignment method (GAP program) will result in an alignment that spans at least 50 contiguous amino acids of the target polypeptide.

[0204] For example, using the computer algorithm GAP (Genetics Computer Group, University of Wisconsin, Madison, Wis.), two polypeptides for which the percent sequence identity is to be determined are aligned for optimal matching of their respective amino acids (the "matched span", as determined by the algorithm). A gap opening penalty (which is calculated as 3.times. the average diagonal; the "average diagonal" is the average of the diagonal of the comparison matrix being used; the "diagonal" is the score or number assigned to each perfect amino acid match by the particular comparison matrix) and a gap extension penalty (which is usually {fraction (1/10)} times the gap opening penalty), as well as a comparison matrix such as PAM 250 or BLOSUM 62 are used in conjunction with the algorithm. A standard comparison matrix (see Dayhoff et al., Atlas of Protein Sequence and Structure, vol. 5, supp. 3 (1978) for the PAM 250 comparison matrix; Henikoff et al., Proc. Natl. Acad. Sci USA, 89:10915-10919 (1992) for the BLOSUM 62 comparison matrix) is also used by the algorithm.

[0205] Preferred parameters for a polypeptide sequence comparison include the following:

[0206] Algorithm: Needleman et al., J. Mol. Biol, 48:443-453 (1970); Comparison matrix: BLOSUM 62 from Henikoff et al., Proc. Natl. Acad. Sci. USA, 89:10915-10919 (1992); Gap Penalty: 12, Gap Length Penalty: 4, Threshold of Similarity: 0.

[0207] The GAP program is useful with the above parameters. The aforementioned parameters are the default parameters for polypeptide comparisons (along with no penalty for end gaps) using the GAP algorithm.

[0208] Preferred parameters for nucleic acid molecule sequence comparisons include the following: Algorithm: Needleman et al., J. Mol Biol., 48:443-453 (1970); Comparison matrix: matches=+10, mismatch=0, Gap Penalty: 50, Gap Length Penalty: 3.

[0209] The GAP program is also useful with the above parameters. The aforementioned parameters are the default parameters for nucleic acid molecule comparisons.

[0210] Other exemplary algorithms, gap opening penalties, gap extension penalties, comparison matrices, thresholds of similarity, etc. may be used, including those set forth in the Program Manual, Wisconsin Package, Version 9, September, 1997. The particular choices to be made will be apparent to those of skill in the art and will depend on the specific comparison to be made, such as DNA to DNA, protein to protein, protein to DNA; and additionally, whether the comparison is between given pairs of sequences (in which case GAP or BestFit are generally preferred) or between one sequence and a large database of sequences (in which case FASTA or BLASTA are preferred).

[0211] The term "Tissue Factor binding affinity", as used herein, means the strength of the binding of a FVII polypeptide to human Tissue Factor. The affinity of a FVII polypeptide is measured by the dissociation constant K_d, defined as [FVII]×[TF]/[FVII−TF] where [FVII−TF] is the molar concentration of the FVII/TF complex, [FVII] is the molar concentration of the unbound FVII polypeptide and [TF] is the molar concentration of the unbound human Tissue Factor. The affinity constant K_a is defined by $1/K_d$.

[0212] The phrase "substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa", as used herein, means an activity more than 50% of the activity of recombinant wild type human Factor VIIa. In one embodiment the activity is more than 60% of the activity of recombinant wild type human Factor VIIa. In one embodiment the activity is more than 70% of the activity of recombinant wild type human Factor VIIa. In one embodiment the activity is more than 80% of the activity of recombinant wild type human Factor VIIa. In another embodiment the activity is more than 90% of the activity of recombinant wild type human Factor VIIa. In a further embodiment the activity is more than 100% of the activity of recombinant wild type human Factor VIIa. In a further embodiment the activity is more than 120% of the activity of recombinant wild type human Factor VIIa. In a further embodiment the activity is more than 200% of the activity of recombinant wild type human Factor VIIa. In a further embodiment the activity is more than 400% of the activity of recombinant wild type human Factor VIIa.

[0213] The term "activity" as used herein means the ability of a Factor VII polypeptide to convert its substrate Factor X to the active Factor Xa. The activity of a Factor VI I polypeptide may be measured with the "In Vitro Proteolysis Assay".

[0214] The phrase "a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa", as used herein, means an binding affinity lower than that of recombinant wild type human Factor VIIa as measured in a TF binding affinity assay, such as the Biosensor assay as described in Example 5. In one embodiment the Tissue Factor binding affinity is less than 90% of the Tissue Factor binding affinity of recombinant wild type human Factor VIIa. In one embodiment the Tissue Factor binding affinity is less than 80% of the Tissue Factor binding affinity of recombinant wild type human Factor VIIa. In one embodiment the Tissue Factor binding affinity is less than 70% of the Tissue Factor binding affinity of recombinant wild type human Factor VIIa. In one embodiment the Tissue Factor binding affinity is less than 60% of the Tissue Factor binding affinity of recombinant wild type human Factor VIIa. In one embodiment the Tissue Factor binding affinity is less than 50% of the Tissue Factor binding affinity of recombinant wild type human Factor VIIa. In one embodiment the Tissue Factor binding affinity is less than 40% of the Tissue Factor binding affinity of recombinant wild type human Factor VIIa. In one embodiment the Tissue Factor binding affinity is less than 30% of the Tissue Factor binding affinity of recombinant wild type human Factor VIIa. In one embodiment the Tissue Factor binding affinity is less than 20% of the Tissue Factor binding affinity of recombinant wild type human Factor VIIa. In one embodiment the Tissue Factor binding affinity is less than 10% of the Tissue Factor binding affinity of recombinant wild type human Factor VIIa. In one embodiment the Tissue Factor binding affinity is less than 5% of the Tissue Factor binding affinity of recombinant wild type human Factor VIIa. In one embodiment the Tissue Factor binding affinity is less than 1% of the Tissue Factor binding affinity of recombinant wild type human Factor VIIa. In one embodiment the Tissue Factor binding affinity is less than 0.1% of the Tissue Factor binding affinity of recombinant wild type human Factor VIIa. In one embodiment the Tissue Factor binding affinity is less than 0.01% of the Tissue Factor binding affinity of recombinant wild type human Factor VIIa. In one embodiment the Tissue Factor binding affinity is less than 0.001% of the Tissue Factor binding affinity of recombinant wild type human Factor VIIa.

[0215] The term "polyethylene glycol" or "PEG" means a polyethylene glycol compound or a derivative thereof, with or without coupling agents, coupling or activating moieties (e.g., with thiol, triflate, tresylate, azirdine, oxirane, or preferably with a maleimide moiety). Compounds such as maleimido monomethoxy PEG are exemplary of activated PEG compounds of the invention.

[0216] In one embodiment of the invention, the Factor VII polypeptides are Factor VII polypeptides, wherein one or more amino acid corresponding to amino acids at positions selected from K18, R36, S43, K62, Q64, L65, I69, F71, L73, P74, E77, G78, R79, K85, Q88, N93, F275, R277, M306, T307, Q308, D309, or R379 of SEQ ID NO: 1 have been substituted with a different amino acid, which different amino acid decreases the Tissue Factor binding affinity.

[0217] The term "a different amino acid" as used herein means one or more amino acids that are different from that amino acid naturally present at that position. This includes but is not limited to amino acids that can be encoded by a polynucleotide. Preferably the different amino acid is in natural L-form and can be encoded by a polynucleotide. A specific example being L-Glutamic acid (L-Glu).

[0218] In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position K18 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position K18 of SEQ ID NO: 1 is selected from the list consisting of E, D, A, and F.

[0219] In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position R36 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position R36 of SEQ ID NO: 1 is selected from the list consisting of E, D, A, and F.

[0220] In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position S43 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position S43 of SEQ ID NO: 1 is selected from the list consisting of E, D, K, R, A, F, and N. In one embodiment this different amino acid is selected from the list consisting of E, D, K, R, A, and F. In one embodiment this different amino acid is an N.

[0221] In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position K62 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position K62 of SEQ ID NO: 1 is selected from the list consisting of E, D, A, and F. In one embodiment this different amino acid is selected from the list consisting of D, A, and F.

[0222] In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position Q64 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position Q64 of SEQ ID NO: 1 is selected from the list consisting of E, D, K, R, A, and F.

[0223] In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position L65 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position L65 of SEQ ID NO: 1 is selected from the list consisting of E, D, K, R, A, and F.

[0224] In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position 169 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position 169 of SEQ ID NO: 1 is selected from the list consisting of E, D, K, R, A, and F. In one embodiment this different amino acid is selected from the list consisting of E, D, K, R, and F.

[0225] In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position F71 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position F71 of SEQ ID NO: 1 is selected from the list consisting of E, D, K, R, and A. In one embodiment this different amino acid is selected from the list consisting of K, R, and A.

[0226] In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position L73 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position L73 of SEQ ID NO: 1 is selected from the list consisting of E, D, K, R, A, and F.

[0227] In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position P74 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position P74 of SEQ ID NO: 1 is selected from the list consisting of E, D, K, R, A, and F.

[0228] In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position E77 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position E77 of SEQ ID NO: 1 is selected from the list consisting of K, R, and A.

[0229] In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position G78 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position G78 of SEQ ID NO: 1 is selected from the list consisting of E, D, K, R, A, and F.

[0230] In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position R79 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position R79 of SEQ ID NO: 1 is selected from the list consisting of E, D, and A.

[0231] In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position K85 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position K85 of SEQ ID NO: 1 is selected from the list consisting of E, D, and A.

[0232] In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position Q88 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position Q88 of SEQ ID NO: 1 is selected from the list consisting of E, D, K, R, A, and F.

[0233] In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position N93 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position N93 of SEQ ID NO: 1 is selected from the list consisting of E, D, K, R, A, and F.

[0234] In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position F275 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position F275 of SEQ ID NO: 1 is selected from the list consisting of E, D, K, R, and A.

[0235] In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position R277 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position R277 of SEQ ID NO: 1 is selected from the list consisting of E, D, A, and F.

[0236] In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position T307 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position T307 of SEQ ID NO: 1 is selected from the list consisting of E, D, K, R, A, F, and N.

[0237] In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position Q308 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position Q308 of SEQ ID NO: 1 is selected from the list consisting of E, D, K, R, A, F, and N.

[0238] In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position R379 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position R379 of SEQ ID NO: 1 is selected from the list consisting of E, D, A, and F.

[0239] In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position M306 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position M306 of SEQ ID NO: 1 is selected from the list consisting of E, D, K, R, A, and F. In one embodiment this different amino acid is selected from the list consisting of E, K, R, A, and F.

[0240] In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position D309 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position D309 of SEQ ID NO: 1 is selected from the list consisting of K, R, and A.

[0241] In a further embodiment, the Factor VII polypeptide of the invention is a Factor VII polypeptide selected from the list consisting of S43N-FVII, K62E-FVII, Q64E-FVII, I69F-FVII, K62E/I69A-FVII, Q64E/I69A-FVII, K62E/Q64E/I69A-FVII, K62E/I69F-FVII, Q64E/I69F-FVII, and K62E/Q64E/I69F-FVII.

[0242] In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides further comprising amino acid substitutions selected from the list consisting of L305V, L305V/M306D/D309S, L3051, L305T, F374P, V158T/M298Q, V158D/E296V/M298Q, K337A, M298Q, V158D/M298Q, L305V/K337A, V158D/E296V/M298Q/L305V, V158D/E296V/M298Q/K337A, V158D/E296V/M298Q/L305V/K337A, K157A, E296V, E296V/M298Q, V158D/E296V, V158D/M298K, and S336G, L305V/K337A, L305V/V158D, L305V/E296V, L305V/M298Q, L305V/V158T, L305V/K337A/V158T, L305V/K337A/M298Q, L305V/K337A/E296V, L305V/K337A/V158D, L305V/V158D/M298Q, L305V/V158D/E296V, L305V/V158T/M298Q, L305V/V158T/E296V, L305V/E296V/M298Q, L305V/V158D/E296V/M298Q, L305V/V158T/E296V/M298Q, L305V/V158T/K337A/M298Q, L305V/V158T/E296V/K337A, L305V/V158D/K337A/M298Q, L305V/V158D/E296V/K337A, L305V/V158D/E296V/M298Q/K337A, L305V/V158T/E296V/M298Q/K337A, S314E/K316H, S314E/K316Q, S314E/L305V, S314E/K337A, S314E/V158D, S314E/E296V, S314E/M298Q, S314E/V158T, K316H/L305V, K316H/K337A, K316H/V158D, K316H/E296V, K316H/M298Q, K316H/V158T, K316Q/L305V, K316Q/K337A, K316Q/V158D, K316Q/E296V, K316Q/M298Q, K316Q/V158T, S314E/L305V/K337A, S314E/L305V/V158D, S314E/L305V/E296V, S314E/L305V/M298Q, S314E/L305V/V158T, S314E/L305V/K337A/V158T, S314E/L305V/K337A/M298Q, S314E/L305V/K337A/E296V, S314E/L305V/K337A/V158D, S314E/L305V/V158D/M298Q, S314E/L305V/V158D/E296V, S314E/L305V/V158T/M298Q, S314E/L305V/V158T/E296V, S314E/L305V/E296V/M298Q, S314E/L305V/V158D/E296V/M298Q, S314E/L305V/V158T/E296V/M298Q, S314E/L305V/V158T/K337A/M298Q, S314E/L305V/V158T/E296V/K337A, S314E/L305V/V158D/K337A/M298Q, S314E/L305V/V158D/E296V/K337A, S314E/L305V/V158D/E296V/M298Q/K337A, S314E/L305V/V158T/E296V/M298Q/K337A, K316H/L305V/K337A, K316H/L305V/V158D, K316H/L305V/E296V, K316H/L305V/M298Q, K316H/L305V/V158T, K316H/L305V/K337A/V158T, K316H/L305V/K337A/M298Q, K316H/L305V/K337A/E296V, K316H/L305V/K337A/V158D, K316H/L305V/V158D/M298Q, K316H/L305V/V158D/E296V, K316H/L305V/V158T/M298Q, K316H/L305V/V158T/E296V, K316H/L305V/E296V/M298Q, K316H/L305V/V158D/E296V/M298Q, K316H/L305V/V158T/E296V/M298Q, K316H/L305V/V158T/K337A/M298Q, K316H/L305V/V158T/E296V/K337A, K316H/L305V/V158D/E296V/M298Q/K337A, K316H/L305V/V158T/E296V/M298Q/K337A, K316Q/L305V/K337A, K316Q/L305V/V158D, K316Q/L305V/E296V, K316Q/L305V/M298Q, K316Q/L305V/V158T, K316Q/L305V/K337A/V158T, K316Q/L305V/K337A/M298Q, K316Q/L305V/K337A/E296V, K316Q/L305V/K337A/V158D, K316Q/L305V/V158D/M298Q, K316Q/L305V/V158D/E296V, K316Q/L305V/V158T/M298Q, K316Q/L305V/V158T/E296V, K316Q/L305V/E296V/M298Q, K316Q/L305V/V158D/E296V/M298Q, K316Q/L305V/V158T/E296V/M298Q, K316Q/L305V/V158T/K337A/M298Q, K316Q/L305V/V158T/E296V/K337A, K316Q/L305V/V158D/K337A/M298Q, K316Q/L305V/V158D/E296V/K337A, K316Q/L305V/V158T/E296V/M298Q/K337A, F374Y/K337A, F374Y/V158D, F374Y/E296V, F374Y/M298Q, F374Y/V158T, F374Y/S314E, F374Y/L305V, F374Y/

L305V/K337A, F374Y/L305V/V158D, F374Y/L305V/E296V, F374Y/L305V/M298Q, F374Y/L305V/V158T, F374Y/L305V/S314E, F374Y/K337A/S314E, F374Y/K337A/V158T, F374Y/K337A/M298Q, F374Y/K337A/E296V, F374Y/K337A/V158D, F374Y/V158D/S314E, F374Y/V158D/M298Q, F374Y/V158D/E296V, F374Y/V158T/S314E, F374Y/V158T/M298Q, F374Y/V158T/E296V, F374Y/E296V/S314E, F374Y/S314E/M298Q, F374Y/E296V/M298Q, F374Y/L305V/K337A/V158D, F374Y/L305V/K337A/E296V, F374Y/L305V/K337A/M298Q, F374Y/L305V/K337A/V158T, F374Y/L305V/K337A/S314E, F374Y/L305V/V158D/E296V, F374Y/L305V/V158D/M298Q, F374Y/L305V/V158D/S314E, F374Y/L305V/E296V/M298Q, F374Y/L305V/E296V/V158T, F374Y/L305V/E296V/S314E, F374Y/L305V/M298Q/V158T, F374Y/L305V/M298Q/S314E, F374Y/L305V/V158T/S314E, F374Y/K337A/S314E/V158T, F374Y/K337A/S314E/M298Q, F374Y/K337A/S314E/E296V, F374Y/K337A/S314E/V158D, F374Y/K337A/V158T/M298Q, F374Y/K337A/V158T/E296V, F374Y/K337A/M298Q/E296V, F374Y/K337A/M298Q/V158D, F374Y/K337A/E296V/V158D, F374Y/V158D/S314E/M298Q, F374Y/V158D/S314E/E296V, F374Y/V158D/M298Q/E296V, F374Y/V158T/S314E/E296V, F374Y/V158T/S314E/M298Q, F374Y/V158T/M298Q/E296V, F374Y/E296V/S314E/M298Q, F374Y/L305V/M298Q/K337A/S314E, F374Y/L305V/E296V/K337A/S314E, F374Y/E296V/M298Q/K337A/S314E, F374Y/L305V/E296V/M298Q/K337A, F374Y/L305V/E296V/M298Q/S314E, F374Y/V158D/E296V/M298Q/K337A, F374Y/V158D/E296V/M298Q/S314E, F374Y/L305V/V158D/K337A/S314E, F374Y/V158D/M298Q/K337A/S314E, F374Y/V158D/E296V/K337A/S314E, F374Y/L305V/V158D/E296V/M298Q, F374Y/L305V/V158D/M298Q/K337A, F374Y/L305V/V158D/E296V/K337A, F374Y/L305V/V158D/M298Q/S314E, F374Y/L305V/V158D/E296V/S314E, F374Y/V158T/E296V/M298Q/K337A, F374Y/V158T/E296V/M298Q/S314E, F374Y/L305V/V158T/K337A/S314E, F374Y/V158T/M298Q/K337A/S314E, F374Y/V158T/E296V/K337A/S314E, F374Y/L305V/V158T/E296V/M298Q, F374Y/L305V/V158T/M298Q/K337A, F374Y/L305V/V158T/E296V/K337A, F374Y/L305V/V158T/M298Q/S314E, F374Y/L305V/V158T/E296V/S314E, F374Y/E296V/M298Q/K337A/V158T/S314E, F374Y/V158D/E296V/M298Q/K337A/S314E, F374Y/L305V/V158D/E296V/M298Q/S314E, F374Y/L305V/E296V/M298Q/V158T/S314E, F374Y/L305V/E296V/M298Q/K337A/V158T, F374Y/L305V/E296V/K337A/V158T/S314E, F374Y/L305V/M298Q/K337A/V158T/S314E, F374Y/L305V/V158D/E296V/M298Q/K337A, F374Y/L305V/V158D/E296V/K337A/S314E, F374Y/L305V/V158D/M298Q/K337A/S314E, F374Y/L305V/E296V/M298Q/K337A/V158T/S314E, F374Y/L305V/V158D/E296V/M298Q/K337A/S314E, S52A, S60A; R152E, S344A, P11Q/K33E, T106N, K143N/N145T, V253N, R290N/A292T, G291N, R315N/V317T, and K143N/N145T/R315N/V317T; or having substitutions, additions or deletions in the amino acid sequence from 233Thr to 240Asn, or having substitutions, additions or deletions in the amino acid sequence from 304Arg to 329Cys.

[0243] In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the dissociation constant K_d of said Factor VII polypeptide is higher than 5 nM, such as higher than 7 nM, such as higher than 10 nM, such as higher than 20 nM, such as higher than 30 nM, such as higher than 50 nM, such as higher than 100 nM, such as higher than 200 nM, such as higher than 300 nM, such as higher than 400 nM, such as higher than 500 nM, such as higher than 1 μM.

[0244] In a further embodiment, the Factor VII polypeptides of the invention comprises one or more amino acid substitution selected from $K18X_1$, $R36X_2$, $S43X_3$, $K62X_4$, $Q64X_5$, $L65X_6$, $I69X_7$, $F71X_8$, $L73X_9$, $P74X_{10}$, $E77X_{11}$, $G78X_{12}$, $R79X_{13}$, $K85X_{14}$, $Q88X_{15}$, $N93X_{16}$, $F275X_{17}$, $R277X_{18}$, $M306X_{19}$, $T307X_{20}$, $Q308X_{21}$, $D309X_{22}$, or $R379X_{23}$ corresponding to amino acids at positions of SEQ ID NO: 1, which different amino acid decreases the Tissue Factor binding affinity, wherein

[0245] X_1 is E, D, A, or F;

[0246] X_2 is E, D, K, A, F, or N;

[0247] X_3 is E, D, K, R, A, or F;

[0248] X_4 is D, A, or F;

[0249] X_5 is E, D, K, R, A, F, or N;

[0250] X_6 is E, D, K, R, A, or F;

[0251] X_7 is C, D, E, F, G, H, K, L, M, N, P, Q, R, S, T, V, W, or Y;

[0252] X_8 is A, G, H, I, K, L, M, P, R, S, T, V, or W;

[0253] X_9 is A, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y;

[0254] X_{10} is A, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, or Y;

[0255] X_{11} is A, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

[0256] X_{12} is A, D, E, F, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

[0257] X_{13} is A, D, E, F, G, H, I, K, L, M, N, P, Q, S, T, V, W, or Y;

[0258] X_{14} is A, D, E, F, G, I, L, M, P, S, T, V, W, or Y;

[0259] X_{15} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;

[0260] X_{16} is A, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y;

[0261] X_{17} is A, D, E, G, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

[0262] X_{18} is A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y;

[0263] X_{19} is A, E, F, G, H, I, K, L, P, Q, R, S, T, V, W, or Y;

[0264] X_{20} is A, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, V, W, or Y;

[0265] X_{21} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;

[0266] X_{22} is A, F, G, H, I, K, L, M, N, P, Q, R, V, W, or Y; and

[0267] X_{23} is A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y.

[0268] In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein one or more amino acid corresponding to amino acids at positions selected from K18, R36, K62, Q64, L65, I69, F71, L73, P74, E77, G78, R79, K85, Q88, N93, F275, R277, M306, Q308, D309, or R379 of SEQ ID NO: 1 have been substituted with a cysteine amino acid residue, which cysteine amino acid residue decreases the Tissue Factor binding affinity, and which cysteine amino acid residue is optionally conjugated with a chemical group, which chemical group increases the molecular weight of said Factor VII polypeptide. In one embodiment K18 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment R36 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment K62 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment Q64 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment L65 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment I69 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment F71 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment L73 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment P74 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment E77 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment G78 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment R79 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment K85 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment Q88 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment N93 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment F275 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment R277 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment M306 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment Q308 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment D309 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment R379 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue.

[0269] In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide comprising one or more N-glycosylation site N-Xaa-S/T introduced by amino acid substitutions corresponding to amino acids starting at positions selected from R36, Q64, I69, F71, P74, E77, G78, Q88, N93, F275, M306, T307, or D309 of SEQ ID NO: 1, wherein Xaa is any amino acid except P, which introduced N-glycosylation site decreases the Tissue Factor binding affinity. In one embodiment an N-glycosylation site N-Xaa-S/T is introduced at a position starting at R36 of SEQ ID NO: 1. In one embodiment an N-glycosylation site N-Xaa-S/T is introduced at a position starting at R36 of SEQ ID NO: 1. In one embodiment an N-glycosylation site N-Xaa-S/T is introduced at a position starting at Q64 of SEQ ID NO: 1.

[0270] In one embodiment an N-glycosylation site N-Xaa-S/T is introduced at a position starting at 169 of SEQ ID NO: 1. In one embodiment an N-glycosylation site N-Xaa-S/T is introduced at a position starting at F71 of SEQ ID NO: 1. In one embodiment an N-glycosylation site N-Xaa-S/T is introduced at a position starting at P74 of SEQ ID NO: 1. In one embodiment an N-glycosylation site N-Xaa-S/T is introduced at a position starting at E77 of SEQ ID NO: 1.

[0271] In one embodiment an N-glycosylation site N-Xaa-S/T is introduced at a position starting at G78 of SEQ ID NO: 1.In one embodiment an N-glycosylation site N-Xaa-S/T is introduced at a position starting at Q88 of SEQ ID NO: 1.In one embodiment an N-glycosylation site N-Xaa-S/T is introduced at a position starting at N93 of SEQ ID NO: 1.In one embodiment an N-glycosylation site N-Xaa-S/T is introduced at a position starting at F275 of SEQ ID NO: 1.

[0272] In one embodiment an N-glycosylation site N-Xaa-S/T is introduced at a position starting at M306 of SEQ ID NO: 1.In one embodiment an N-glycosylation site N-Xaa-S/T is introduced at a position starting at T307 of SEQ ID NO: 1. In one embodiment an N-glycosylation site N-Xaa-S/T is introduced at a position starting at D309 of SEQ ID NO: 1.

[0273] In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position K18 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of E, D, A, and F.

[0274] In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position R36 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of E, D, K, A, F, and N.

[0275] In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position S43 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of E, D, K, R, A, and F.

[0276] In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position K62 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of D, A, and F.

[0277] In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position Q64 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of E, D, K, R, A, F, and N.

[0278] In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position L65 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of E, D, K, R, A, and F.

[0279] In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein

the amino acid corresponding to the amino acid at a position 169 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of C, D, E, F, G, H, K, L, M, N, P, Q, R, S, T, V, W, and Y.

[0280] In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position F71 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, G, H, I, K, L, M, P, R, S, T, V, and W.

[0281] In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position L73 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, and Y.

[0282] In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position P74 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, and Y.

[0283] In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position E77 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, and Y.

[0284] In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position G78 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, D, E, F, H, I, K, L, M, N, P, Q, R, S, T, V, W, and Y.

[0285] In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position R79 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, D, E, F, G, H, I, K, L, M, N, P, Q, S, T, V, W, and Y.

[0286] In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position K85 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, D, E, F, G, I, L, M, P, S, T, V, W, and Y.

[0287] In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position Q88 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, and Y.

[0288] In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position N93 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, and Y.

[0289] In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position F275 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, D, E, G, I, K, L, M, N, P, Q, R, S, T, V, W, and Y.

[0290] In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position R277 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, and Y.

[0291] In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position M306 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, E, F, G, H, I, K, L, P, Q, R, S, T, V, W, and Y.

[0292] In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position T307 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, V, W, and Y.

[0293] In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position Q308 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, and Y.

[0294] In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position D309 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, F, G, H, I, K, L, M, N, P, Q, R, V, W, and Y.

[0295] In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position R379 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y.

[0296] In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, which is selected from the list consisting of FVII-(S43N), FVII-(K62E), FVII-(Q64E), FVII-(I69F), FVII-(K62E/I69A), FVII-(Q64E/I69A), FVII-(Q62E/Q64E/I69A), FVII-(K62E/I69F), FVII-(Q64E/I69F), FVII-(K62E/Q64E/I69F), FVII-(S43C), FVII-(I69C), FVII-(Q64C), FVII-(M306C), FVII-(R277C), FVII-(I69N/F71T), FVII-(F71N/L73T), FVII-(R277N), and FVII-(D309N/L311T).

[0297] In one embodiment the polynucleotide construct of the invention is a vector.

[0298] The term "a polynucleotide" denotes a single- or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. Polynucleotides include RNA and DNA, and may be isolated from natural sources, synthesized in vitro, or prepared from a combination of natural and synthetic molecules. The length of a polynucleotide molecule is given herein in terms of nucleotides (abbreviated "nt") or base pairs (abbreviated "bp"). The term "nucleotides" is used for both single- and

double-stranded molecules where the context permits. When the term is applied to double-stranded molecules it is used to denote overall length and will be understood to be equivalent to the term "base pairs". It will be recognized by those skilled in the art that the two strands of a double-stranded polynucleotide may differ slightly in length and that the ends thereof may be staggered as a result of enzymatic cleavage; thus all nucleotides within a double-stranded polynucleotide molecule may not be paired. Such unpaired ends will in general not exceed 20 nt in length.

[0299] The term "vector", as used herein, means any nucleic acid entity capable of the amplification in a host cell. Thus, the vector may be an autonomously replicating vector, i.e. a vector, which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated. The choice of vector will often depend on the host cell into which it is to be introduced. Vectors include, but are not limited to plasmid vectors, phage vectors, viruses or cosmid vectors. Vectors usually contain a replication origin and at least one selectable gene, i.e., a gene which encodes a product which is readily detectable or the presence of which is essential for cell growth.

[0300] In one embodiment, the eukaryotic host cell of the invention is of mammalian origin.

[0301] In a further embodiment, the eukaryotic host cell of the invention is selected from the group consisting of CHO cells, BHK cells or HEK cells.

[0302] The term "a eukaryotic host cell", as used herein, represent any cell, including hybrid cells, in which heterologous DNA can be expressed. Typical host cells includes, but are not limited to insect cells, yeast cells, mammalian cells, including human cells, such as BHK, CHO, HEK, and COS cells. In practicing the present invention, the host cells being cultivated are preferably mammalian cells, more preferably an established mammalian cell line, including, without limitation, CHO (e.g., ATCC CCL 61), COS-1 (e.g., ATCC CRL 1650), baby hamster kidney (BHK) and HEK293 (e.g., ATCC CRL 1573; Graham et al., *J. Gen. Virol.* 36:59-72, 1977) cell lines.

[0303] A preferred BHK cell line is the tk^- ts13 BHK cell line (Waechter and Baserga, *Proc. Natl. Acad. Sci. USA* 79:1106-1110, 1982), hereinafter referred to as BHK 570 cells. The BHK 570 cell line is available from the American Type Culture Collection, 12301 Parklawn Dr., Rockville, Md. 20852, under ATCC accession number CRL 10314. A tk^- ts13 BHK cell line is also available from the ATCC under accession number CRL 1632.

[0304] Other suitable cell lines include, without limitation, Rat Hep I (Rat hepatoma; ATCC CRL 1600), Rat Hep II (Rat hepatoma; ATCC CRL 1548), TCMK (ATCC CCL 139), Human lung (ATCC HB 8065), NCTC 1469 (ATCC CCL 9.1) and DUKX cells (Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA* 77:4216-4220, 1980). Also useful are 3T3 cells, Namalwa cells, myelomas and fusions of myelomas with other cells. In one embodiment the eucaryotic host cell is of mammalian origin. In a further embodiment the eucaryotic host cell is selected from the group consisting of CHO cells, BHK cells or HEK cells.

[0305] The term "treatment", as used herein, means the administration of an effective amount of a therapeutically active compound of the invention with the purpose of preventing any symptoms or disease state to develop or with the purpose of curing or easing such symptoms or disease states already developed. The term "treatment" is thus meant to include prophylactic treatment.

[0306] The term "enhancement of the normal haemostatic system" means an enhancement of the ability to generate thrombin.

[0307] As used herein the term "bleeding disorders" reflects any defect, congenital, acquired or induced, of cellular or molecular origin that is manifested in bleedings. Examples are clotting factor deficiencies (e.g. haemophilia A and B or deficiency of coagulation Factors XI or VII), clotting factor inhibitors, defective platelet function, thrombocytopenia or von Willebrand's disease.

[0308] The term "bleeding episodes" is meant to include uncontrolled and excessive bleeding which is a major problem both in connection with surgery and other forms of tissue damage. Uncontrolled and excessive bleeding may occur in subjects having a normal coagulation system and subjects having coagulation or bleeding disorders. Clotting factor deficiencies (haemophilia A and B, deficiency of coagulation factors XI or VII) or clotting factor inhibitors may be the cause of bleeding disorders. Excessive bleedings also occur in subjects with a normally functioning blood clotting cascade (no clotting factor deficiencies or -inhibitors against any of the coagulation factors) and may be caused by a defective platelet function, thrombocytopenia or von Willebrand's disease. In such cases, the bleedings may be similar to those bleedings caused by haemophilia because the haemostatic system, as in haemophilia, lacks or has abnormal essential clotting "compounds" (such as platelets or von Willebrand factor protein) that causes major bleedings. In subjects who experience extensive tissue damage in association with surgery or vast trauma, the normal haemostatic mechanism may be overwhelmed by the demand of immediate haemostasis and they may develop bleeding in spite of a normal haemostatic mechanism. Achieving satisfactory haemostasis also is a problem when bleedings occur in organs such as the brain, inner ear region and eyes with limited possibility for surgical haemostasis. The same problem may arise in the process of taking biopsies from various organs (liver, lung, tumour tissue, gastrointestinal tract) as well as in laparoscopic surgery. Common for all these situations is the difficulty to provide haemostasis by surgical techniques (sutures, clips, etc.) which also is the case when bleeding is diffuse (haemorrhagic gastritis and profuse uterine bleeding). Acute and profuse bleedings may also occur in subjects on anticoagulant therapy in whom a defective haemostasis has been induced by the therapy given. Such subjects may need surgical interventions in case the anticoagulant effect has to be counteracted rapidly. Radical retropubic prostatectomy is a commonly performed procedure for subjects with localized prostate cancer. The operation is frequently complicated by significant and sometimes massive blood loss. The considerable blood loss during prostatectomy is mainly related to the complicated anatomical situation, with various densely vascularized sites that are not easily accessible for surgical haemostasis, and which may result in diffuse bleeding from a large area. Another situation that may cause problems in the case of unsatisfactory

haemostasis is when subjects with a normal haemostatic mechanism are given anticoagulant therapy to prevent thromboembolic disease. Such therapy may include heparin, other forms of proteoglycans, warfarin or other forms of vitamin K-antagonists as well as aspirin and other platelet aggregation inhibitors.

[0309] In one embodiment of the invention, the bleeding is associated with haemophilia A or B. In another embodiment, the bleeding is associated with haemophilia with aquired inhibitors. In another embodiment, the bleeding is associated with thrombocytopenia. In another embodiment, the bleeding is associated with von Willebrand's disease. In another embodiment, the bleeding is associated with severe tissue damage. In another embodiment, the bleeding is associated with severe trauma. In another embodiment, the bleeding is associated with surgery. In another embodiment, the bleeding is associated with laparoscopic surgery. In another embodiment, the bleeding is associated with haemorrhagic gastritis. In another embodiment, the bleeding is profuse uterine bleeding. In another embodiment, the bleeding is occurring in organs with a limited possibility for mechanical haemostasis. In another embodiment, the bleeding is occurring in the brain, inner ear region or eyes. In another embodiment, the bleeding is associated with the process of taking biopsies. In another embodiment, the bleeding is associated with anticoagulant therapy.

[0310] The term "subject" as used herein is intended to mean any animal, in particular mammals, such as humans, and may, where appropriate, be used interchangeably with the term "patient".

[0311] The terminology for amino acid substitutions used herein is as follows. The first letter represent the amino acid naturally present at a position of SEQ ID NO: 1. The following number represent the position in SEQ ID NO: 1. The second letter represent the different amino acid substituting for the natural amino acid. An example is S43N, where a serine at position 43 of SEQ ID NO: 1 is replaced by an Asparagine. In another example, K62E/I69A, the lysine in position 62 of SEQ ID NO: 1 is replaced by a glutamic acid and the isoleucine in position 69 of SEQ ID NO: 1 is replaced by an alanine in the same Factor VII polypeptide. I69A-FVII thus means human wild type FVII, wherein a isoleucine in position 69 of SEQ ID NO: 1 has been replaced by an alanine.

[0312] In one aspect the present invention relates to a composition comprising wild type human FVIIa and a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa.

[0313] In a further aspect the present invention relates to a method for the treatment of bleeding episodes in a subject or for the enhancement of the normal haemostatic system, the method comprising administering to a subject in need thereof a therapeutically or prophylactically effective amount of:

[0314] a) composition comprising wild type human FVIIa and a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa; or

[0315] b) a first composition comprising wild type human FVIIa and a second composition comprising a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa.

[0316] In a further aspect the present invention relates to a process for preparing a composition comprising wild type human FVIIa and a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa, wherein the process comprises the step of: mixing wild type human FVIIa with a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa in an aqueous medium.

[0317] In a further aspect the present invention relates to a use of a composition comprising wild type human FVIIa and a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa, for the preparation of a medicament for the treatment of bleeding episodes in a subject or for the enhancement of the normal haemostatic system.

[0318] In a further aspect the present invention relates to the use of a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa; in the therapeutic treatment of bleedings, where the exposure of TF is high, such as indications where there is a risk of plaque rupture, plaque disruption after percutaneous transluminal coronary angioplasty (PTCA) procedures, sepsis, or other bleeding complications, where inflammatory conditions where increased quantities of tissue factor is seen. In one embodiment the bleedings, where the exposure of TF is high is a coagulation disorder, such as disseminated intravascular coagulation (DIC).

[0319] The present invention also encompasses methods and compositions that provide combination therapies in which the Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa is administered with another procoagulant compound, antifibrinolytic compound or regulator of anticoagulant compounds. Suitable compounds include, without limitation, FVIII (such as Refacto® (Genetics Institute), Kogenate FS® (Bayer), Monoclate-P® (Aventis Behring)), Factor XIII (see, e.g., WO 01/85198); inhibitors of tissue factor pathway inhibitor (TFPI inhibitors) (see, e.g., WO 01/85199); Factor IX (see, e.g., WO 02/062376); thrombin activatable fibrinolysis inhibitor (TAFI) (see, e.g., PCT/DK02/00734; PAI-1 (see, e.g., PCT/DK02/00735; Factor V (see, e.g., PCT/DK02/00736); protein C inhibitors (see, e.g., PCT/DK02/00737); thrombomodulin (see, e.g., PCT/DK02/00738); protein S inhibitors (see, e.g., PCT/DK02/00739); tissue plasminogen activator inhibitors (see, e.g., PCT/DK02/00740); alpha2-antiplasmin (see, e.g., PCT/DK02/

00741); aprotinin (see, e.g., PCT/DK02/00742); tranexamic acid (see, e.g., PCT/DK02/00751); epsilon-aminocaproic acid (see, e.g., PCT/DK02/00752); prothrombin, thrombin, Factor VII, Factor X, Factor XI and fibrinogen, artificial oxygen carriers (such as POLYHEME®, Northfield lab), colloids (such as Hextend, BioTime, Inc), Desmopressin (such as DDAVP® Tablets (desmopressin acetate) Adventis Pharmaceuticals), activated prothrombin complex concentrates (i.e., Bebulin, Proplex-T, Profiline, Autoplex, FEIBA), Clopidogrel, Ticlopidine, Glycoprotein IIB/IIIA antagonists (Abciximab), LMWH, Warfarin. Streptokinase, Tissue plasminogen activator (tPA)/tPA mutants.

[0320] In one embodiment the Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa is as disclosed in any of international patent applications WO 99/20767, WO 00/66753, WO 01/58935, WO 03/93465 and WO 04/029091, each of which is hereby incorporated by reference in its entirety.

[0321] In the present specification, amino acids are represented using abbreviations, as indicated in table 1, approved by IUPAC-IUB Commission on Biochemical Nomenclature (CBN). Amino acid and the like having isomers represented by name or the following abbreviations are in natural L-form unless otherwise indicated. Further, the left and right ends of an amino acid sequence of a peptide are, respectively, the N- and C-termini unless otherwise specified.

TABLE 1

Abbreviations for amino acids:

Amino acid	Tree-letter code	One-letter code
Glycine	Gly	G
Proline	Pro	P
Alanine	Ala	A
Valine	Val	V
Leucine	Leu	L
Isoleucine	Ile	I
Methionine	Met	M
Cysteine	Cys	C
Phenylalanine	Phe	F
Tyrosine	Tyr	Y
Tryptophan	Trp	W
Histidine	His	H
Lysine	Lys	K
Arginine	Arg	R
Glutamine	Gln	Q
Asparagine	Asn	N
Glutamic Acid	Glu	E
Aspartic Acid	Asp	D
Serine	Ser	S
Threonine	Thr	T

[0322] The invention also relates to a method of preparing human Factor VII polypeptides as mentioned above. The human Factor VII polypeptides are preferably produced by recombinant DNA techniques. To this end, DNA sequences encoding human Factor VII may be isolated by preparing a genomic or cDNA library and screening for DNA sequences coding for all or part of the protein by hybridization using synthetic oligonucleotide probes in accordance with standard techniques (cf. Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989). For the present purpose, the DNA sequence encoding the protein is preferably of human origin, i.e. derived from a human genomic DNA or cDNA library.

[0323] The DNA sequences encoding the human Factor VII polypeptides may also be prepared synthetically by established standard methods, e.g. the phosphoamidite method described by Beaucage and Caruthers, *Tetrahedron Letters* 22 (1981), 1859-1869, or the method described by Matthes et al., *EMBO Journal* 3 (1984), 801-805. According to the phosphoamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in suitable vectors.

[0324] The DNA sequences may also be prepared by polymerase chain reaction using specific primers, for instance as described in U.S. Pat. No. 4,683,202, Saiki et al., *Science* 239 (1988), 487-491, or Sambrook et al., supra.

[0325] The DNA sequences encoding the human Factor VII polypeptides are usually inserted into a recombinant vector which may be any vector, which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector, which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

[0326] The vector is preferably an expression vector in which the DNA sequence encoding the human Factor VII polypeptides is operably linked to additional segments required for transcription of the DNA. In general, the expression vector is derived from plasmid or viral DNA, or may contain elements of both. The term, "operably linked" indicates that the segments are arranged so that they function in concert for their intended purposes, e.g. transcription initiates in a promoter and proceeds through the DNA sequence coding for the polypeptide.

[0327] The promoter may be any DNA sequence, which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell.

[0328] Examples of suitable promoters for directing the transcription of the DNA encoding the human Factor VII polypeptide in mammalian cells are the SV40 promoter (Subramani et al., *Mol. Cell Biol.* 1 (1981), 854-864), the MT-1 (metallothionein gene) promoter (Palmiter et al., *Science* 222 (1983), 809-814), the CMV promoter (Boshart et al., *Cell* 41:521-530, 1985) or the adenovirus 2 major late promoter (Kaufman and Sharp, *Mol. Cell. Biol,* 2:1304-1319, 1982).

[0329] An example of a suitable promoter for use in insect cells is the polyhedrin promoter (U.S. Pat. No. 4,745,051; Vasuvedan et al., *FEBS Lett.* 311, (1992) 7-11), the P10 promoter (J. M. Vlak et al., *J. Gen. Virology* 69, 1988, pp. 765-776), the *Autographa californica* polyhedrosis virus basic protein promoter (EP 397 485), the baculovirus immediate early gene 1 promoter (U.S. Pat. No. 5,155,037; U.S. Pat. No. 5,162,222), or the baculovirus 39K delayed-early gene promoter (U.S. Pat. No. 5,155,037; U.S. Pat. No. 5,162,222).

[0330] Examples of suitable promoters for use in yeast host cells include promoters from yeast glycolytic genes (Hitzeman et al., *J. Biol. Chem.* 255 (1980), 12073-12080; Alber and Kawasaki, *J. Mol. Appl Gen.* 1 (1982), 419-434) or alcohol dehydrogenase genes (Young et al., in *Genetic Engineering of Microorganisms for Chemicals* (Hollaender et al, eds.), Plenum Press, New York, 1982), or the TPI1 (U.S. Pat. No. 4,599,311) or ADH2-4c (Russell et al., *Nature* 304 (1983), 652-654) promoters.

[0331] Examples of suitable promoters for use in filamentous fungus host cells are, for instance, the ADH3 promoter (McKnight et al., *The EMBO J.* 4 (1985), 2093-2099) or the tpiA promoter. Examples of other useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral α-amylase, *A. niger* acid stable α-amylase, *A. niger* or *A. awamori* glucoamylase (gluA), *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase. Preferred are the TAKA-amylase and gluA promoters. Suitable promoters are mentioned in, e.g. EP 238 023 and EP 383 779.

[0332] The DNA sequences encoding the human Factor VII polypeptides may also, if necessary, be operably connected to a suitable terminator, such as the human growth hormone terminator (Palmiter et al., *Science* 222, 1983, pp. 809-814) or the TPI1 (Alber and Kawasaki, *J. Mol. Appl. Gen.* 1, 1982, pp. 419-434) or ADH3 (McKnight et al., *The EMBO J.* 4, 1985, pp. 2093-2099) terminators. The vector may also contain a set of RNA splice sites located downstream from the promoter and upstream from the insertion site for the Factor VII sequence itself. Preferred RNA splice sites may be obtained from adenovirus and/or immunoglobulin genes. Also contained in the expression vectors is a polyadenylation signal located downstream of the insertion site. Particularly preferred polyadenylation signals include the early or late polyadenylation signal from SV40 (Kaufman and Sharp, ibid.), the polyadenylation signal from the adenovirus 5 E1b region, the human growth hormone gene terminator (DeNoto et al. *Nuc. Acids Res.* 9:3719-3730, 1981) or the polyadenylation signal from the human Factor VII gene or the bovine Factor VII gene. The expression vectors may also include a noncoding viral leader sequence, such as the adenovirus 2 tripartite leader, located between the promoter and the RNA splice sites; and enhancer sequences, such as the SV40 enhancer.

[0333] The recombinant vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. An example of such a sequence (when the host cell is a mammalian cell) is the SV40 origin of replication.

[0334] When the host cell is a yeast cell, suitable sequences enabling the vector to replicate are the yeast plasmid 2μ replication genes REP 1-3 and origin of replication.

[0335] The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or the *Schizosaccharomyces pombe* TPI gene (described by P. R. Russell, *Gene* 40, 1985, pp. 125-130), or one which confers resistance to a drug, e.g. ampicillin, kanamycin, tetracyclin, chloramphenicol, neomycin, hygromycin or methotrexate. For filamentous fungi, selectable markers include amdS, pyrG, argB, niaD or sC.

[0336] To direct the human Factor VII polypeptides of the present invention into the secretory pathway of the host cells, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence) may be provided in the recombinant vector. The secretory signal sequence is joined to the DNA sequences encoding the human Factor VII polypeptides in the correct reading frame. Secretory signal sequences are commonly positioned 5' to the DNA sequence encoding the peptide. The secretory signal sequence may be that, normally associated with the protein or may be from a gene encoding another secreted protein.

[0337] For secretion from yeast cells, the secretory signal sequence may encode any signal peptide, which ensures efficient direction of the expressed human Factor VII polypeptides into the secretory pathway of the cell. The signal peptide may be naturally occurring signal peptide, or a functional part thereof, or it may be a synthetic peptide. Suitable signal peptides have been found to be the α-factor signal peptide (cf. U.S. Pat. No. 4,870,008), the signal peptide of mouse salivary amylase (cf. O. Hagenbuchle et al., *Nature* 289, 1981, pp. 643-646), a modified carboxypeptidase signal peptide (cf. L. A. Valls et al., *Cell* 48, 1987, pp. 887-897), the yeast BAR1 signal peptide (cf. WO 87/02670), or the yeast aspartic protease 3 (YAP3) signal peptide (cf. M. Egel-Mitani et al., *Yeast* 6, 1990, pp. 127-137).

[0338] For efficient secretion in yeast, a sequence encoding a leader peptide may also be inserted downstream of the signal sequence and upstream of the DNA sequence encoding the human Factor VII polypeptides. The function of the leader peptide is to allow the expressed peptide to be directed from the endoplasmic reticulum to the Golgi apparatus and further to a secretory vesicle for secretion into the culture medium (i.e. exportation of the human Factor VII polypeptides across the cell wall or at least through the cellular membrane into the periplasmic space of the yeast cell). The leader peptide may be the yeast alpha-factor leader (the use of which is described in e.g. U.S. Pat. No. 4,546,082, U.S. Pat. No. 4,870,008, EP 16 201, EP 123 294, EP 123 544 and EP 163 529). Alternatively, the leader peptide may be a synthetic leader peptide, which is to say a leader peptide not found in nature. Synthetic leader peptides may, for instance, be constructed as described in WO 89/02463 or WO 92/11378.

[0339] For use in filamentous fungi, the signal peptide may conveniently be derived from a gene encoding an *Aspergillus* sp. amylase or glucoamylase, a gene encoding a *Rhizomucor miehei* lipase or protease or a *Humicola lanuginosa* lipase. The signal peptide is preferably derived from a gene encoding *A. oryzae* TAKA amylase, *A. niger* neutral α-amylase, *A. niger* acid-stable amylase, or *A. niger* glucoamylase. Suitable signal peptides are disclosed in, e.g. EP 238 023 and EP 215 594.

[0340] For use in insect cells, the signal peptide may conveniently be derived from an insect gene (cf. WO 90/05783), such as the lepidopteran *Manduca sexta* adipokinetic hormone precursor signal peptide (cf. U.S. Pat. No. 5,023,328).

[0341] The procedures used to ligate the DNA sequences coding for the human Factor VII polypeptides, the promoter and optionally the terminator and/or secretory signal

sequence, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, N.Y., 1989).

[0342] Methods of transfecting mammalian cells and expressing DNA sequences introduced in the cells are described in e.g. Kaufman and Sharp, *J. Mol. Biol.* 159 (1982), 601-621; Southern and Berg, *J. Mol. Appl. Genet.* 1 (1982), 327-341; Loyter et al., *Proc. Natl. Acad. Sci. USA* 79 (1982), 422-426; Wigler et al., *Cell* 14 (1978), 725; Corsaro and Pearson, *Somatic Cell Genetics* 7 (1981), 603, Graham and van der Eb, *Virology* 52 (1973), 456; and Neumann et al., *EMBO J.* 1 (1982), 841-845.

[0343] Selectable markers may be introduced into the cell on a separate plasmid at the same time as the gene of interest, or they may be introduced on the same plasmid. If on the same plasmid, the selectable marker and the gene of interest may be under the control of different promoters or the same promoter, the latter arrangement producing a dicistronic message. Constructs of this type are known in the art (for example, Levinson and Simonsen, U.S. Pat. No. 4,713,339). It may also be advantageous to add additional DNA, known as "carrier DNA," to the mixture that is introduced into the cells.

[0344] After the cells have taken up the DNA, they are grown in an appropriate growth medium, typically 1-2 days, to begin expressing the gene of interest. As used herein the term "appropriate growth medium" means a medium containing nutrients and other components required for the growth of cells and the expression of the human Factor VII polypeptides of interest. Media generally include a carbon source, a nitrogen source, essential amino acids, essential sugars, vitamins, salts, phospholipids, protein and growth factors. For production of gamma-carboxylated proteins, the medium will contain vitamin K, preferably at a concentration of about 0.1 μg/ml to about 5 μg/ml. Drug selection is then applied to select for the growth of cells that are expressing the selectable marker in a stable fashion. For cells that have been transfected with an amplifiable selectable marker the drug concentration may be increased to select for an increased copy number of the cloned sequences, thereby increasing expression levels. Clones of stably transfected cells are then screened for expression of the human Factor VII polypeptide of interest.

[0345] The host cell into which the DNA sequences encoding the human Factor VII polypeptides is introduced may be any cell, which is capable of producing the post-translational modified human Factor VII polypeptides and includes yeast, fungi and higher eucaryotic cells.

[0346] Examples of mammalian cell lines for use in the present invention are the COS-1 (ATCC CRL 1650), baby hamster kidney (BHK) and 293 (ATCC CRL 1573; Graham et al., *J. Gen. Virol.* 36:59-72, 1977) cell lines. A preferred BHK cell line is the tk^- ts13 BHK cell line (Waechter and Baserga, *Proc. Natl. Acad. Sci. USA* 79:1106-1110, 1982, incorporated herein by reference), hereinafter referred to as BHK 570 cells. The BHK 570 cell line has been deposited with the American Type Culture Collection, 12301 Parklawn Dr., Rockville, Md. 20852, under ATCC accession number CRL 10314. A tk^- ts13 BHK cell line is also available from the ATCC under accession number CRL 1632. In addition, a number of other cell lines may be used within the present invention, including Rat Hep I (Rat hepatoma; ATCC CRL 1600), Rat Hep II (Rat hepatoma; ATCC CRL 1548), TCMK (ATCC CCL 139), Human lung (ATCC HB 8065), NCTC 1469 (ATCC CCL 9.1), CHO (ATCC CCL 61) and DUKX cells (Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA* 77:4216-4220, 1980).

[0347] Examples of suitable yeasts cells include cells of *Saccharomyces* spp. or *Schizosaccharomyces* spp., in particular strains of *Saccharomyces cerevisiae* or *Saccharomyces kluyveri*. Methods for transforming yeast cells with heterologous DNA and producing heterologous polypeptides there from are described, e.g. in U.S. Pat. No. 4,599,311, U.S. Pat. No. 4,931,373, U.S. Pat. Nos. 4,870,008, 5,037,743, and U.S. Pat. No. 4,845,075, all of which are hereby incorporated by reference. Transformed cells are selected by a phenotype determined by a selectable marker, commonly drug resistance or the ability to grow in the absence of a particular nutrient, e.g. leucine. A preferred vector for use in yeast is the POT1 vector disclosed in U.S. Pat. No. 4,931,373. The DNA sequences encoding the human Factor VII polypeptides may be preceded by a signal sequence and optionally a leader sequence, e.g. as described above. Further examples of suitable yeast cells are strains of *Kluyveromyces*, such as *K. lactis, Hansenula*, e.g. *H. polymorpha*, or *Pichia*, e.g. *P. pastoris* (cf. Gleeson et al., *J. Gen. Microbiol.* 132, 1986, pp. 3459-3465; U.S. Pat. No. 4,882,279).

[0348] Examples of other fungal cells are cells of filamentous fungi, e.g. *Aspergillus* spp., *Neurospora* spp., *Fusarium* spp. or *Trichoderma* spp., in particular strains of *A. oryzae, A. nidulans* or *A. niger*. The use of *Aspergillus* spp. for the expression of proteins is described in, e.g., EP 272 277, EP 238 023, EP 184 438 The transformation of *F. oxysporum* may, for instance, be carried out as described by Malardier et al., 1989, *Gene* 78: 147-156. The transformation of *Trichoderma* spp. may be performed for instance as described in EP 244 234.

[0349] When a filamentous fungus is used as the host cell, it may be transformed with the DNA construct of the invention, conveniently by integrating the DNA construct in the host chromosome to obtain a recombinant host cell. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, e.g. by homologous or heterologous recombination.

[0350] Transformation of insect cells and production of heterologous polypeptides therein may be performed as described in U.S. Pat. No. 4,745,051; U.S. Pat. No. 4,879,236; U.S. Pat. Nos. 5,155,037; 5,162,222; EP 397,485) all of which are incorporated herein by reference. The insect cell line used as the host may suitably be a *Lepidoptera* cell line, such as *Spodoptera frugiperda* cells or *Trichoplusia ni* cells (cf. U.S. Pat. No. 5,077,214). Culture conditions may suitably be as described in, for instance, WO 89/01029 or WO 89/01028, or any of the aforementioned references.

[0351] The transformed or transfected host cell described above is then cultured in a suitable nutrient medium under conditions permitting expression of the human Factor VII polypeptide after which all or part of the resulting peptide may be recovered from the culture. The medium used to

culture the cells may be any conventional medium suitable for growing the host cells, such as minimal or complex media containing appropriate supplements. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. in catalogues of the American Type Culture Collection). The human Factor VII polypeptide produced by the cells may then be recovered from the culture medium by conventional procedures including separating the host cells from the medium by centrifugation or filtration, precipitating the proteinaqueous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulphate, purification by a variety of chromatographic procedures, e.g. ion exchange chromatography, gel-filtration chromatography, affinity chromatography, or the like, dependent on the type of polypeptide in question.

[0352] For the preparation of recombinant human Factor VII polypeptides, a cloned wild-type Factor VII DNA sequence is used. This sequence may be modified to encode a desired Factor VII variant. The complete nucleotide and amino acid sequences for human Factor VII are known. See U.S. Pat. No. 4,784,950, which is incorporated herein by reference, where the cloning and expression of recombinant human Factor VII is described. The bovine Factor VII sequence is described in Takeya et al., *J. Biol. Chem,* 263:14868-14872 (1988), which is incorporated by reference herein.

[0353] The amino acid sequence alterations may be accomplished by a variety of techniques. Modification of the DNA sequence may be by site-specific mutagenesis. Techniques for site-specific mutagenesis are well known in the art and are described by, for example, Zoller and Smith (*DNA* 3:479-488, 1984). Thus, using the nucleotide and amino acid sequences of Factor VII, one may introduce the alterations of choice.

[0354] DNA sequences for use within the present invention will typically encode a pre-pro peptide at the amino-terminus of the Factor VII protein to obtain proper post-translational processing (e.g. gamma-carboxylation of glutamic acid residues) and secretion from the host cell. The pre-pro peptide may be that of Factor VII or another vitamin K-dependent plasma protein, such as factor IX, factor X, prothrombin, protein C or protein S. As will be appreciated by those skilled in the art, additional modifications can be made in the amino acid sequence of Factor VII where those modifications do not significantly impair the ability of the protein to act as a coagulation factor. For example, Factor VII in the catalytic triad can also be modified in the activation cleavage site to inhibit the conversion of zymogen Factor VII into its activated two-chain form, as generally described in U.S. Pat. No. 5,288,629, incorporated herein by reference.

[0355] Within the present invention, transgenic animal technology may be employed to produce the human Factor VII polypeptide. It is preferred to produce the proteins within the mammary glands of a host female mammal. Expression in the mammary gland and subsequent secretion of the protein of interest into the milk overcomes many difficulties encountered in isolating proteins from other sources. Milk is readily collected, available in large quantities, and well characterized biochemically. Furthermore, the major milk proteins are present in milk at high concentrations (typically from about 1 to 15 g/l). From a commercial point of view, it is clearly preferable to use as the host a species that has a large milk yield. While smaller animals such as mice and rats can be used (and are preferred at the proof of principle stage), within the present invention it is preferred to use livestock mammals including, but not limited to, pigs, goats, sheep and cattle. Sheep are particularly preferred due to such factors as the previous history of transgenesis in this species, milk yield, cost and the ready availability of equipment for collecting sheep milk. See WIPO Publication WO 88/00239 for a comparison of factors influencing the choice of host species. It is generally desirable to select a breed of host animal that has been bred for dairy use, such as East Friesland sheep, or to introduce dairy stock by breeding of the transgenic line at a later date. In any event, animals of known, good health status should be used.

[0356] To obtain expression in the mammary gland, a transcription promoter from a milk protein gene is used. Milk protein genes include those genes encoding caseins (see U.S. Pat. No. 5,304,489, incorporated herein by reference), beta-lactoglobulin, alpha-lactalbumin, and whey acidic protein. The beta-lactoglobulin (BLG) promoter is preferred. In the case of the ovine beta-lactoglobulin gene, a region of at least the proximal 406 bp of 5' flanking sequence of the gene will generally be used, although larger portions of the 5' flanking sequence, up to about 5 kbp, are preferred, such as about 4.25 kbp DNA segment encompassing the 5' flanking promoter and non-coding portion of the beta-lactoglobulin gene. See Whitelaw et al., *Biochem J.* 286: 31-39 (1992). Similar fragments of promoter DNA from other species are also suitable.

[0357] Other regions of the beta-lactoglobulin gene may also be incorporated in constructs, as may genomic regions of the gene to be expressed. It is generally accepted in the art that constructs lacking introns, for example, express poorly in comparison with those that contain such DNA sequences (see Brinster et al., *Proc. Natl. Acad. Sci. USA* 85: 836-840 (1988); Palmiter et al., *Proc. Natl. Acad. Sci. USA* 88: 478-482 (1991); Whitelaw et al., *Transgenic Res.* 1: 3-13 (1991); WO 89/01343; and WO 91/02318, each of which is incorporated herein by reference). In this regard, it is generally preferred, where possible, to use genomic sequences containing all or some of the native introns of a gene encoding the protein or polypeptide of interest, thus the further inclusion of at least some introns from, e.g, the beta-lactoglobulin gene, is preferred. One such region is a DNA segment which provides for intron splicing and RNA polyadenylation from the 3' non-coding region of the ovine beta-lactoglobulin gene. When substituted for the natural 3' non-coding sequences of a gene, this ovine beta-lactoglobulin segment can both enhance and stabilize expression levels of the protein or polypeptide of interest. Within other embodiments, the region surrounding the initiation ATG of the sequence encoding the human Factor VII polypeptide is replaced with corresponding sequences from a milk specific protein gene. Such replacement provides a putative tissue-specific initiation environment to enhance expression. It is convenient to replace the entire pre-pro sequence of the human Factor VII polypeptide and 5' non-coding sequences with those of, for example, the BLG gene, although smaller regions may be replaced.

[0358] For expression of a human Factor VII polypeptide in transgenic animals, a DNA segment encoding the human Factor VII polypeptide is operably linked to additional DNA

segments required for its expression to produce expression units. Such additional segments include the above-mentioned promoter, as well as sequences which provide for termination of transcription and polyadenylation of mRNA. The expression units will further include a DNA segment encoding a secretory signal sequence operably linked to the segment encoding the human Factor VII polypeptide. The secretory signal sequence may be a native secretory signal sequence of the human Factor VII polypeptide or may be that of another protein, such as a milk protein. See, for example, von Heinje, *Nuc. Acids Res.* 14: 4683-4690 (1986); and Meade et al., U.S. Pat. No. 4,873,316, which are incorporated herein by reference.

[0359] Construction of expression units for use in transgenic animals is conveniently carried out by inserting a sequence encoding the human Factor VII polypeptide into a plasmid or phage vector containing the additional DNA segments, although the expression unit may be constructed by essentially any sequence of ligations. It is particularly convenient to provide a vector containing a DNA segment encoding a milk protein and to replace the coding sequence for the milk protein with that of the human Factor VII polypeptide, thereby creating a gene fusion that includes the expression control sequences of the milk protein gene. In any event, cloning of the expression units in plasmids or other vectors facilitates the amplification of the human Factor VII polypeptide. Amplification is conveniently carried out in bacterial (e.g. *E. coli*) host cells, thus the vectors will typically include an origin of replication and a selectable marker functional in bacterial host cells.

[0360] The expression unit is then introduced into fertilized eggs (including early-stage embryos) of the chosen host species. Introduction of heterologous DNA can be accomplished by one of several routes, including microinjection (e.g. U.S. Pat. No. 4,873,191), retroviral infection (Jaenisch, *Science* 240: 1468-1474 (1988)) or site-directed integration using embryonic stem (ES) cells (reviewed by Bradley et al., *Bio/Technology* 10: 534-539 (1992)). The eggs are then implanted into the oviducts or uteri of pseudopregnant females and allowed to develop. Offspring carrying the introduced DNA in their germ line can pass the DNA on to their progeny in the normal, Mendelian fashion, allowing the development of transgenic herds.

[0361] General procedures for producing transgenic animals are known in the art. See, for example, Hogan et al., *Manipulating the Mouse Embryo: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1986; Simons et al., *Bio/Technology* 6: 179-183 (1988); Wall et al., Biol. Reprod. 32: 645-651 (1985); Buhler et al., *Bio/Technology* 8: 140-143 (1990); Ebert et al., *Bio/Technology* 9: 835-838 (1991); Krimpenfort et al., *Bio/Technology* 9: 844-847 (1991); Wall et al., *J. Cell. Biochem.* 49: 113-120 (1992); U.S. Pat. Nos. 4,873,191 and 4,873,316; WIPO publications WO 88/00239, WO 90/05188, WO 92/11757; and GB 87/00458, which are incorporated herein by reference. Techniques for introducing foreign DNA sequences into mammals and their germ cells were originally developed in the mouse. See, e.g., Gordon et al., *Proc. Natl. Acad. Sci. USA* 77: 7380-7384 (1980); Gordon and Ruddle, *Science* 214: 1244-1246 (1981); Palmiter and Brinster, *Cell* 41: 343-345 (1985); and Brinster et al., *Proc. Natl. Acad. Sci. USA* 82: 4438-4442 (1985). These techniques were subsequently adapted for use with larger animals, including livestock species (see e.g., WIPO publications WO 88/00239, WO 90/05188, and WO 92/11757; and Simons et al., *Bio/Technology* 6: 179-183 (1988). To summarize, in the most efficient route used to date in the generation of transgenic mice or livestock, several hundred linear molecules of the DNA of interest are injected into one of the pro-nuclei of a fertilized egg according to established techniques. Injection of DNA into the cytoplasm of a zygote can also be employed. Production in transgenic plants may also be employed. Expression may be generalized or directed to a particular organ, such as a tuber. See, Hiatt, *Nature* 344:469-479 (1990); Edelbaum et al., *J. Interferon Res.* 12:449-453 (1992); Sijmons et al., *Bio/Technology* 8:217-221 (1990); and European Patent Office Publication EP 255,378.

[0362] Factor VII produced according to the present invention may be purified by affinity chromatography on an anti-Factor VII antibody column. It is preferred that the immunoadsorption column comprise a high-specificity monoclonal antibody. The use of calcium-dependent monoclonal antibodies, as described by Wakabayashi et al., *J. Biol. Chem,* 261:11097-11108, (1986) and Thim et al., *Biochem.* 27: 7785-7793, (1988), incorporated by reference herein, is particularly preferred. Additional purification may be achieved by conventional chemical purification means, such as high performance liquid chromatography. Other methods of purification, including barium citrate precipitation, are known in the art, and may be applied to the purification of the Factor VII described herein (see, generally, Scopes, R., *Protein Purification*, Springer-Verlag, N.Y., 1982). Substantially pure Factor VII of at least about 90 to 95% homogeneity is preferred, and 98 to 99% or more homogeneity most preferred, for pharmaceutical uses. Once purified, partially or to homogeneity as desired, the Factor VII may then be used therapeutically.

[0363] Conversion of single-chain Factor VII to active two-chain Factor VIIa may be achieved using factor XIIa as described by Hedner and Kisiel (1983, *J. Clin. Invest.* 71: 1836-1841), or with other proteases having trypsin-like specificity (Kisiel and Fujikawa, *Behring Inst. Mitt.* 73: 29-42, 1983). Alternatively Factor VII may be autoactivated by passing it through an ion-exchange chromatography column, such as mono Q®. (Pharmacia Fire Chemicals) or the like (Bjoern et al., 1986, *Research Disclosures* 269:564-565). The Factor VII molecules of the present invention and pharmaceutical compositions thereof are particularly useful for administration to humans to treat a variety of conditions involving intravascular coagulation.

[0364] The invention also provides suitable assays for selecting preferred Factor VIIa polypeptides and Factor VIIa derivatives according to the invention. These assays can be performed as a simple preliminary in vitro test.

[0365] Thus, Example 3 herein discloses a simple test (entitled "In Vitro Hydrolysis Assay") for the activity of Factor VIIa polypeptides of the invention. Based thereon, Factor VIIa polypeptides which are of particular interest are such polypeptides where the ratio between the activity of the variant and the activity of native human Factor VII shown in **FIG. 1** is about 1.0 or higher, when tested in the "In Vitro Hydrolysis Assay" defined herein.

[0366] The activity of the polypeptides can also be measured using a physiological substrate such as factor X ("In Vitro Proteolysis Assay") (see Example 4), suitably at a

concentration of 100-1000 nM, where the factor Xa generated is measured after the addition of a suitable chromogenic substrate (e.g. S-2765). In addition, the activity assay may be run at physiological temperature.

[0367] The ability of the procoagulant Factor VIIa polypeptides to generate thrombin can also be measured in an assay comprising all relevant coagulation factors and inhibitors at physiological concentrations (minus factor VIII when mimicking haemophilia A conditions) and activated platelets (as described on p. 543 in Monroe et al. (1997) *Brit. J. Haematol.* 99, 542-547 which is hereby incorporated as reference).

[0368] The procoagulant Factor VII polypeptides according to the present invention may be used to control bleeding disorders which have several causes such as clotting factor deficiencies (e.g. haemophilia A and B or deficiency of coagulation factors XI or VII) or clotting factor inhibitors, or they may be used to control excessive bleeding occurring in subjects with a normally functioning blood clotting cascade (no clotting factor deficiencies or inhibitors against any of the coagulation factors). The bleedings may be caused by a defective platelet function, thrombocytopenia or von Willebrand's disease. They may also be seen in subjects in whom an increased fibrinolytic activity has been induced by various stimuli.

[0369] In subjects who experience extensive tissue damage in association with surgery or vast trauma, the haemostatic mechanism may be overwhelmed by the demand of immediate haemostasis and they may develop bleedings in spite of a normal haemostatic mechanism. Achieving satisfactory haemostasis is also a problem when bleedings occur in organs such as the brain, inner ear region and eyes and may also be a problem in cases of diffuse bleedings (haemorrhagic gastritis and profuse uterine bleeding) when it is difficult to identify the source. The same problem may arise in the process of taking biopsies from various organs (liver, lung, tumour tissue, gastrointestinal tract) as well as in laparoscopic surgery. These situations share the difficulty of providing haemostasis by surgical techniques (sutures, clips, etc.). Acute and profuse bleedings may also occur in subjects on anticoagulant therapy in whom a defective haemostasis has been induced by the therapy given. Such subjects may need surgical interventions in case the anticoagulant effect has to be counteracted rapidly. Another situation that may cause problems in the case of unsatisfactory haemostasis is when subjects with a normal haemostatic mechanism are given anticoagulant therapy to prevent thromboembolic disease. Such therapy may include heparin, other forms of proteoglycans, warfarin or other forms of vitamin K-antagonists as well as aspirin and other platelet aggregation inhibitors.

[0370] A systemic activation of the coagulation cascade may lead to disseminated intravascular coagulation (DIC). However, such complications have not been seen in subjects treated with high doses of recombinant Factor VIIa because of a localised haemostatic process of the kind induced by the complex formation between Factor VIIa and TF exposed at the site of vessel wall injury. The procoagulant Factor VII polypeptides according to the invention may thus also be used in their activated form to control such excessive bleedings associated with a normal haemostatic mechanism.

[0371] For treatment in connection with deliberate interventions, the procoagulant Factor VII polypeptides of the invention will typically be administered within about 24 hours prior to performing the intervention, and for as much as 7 days or more thereafter. Administration as a coagulant can be by a variety of routes as described herein.

[0372] The dose of the Factor VII polypeptides ranges from about 0.05 mg to 500 mg/day, preferably from about 1 mg to 200 mg/day, and more preferably from about 10 mg to about 175 mg/day for a 70 kg subject as loading and maintenance doses, depending on the severity of the condition.

[0373] The pharmaceutical compositions are primarily intended for parenteral administration for prophylactic and/or therapeutic treatment. Preferably, the pharmaceutical compositions are administered parenterally, i.e., intravenously, subcutaneously, or intramuscularly, or it may be administered by continuous or pulsatile infusion. The compositions for parenteral administration comprise the Factor VII polypeptide of the invention in combination with, preferably dissolved in, a pharmaceutically acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, such as water, buffered water, 0.4% saline, 0.3% glycine and the like. The Factor VII polypeptides of the invention can also be formulated into liposome preparations for delivery or targeting to the sites of injury. Liposome preparations are generally described in, e.g., U.S. Pat. No. 4,837,028, U.S. Pat. No. 4,501,728, and U.S. Pat. No. 4,975,282. The compositions may be sterilised by conventional, well-known sterilisation techniques. The resulting aqueous solutions may be packaged for use or filtered under aseptic conditions and lyophilised, the lyophilised preparation being combined with a sterile aqueous solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, etc.

[0374] The concentration of Factor VII polypeptide in these formulations can vary widely, i.e., from less than about 0.5% by weight, usually at or at least about 1% by weight to as much as 15 or 20% by weight and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

[0375] Thus, a typical pharmaceutical composition for intravenous infusion could be made up to contain 250 ml of sterile Ringer's solution and 10 mg of the Factor VII polypeptide. Actual methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art and are described in more detail in, for example, *Remington's Pharmaceutical Sciences,* 18th ed., Mack Publishing Company, Easton, Pa. (1990).

[0376] The compositions containing the Factor VII polypeptides of the present invention can be administered for prophylactic and/or therapeutic treatments. In therapeutic applications, compositions are administered to a subject already suffering from a disease, as described above, in an amount sufficient to cure, alleviate or partially arrest the disease and its complications. An amount adequate to accomplish this is defined as "therapeutically effective amount". As will be understood by the person skilled in the art amounts effective for this purpose will depend on the severity of the disease or injury as well as the weight and

general state of the subject. In general, however, the effective amount will range from about 0.05 mg up to about 500 mg of the Factor VII polypeptide per day for a 70 kg subject, with dosages of from about 1.0 mg to about 200 mg of the Factor VII polypeptide per day being more commonly used.

[0377] It must be kept in mind that the materials of the present invention may generally be employed in serious disease or injury states, that is, life threatening or potentially life threatening situations. In such cases, in view of the minimisation of extraneous substances and general lack of immunogenicity of human Factor VII polypeptides in humans, it is possible and may be felt desirable by the treating physician to administer a substantial excess of these variant Factor VII compositions.

[0378] In prophylactic applications, compositions containing the Factor VII polypeptide of the invention are administered to a subject susceptible to or otherwise at risk of a disease state or injury to enhance the subject's own coagulative capability. Such an amount is defined to be a "prophylactically effective dose." In prophylactic applications, the precise amounts once again depend on the subject's state of health and weight, but the dose generally ranges from about 0.05 mg to about 500 mg per day for a 70-kilogram subject, more commonly from about 1.0 mg to about 200 mg per day for a 70-kilogram subject.

[0379] Single or multiple administrations of the compositions can be carried out with dose levels and patterns being selected by the treating physician. For ambulatory subjects requiring daily maintenance levels, the Factor VII polypeptides may be administered by continuous infusion using e.g. a portable pump system.

[0380] Local delivery of the Factor VII polypeptide of the present invention, such as, for example, topical application may be carried out, for example, by means of a spray, perfusion, double balloon catheters, stent, incorporated into vascular grafts or stents, hydrogels used to coat balloon catheters, or other well established methods. In any event, the pharmaceutical compositions should provide a quantity of Factor VII polypeptide sufficient to effectively treat the subject.

[0381] Inactivated Factor VII polypeptides of the present invention are able to bind to cell-surface tissue factor. For example, DEGR-Factor VIIa binds cell-surface tissue factor with an equivalent or higher affinity than wild-type Factor VIIa. DEGR-Factor VIIa, however, has no enzymatic activity, yet it binds to tissue factor and acts as a competitive antagonist for wild-type Factor VIIa, thereby inhibiting the subsequent steps in the extrinsic pathway of coagulation leading to the generation of thrombin.

[0382] Inactivated Factor VII polypeptides are particularly useful for administration to humans to treat a variety of conditions involving intravascular coagulation. For example, although deep vein thrombosis and pulmonary embolism can be treated with conventional anticoagulants, the inactivated Factor VII polypeptides described herein may be used to prevent the occurrence of thromboembolic complications in identified high risk patients, such as those undergoing surgery or those with congestive heart failure. In addition, inactivated Factor VII polypeptides may act as an antagonist for tissue factor-mediated induction of coagulation, thus blocking the production of thrombin and the subsequent deposition of fibrin. As such, inactivated Factor VII polypeptides may be useful for inhibiting tissue factor activity resulting in, for example, the inhibition of blood coagulation, thrombosis or platelet deposition.

[0383] The inactivated Factor VII polypeptides may be particularly useful in the treatment of intimal hyperplasia, restenosis due to acute vascular injury, deep venous thrombosis, arterial thrombosis, post surgical thrombosis, coronary artery bypass graft (CABG), percutaneous transdermal coronary angioplastry (PTCA), stroke, cancer, tumour metastasis, angiogenesis, ischemia/reperfusion, rheumatoid arthritis, thrombolysis, arteriosclerosis and restenosis following angioplastry, acute and chronic indications such as inflammation, septic chock, septicemia, hypotension, adult respiratory distress syndrome (ARDS), disseminated intravascular coagulopathy (DIC), pulmonary embolism, platelet deposition, myocardial infarction, or the prophylactic treatment of mammals with atherosclerotic vessels at risk for thrombosis. Acute vascular injuries are those which occur rapidly (i.e. over days to months), in contrast to chronic vascular injuries (e.g. atherosclerosis) which develop over a lifetime. Acute vascular injuries often result from surgical procedures such as vascular reconstruction, wherein the techniques of angioplasty, endarterectomy, atherectomy, vascular graft emplacement or the like are employed. Hyperplasia may also occur as a delayed response in response to, e.g., graft emplacement or organ transplantation. Since inactivated Factor VII polypeptides is more selective than heparin, generally binding only tissue factor which has been exposed at sites of injury, and because inactivated Factor VII polypeptides does not destroy other coagulation proteins, it will be more effective and less likely to cause bleeding complications than heparin when used prophylactically for the prevention of deep vein thrombosis.

[0384] Inactivated Factor VII polypeptides which maintain tissue factor binding inhibit platelet accumulation at the site of vascular injury by blocking the production of thrombin and the subsequent deposition of fibrin.

[0385] Due to the ability of DEGR-Factor VII to block thrombin generation and limit platelet deposition at sites of acute vascular injury, inactivated Factor VII polypeptides which maintain tissue factor binding activity but lack Factor VIIa enzymatic activity can be used to inhibit vascular restenosis.

[0386] Compositions comprising inactivated Factor VII polypeptides are particularly useful in methods for treating patients when formulated into pharmaceutical compositions, where they may be given to individuals suffering from a variety of disease states to treat coagulation-related conditions. Such inactivated Factor VII polypeptides, capable of binding tissue factor but having a substantially reduced ability to catalyze activation of other factors in the clotting cascade, may possess a longer plasma half-life and thus a correspondingly longer period of anticoagulative activity when compared to other anticoagulants. Among the medical indications for the subject compositions are those commonly treated with anticoagulants, such as, for example, deep vein thrombosis, pulmonary embolism, stroke, disseminated intravascular coagulation (DIC), fibrin deposition in lungs and kidneys associated with gram-negative endotoxemia, and myocardial infarction. The compositions can be used to inhibit vascular restenosis as occurs following mechanical

vascular injury, such as injury caused by balloon angioplasty, endarterectomy, reductive atherectomy, stent placement, laser therapy or rotablation, or as occurs secondary to vascular grafts, stents, bypass grafts or organ transplants. The compositions can thus be used to inhibit platelet deposition and associated disorders. Thus, a method of inhibiting coagulation, vascular restenosis or platelet deposition, for example, comprises administering to a patient a composition comprising inactivated Factor VII polypeptides, such as that having at least one amino acid substitution in a catalytic triad of Ser344, Asp242 and His193, in an amount sufficient to effectively inhibit coagulation, vascular restenosis or platelet deposition. The methods also find use in the treatment of acute closure of a coronary artery in an individual (e.g. acute myocardial infarction), which comprises administering the inactivated Factor VII polypeptides, which includes DEGR-Factor VII and FFR-Factor VII, in conjunction with tissue plasminogen activator or streptokinase, and can accelerate tPA induced thrombolysis. The inactivated Factor VII polypeptides is given prior to, in conjunction with, or shortly following administration of a thrombolytic agent, such as tissue plasminogen activator.

[0387] Compositions of inactivated Factor VII polypeptides will also have substantial utility in the prevention of cardiogenic emboli and in the treatment of thrombotic strokes. Because of its low potential for causing bleeding complications and its selectivity, Factor VII polypeptides can be given to stroke victims and may prevent the extension of the occluding arterial thrombus. The amount of Factor VII polypeptides administered will vary with each patient depending on the nature and severity of the stroke, but doses will generally be in the range of those suggested below.

[0388] Inactivated Factor VII polypeptides and compositions thereof can also be used to inhibit deleterious events associated with ischemic reperfusion. Severe ischemia to a tissue, organ or limb may be due to a decrease in blood flow and may be associated with trauma, surgical manipulation, or lowered blood pressure. One of the complications associated with severe ischemia is the up-regulation of tissue factor in the arterial system. This increased expression of tissue factor is believed to stimulate a procoagulant response, primarily in the capillary bed. Following reperfusion to the ischemic tissue, thrombi can be generated which may be either occlusive or non-occlusive. The generation of thrombi in the arterial bed, and the deposition of platelets along the thrombus, lead to the secondary generation of ischemia to the tissue. The generation of the thrombi and the presence of platelets can then cause the generation and release of multiple bioactive factors, including those generated from the coagulation pathway, such as thrombin and Factor X, as well as factors released from activated platelets. In turn, these factors may induce the generation of additional factors by the underlying endothelial and smooth muscle cells, or by adjacent mononuclear cells, such as TNF-alpha and IL-1. These factors, in turn, can then activate the endothelial cells leading to the up-regulation of various adhesion molecules associated with monocyte and neutrophil binding. The binding and transmigration of monocytes and neutrophils, the release of bioactive compounds by these cells, including the generation of free-oxygen radicals, can exacerbate the level of endothelial cell activation and damage. Ultimately, if the cascade of events goes unchecked, this can lead to systemic complications and the potential to stimulate multiple organ failure. By blocking tissue factor according to the present invention by administering a specific inhibitor for tissue factor/Factor VII binding (e.g., FFR-FVIIa), and thereby blocking the initiation of the extrinsic pathway of coagulation, the initiation of the cascade of events may be prevented, thereby eliminating, or minimizing the deleterious events associated with ischemia/reperfusion.

[0389] The dose of inactivated Factor VII polypeptides for prevention of deep vein thrombosis is in the range of about 50 μg to 500 mg/day, more typically 1 mg to 200 mg/day, and more preferably 10 to about 175 mg/day for a 70 kg patient, and administration should begin at least about 6 hours prior to surgery and continue at least until the patient becomes ambulatory. The dose of inactivated Factor VII polypeptides in the treatment for restenosis will vary with each patient but will generally be in the range of those suggested above.

[0390] Compositions comprising inactivated Factor VII polypeptides will typically be administered within about 24 hours prior to performing an intervention, and for as much as 7 days or more thereafter. Administration can be by a variety of routes as further described herein. The compositions comprising inactivated Factor VII polypeptides can also be administered systemically or locally for the placement of vascular grafts (e.g., by coating synthetic or modified natural arterial vascular grafts), at sites of anastomosis, surgical endarterectomy (typically carotid artery endarterectomy), bypass grafts, and the like.

[0391] In the treatment of established deep vein thrombosis and/or pulmonary embolism, the dose of Factor VII polypeptides ranges from about 50 μg to 500 mg/day, more typically 1 mg to 200 mg/day, and more preferably 10 mg to about 175 mg/day for a 70 kg patient as loading and maintenance doses, depending on the weight of the patient and the severity of the condition. Because of the lower likelihood of bleeding complications from infusions of inactivated Factor VII polypeptides, inactivated Factor VII polypeptides can replace or lower the dose of heparin during or after surgery in conjunction with thrombectomies or embolectomies.

[0392] In cases of acute bacteremia, endotoxemia or DIC, the patient is given a loading dose of a Factor VII polypeptide of at least about 50 μg to 500 mg/day, more typically 1 mg to 200 mg/day, and more preferably 10 mg to about 175 mg/day for a 70 kg patient, with maintenance doses thereafter in the range of 50 μg to 500 mg/day, typically 1 mg to 200 mg/day for a 70 kg patient.

[0393] Preferably, the Factor VII polypeptide has a half-life ($t_{1/2}$) which is enhanced relative to the half-life of the unconjugated Factor VII from which it was derived. Preferably, the half-life of the Factor VII polypeptide is enhanced by at least 1.5-fold to 2-fold, more preferably by about 2-fold to 3-fold, even more preferably by about 5-fold to 10-fold, optimally about 100-fold, usually about 6-fold relative to the half-life of the unmodified parent Factor VII.

[0394] General methods of attaching polyethylene glycol to proteins are disclosed within U.S. Pat. No. 4,179,337 issued Dec. 18, 1979 (incorporated herein by reference to disclose methods of attaching polyethylene glycol to proteins). Further, other methods of attaching polyethylene glycol are disclosed within U.S. Pat. No. 5,122,614 issued

Jun. 16, 1992, also incorporated herein by reference to disclose methods of attaching polyethylene glycol to proteins. Maleimido-PEG is perhaps the most useful reagent for cysteine-PEGylation, but other chemistries are available for specific cysteine modification.

[0395] The present invention is further illustrated by the following examples which, however, are not to be construed as limiting the scope of protection. The features disclosed in the foregoing description and in the following examples may, both separately and in any combination thereof, be material for realizing the invention in diverse forms thereof.

EXAMPLES

Example 1

[0396] Construction of DNA Encoding FVII-(S43N), FVII-(K62E), FVII-(Q64E), FVII-(I69F), FVII-(K62E/I69A), FVII-(Q64E/I69A), FVII-(Q62E/Q64E/I69A), FVII-(K62E/I69F), FVII-(Q64E/I69F), FVII-(K62E/Q64E/I69F), FVII-(S43C), FVII-(I69C), FVII-(Q64C), FVII-(M306C), FVII-(R277C), FVII-(I69N/F71T), FVII-(F71N/L73T), FVII-(R277N), and FVII-(D309N/L311T):

[0397] DNA constructs encoding FVII-(S43N), FVII-(K62E), FVII-(Q64E), FVII-(I69F), FVII-(K62E/I69A), FVII-(Q64E/I69A), FVII-(Q62E/Q64E/I69A), FVII-(K62E/I69F), FVII-(Q64E/I69F), FVII-(K62E/Q64E/I69F), FVII-(S43C), FVII-(I69C), FVII-(Q64C), FVII-(M306C), FVII-(R277C), FVII-(I69N/F71T), FVII-(F71N/L73T), FVII-(R277N), and FVII-(D309N/L311T) were prepared by site-directed mutagenesis using a supercoiled, double stranded DNA vector with insert of human FVII and two synthetic primers containing the desired mutation. The following primers were used:

For FVII-(S43N):
5'-GGACGAAGCTGTTCTGGATTAACTACAGTGATGGGGACCAG-3' (SEQ ID NO:2)
5'-CTGGTCCCCATCACTGTAGTTAATCCAGAACAGCTTCGTCC-3' (SEQ ID NO:3)

For FVII-(K62E):
5'-GGGGGCTCCTGCGAGGACCAGCTCCAG-3' (SEQ ID NO:4)
5'-CTGGAGCTGGTCCTCGCAGGAGCCCCC-3' (SEQ ID NO:5)

For FVII-(Q64E):
5'-GGGCTCCTGCAAGGACGAGCTCCAGTCCTATATCTGC-3' (SEQ ID NO:6)
5'-GCAGATATAGGACTGGAGCTCGTCCTTGCAGGAGCCC-3' (SEQ ID NO:7)

For FVII-(I69F):
5'-CCAGCTCCAGTCCTATTTCTGCTTCTGCCTCCC-3' (SEQ ID NO:8)
5'-GGGAGGCAGAAGCAGAAATAGGACTGGAGCTGG-3' (SEQ ID NO:9)

For FVII-(K62E/I69A):
5'-GGGGGCTCCTGCGAGGACCAGCTCCAGTCCTATGCCTGCTTCTGCCTC-3' (SEQ ID NO:10)
5'-GAGGCAGAAGCAGGCATAGGACTGGAGCTGGTCCTCGCAGGAGCCCCC-3' (SEQ ID NO:11)

For FVII-(Q64E/I69A):
5'-GGGCTCCTGCAAGGACGAGCTCCAGTCCTATGCCTGCTTCTGCCTCC-3' (SEQ ID NO:12)
5'-GGAGGCAGAAGCAGGCATAGGACTGGAGCTCGTCCTTGCAGGAGCCC-3' (SEQ ID NO:13)

For FVII-(K62E/Q64E/I69A):
5'-GGGGGCTCCTGCGAGGACGAGCTCCAGTCCTATGCCTGCTTCTGCCTCC-3' (SEQ ID NO:14)
5'-GGAGGCAGAAGCAGGCATAGGACTGGAGCTCGTCCTCGCAGGAGCCCCC-3' (SEQ ID NO:15)

For FVII-(S43C):
5'-GGACGAAGCTGTTCTGGATTTGCTACAGTGATGGGGAC-3' (SEQ ID NO:16)
5'-GTCCCCATCACTGTAGCAAATCCAGAACAGCTTCGTCC-3' (SEQ ID NO:17)

For FVII-(I69C)
5'-CCAGCTCCAGTCCTATTGCTGCTTCTGCCTCCCTG-3' (SEQ ID NO:18)
5'-CAGGGAGGCAGAAGCAGCAATAGGACTGGAGCTGG-3' (SEQ ID NO:19)

For FVII-(Q64C)
5'-GGGCTCCTGCAAGGACTGCCTCCAGTCCTATATCTG-3' (SEQ ID NO:20)
5'-CAGATATAGGACTGGAGGCAGTCCTTGCAGGAGCCC-3' (SEQ ID NO:21)

For FVII-(R277C)
5'-GGACGCTGGCCTTCGTGTGCTTCTCATTGGTCAGCG-3' (SEQ ID NO:22)
5'-CGCTGACCAATGAGAAGCACACGAAGGCCAGCGTCC-3' (SEQ ID NO:23)

For FVII-(M306C)
5'-CAACGTGCCCCGGCTGTGCACCCAGGACTGCCTGC-3' (SEQ ID NO:24)
5'-GCAGGCAGTCCTGGGTGCACAGCCGGGGCACGTTG-3' (SEQ ID NO:25)

FVII-(I69N/F71T):
5'-CCAGCTCCAGTCCTATAACTGCACCTGCCTCCCTGCCTTCG-3' (SEQ ID NO:26)
5'-CGAAGGCAGGGAGGCAGGTGCAGTTATAGGACTGGAGCTGG-3' (SEQ ID NO:27)

FVII-(F71N/L73T):
5'-CCAGTCCTATATCTGCAACTGCACCCCTGCCTTCGAGGGCCG-3' (SEQ ID NO:28)

-continued

5'-CGGCCCTCGAAGGCAGGGGTGCAGTTGCAGATATAGGACTGG-3' (SEQ ID NO:29)

FVII-(R277N)

5'-GGACGCTGGCCTTCGTGAACTTCTCATTGGTCAGCGG-3' (SEQ ID NO:30)

5'-CCGCTGACCAATGAGAAGTTCACGAAGGCCAGCGTCC-3' (SEQ ID NO:31)

FVII-(D309N/L311T)

5'-CGGCTGATGACCCAGAACTGCACCCAGCAGTCACGGAAGG-3' (SEQ ID NO:32)

5'-CCTTCCGTGACTGCTGGGTGCAGTTCTGGGTCATCAGCCG-3' (SEQ ID NO:33)

[0398] The oligonucleotide primers, each complementary to opposite strands of the vector insert, were extended during temperature cycling by means of Pfu DNA polymerase. On incorporation of the primers, a mutated plasmid containing staggered nicks was generated. Following temperature cycling, the product was treated with DpnI which is specific for methylated and hemimethylated DNA to digest the parental DNA template and to select for mutation-containing synthesized DNA.

[0399] Procedures for preparing a DNA construct using polymerase chain reaction using specific primers are well known to persons skilled in the art (cf. PCR Protocols, 1990, Academic ress, San Diego, Calif., USA).

Example 2

[0400] Preparation of FVII-(S43N), FVII-(K62E), FVII-(Q64E), FVII-(I69F), FVII-(K62E/I69A), FVII-(Q64E/I69A), FVII-(Q62E/Q64E/I69A), FVII-(K62E/I69F), FVII-(Q64E/I69F), FVII-(K62E/Q64E/I69F), FVII-(S43C), FVII-(I69C), FVII-(Q64C), FVII-(M306C), FVII-(R277C), FVII-(I69N/F71T), FVII-(F71N/L73T), FVII-(R277N), and FVII-(D309N/L311T).

[0401] BHK cells were transfected essentially as previously described (Thim et al. (1988) *Biochemistry* 27, 7785-7793; Persson and Nielsen (1996) *FEBS Lett.* 385, 241-243) to obtain expression of the variant FVII polypeptide. The Factor VII polypeptide was purified as follows:

[0402] Conditioned medium was loaded onto a 50-ml column of Q Sepharose Fast Flow (Pharmacia Biotech) after addition of 5 mM EDTA, and 10 mM Tris, adjustment of pH to 8.0 and adjustment of the conductivity to 10-11 mS/cm by adding water. Elution of the protein was accomplished by a gradient from 10 mM Tris, 50 mM NaCl, pH 8.0 to 10 mM Tris, 50 mM NaCl, 25 mM $CaCl_2$, pH 8.0. The fractions containing the variant FVII polypeptide were pooled, and applied to a 25-ml column containing the monoclonal antibody F1A2 (Novo Nordisk, Bagsvaerd, Denmark) coupled to CNBr-activated Sepharose 4B (Pharmacia Biotech). The column was equilibrated with 50 mM Hepes, pH 7.5, containing 10 mM $CaCl_2$, and 100 mM NaCl. After washing with equilibration buffer and equilibration buffer containing 2 M NaCl, bound material was eluted with equilibration buffer containing 10 mM EDTA instead of $CaCl_2$. Before use or storage, excess $CaCl_2$ over EDTA was added or the variant FVII polypeptide was transferred to a Ca^{2+}-containing buffer. The yield of each step was followed by factor VII ELISA measurements and the purified protein was analysed by SDS-PAGE.

Example 3

[0403] In Vitro Hydrolysis Assay

[0404] Native (wild-type) Factor VIIa and Factor VIIa variant (both hereafter referred to as "Factor VIIa") are assayed in parallel to directly compare their specific activities. The assay is carried out in a microtiter plate (MaxiSorp, Nunc, Denmark). The chromogenic substrate D-Ile-Pro-Arg-p-nitroanilide (S-2288, Chromogenix, Sweden), final concentration 1 mM, is added to Factor VIIa (final concentration 100 nM) in 50 mM Hepes, pH 7.4, containing 0.1 M NaCl, 5 mM $CaCl_2$ and 1 mg/ml bovine serum albumin. The absorbance at 405 nm is measured continuously in a SpectraMax™ 340 plate reader (Molecular Devices, USA). The absorbance developed during a 20-minute incubation, after subtraction of the absorbance in a blank well containing no enzyme, is used to calculate the ratio between the activities of variant and wild-type Factor VIIa:

Ratio=($A_{405\ nm}$ Factor *VIIa* variant)/($A_{405\ nm}$ nm Factor *VIIa* wild-type).

Example 4

[0405] In Vitro Proteolysis Assay

[0406] Native (wild-type) Factor VIIa and Factor VIIa variant (both hereafter referred to as "Factor VIIa") are assayed in parallel to directly compare their specific activities. The assay is carried out in a microtiter plate (MaxiSorp, Nunc, Denmark). Factor VIIa (10 nM) and Factor X (0.8 microM) in 100 microL 50 mM Hepes, pH 7.4, containing 0.1 M NaCl, 5 mM $CaCl_2$ and 1 mg/ml bovine serum albumin, are incubated for 15 min. Factor X cleavage is then stopped by the addition of 50 microL 50 mM Hepes, pH 7.4, containing 0.1 M NaCl, 20 mM EDTA and 1 mg/ml bovine serum albumin. The amount of Factor Xa generated is measured by addition of the chromogenic substrate Z-D-Arg-Gly-Arg-p-nitroanilide (S-2765, Chromogenix, Sweden), final concentration 0.5 mM. The absorbance at 405 nm is measured continuously in a SpectraMax™ 340 plate reader (Molecular Devices, USA). The absorbance developed during 10 minutes, after subtraction of the absorbance in a blank well containing no FVIIa, is used to calculate the ratio between the proteolytic activities of variant and wild-type Factor VIIa:

Ratio=($A_{405\ nm}$ Factor *VIIa* variant)/($A_{405\ nm}$ Factor *VIIa* wild-type).

Example 5

[0407] TF Binding Affinity Assay—Biosensor Assay:

[0408] FVII polypeptides are tested on the Biacore instrument by passing a standard solution of the FVII polypeptide over a chip with immobilized TF. This is followed by different concentrations of sTF in 10 mM hepes pH 7.4 containing 150 mM NaCl, 10 mM $CaCl_2$ and 0.0003% polysorbate 20. Kd's are calculated from the sensorgrams using the integrated Biacore evaluation software.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 1

<210> SEQ ID NO 1
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<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
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<222> LOCATION: (1)..(406)
<223> OTHER INFORMATION: Xaa= 4-carboxyglutamic acid
(gamma-carboxyglutamate)

<400> SEQUENCE: 1

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1               5                   10                  15

Cys Lys Xaa Xaa Gln Cys Ser Phe Xaa Xaa Ala Arg Xaa Ile Phe Lys
            20                  25                  30

Asp Ala Xaa Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp
        35                  40                  45

Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln
    50                  55                  60

Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn
65                  70                  75                  80

Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly
                85                  90                  95

Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys
            100                 105                 110

Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr
        115                 120                 125

Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg
    130                 135                 140

Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro
145                 150                 155                 160

Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln
                165                 170                 175

Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala
            180                 185                 190

His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu
        195                 200                 205

Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg
    210                 215                 220

Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn
225                 230                 235                 240

His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp
                245                 250                 255

His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr
            260                 265                 270

Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu
        275                 280                 285

Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg
    290                 295                 300

Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser
305                 310                 315                 320
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Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser
                325                 330                 335

Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr
            340                 345                 350

Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys
        355                 360                 365

Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile
    370                 375                 380

Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu
385                 390                 395                 400

Leu Arg Ala Pro Phe Pro
                405
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1. A Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa, wherein said Factor VII polypeptide is selected from the group consisting of:

i) a Factor VII polypeptide comprising one or more amino acid substitutions at positions selected from the group consisting of: $K18X_1$, $R36X_2$, $S43X_3$, $K62X_4$, $Q64X_5$, $L65X_6$, $I69X_7$, $F71X_8$, $L73X_9$, $P74X_{10}$, $E77X_{11}$, $G78X_{12}$, $R79X_{13}$, $K85X_{14}$, $Q88X_{15}$, $N93X_{16}$, $F275X_{17}$, $R277X_{18}$, $M306X_{19}$, $T307X_{20}$, $Q308X_{21}$, $D309X_{22}$, or $R379X_{23}$ corresponding to amino acids at positions of SEQ ID NO: 1, wherein

X_1 is E, D, A, or F;

X_2 is E, D, K, A, F, or N;

X_3 is E, D, K, R, A, or F;

X_4 is D, A, or F;

X_5 is E, D, K, R, A, F, or N;

X_6 is E, D, K, R, A, or F;

X_7 is C, D, E, F, G, H, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_8 is A, G, H, I, K, L, M, P, R, S, T, V, or W;

X_9 is A, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y;

X_{10} is A, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, or Y;

X_{11} is A, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_{12} is A, D, E, F, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_{13} is A, D, E, F, G, H, I, K, L, M, N, P, Q, S, T, V, W, or Y;

X_{14} is A, D, E, F, G, I, L, M, P, S, T, V, W, or Y;

X_{15} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;

X_{16} is A, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y;

X_{17} is A, D, E, G, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_{18} is A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y;

X_{19} is A, E, F, G, H, I, K, L, P, Q, R, S, T, V, W, or Y;

X_{20} is A, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, V, W, or Y;

X_{21} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;

X_{22} is A, F, G, H, I, K, L, M, N, P, Q, R, V, W, or Y; and

X_{23} is A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y;

and/or

ii) a Factor VII polypeptide in which one or more amino acids corresponding to amino acids at positions selected from K18, R36, K62, Q64, L65, I69, F71, L73, P74, E77, G78, R79, K85, Q88, N93, F275, R277, M306, Q308, D309, or R379 of SEQ ID NO: 1 have been substituted with a cysteine amino acid residue, and wherein the cysteine amino acid residue may optionally be conjugated with a chemical group that increases the molecular weight of the Factor VII polypeptide;

and/or

iii) a Factor VII polypeptide comprising one or more N-glycosylation site N-Xaa-S/T introduced by amino acid substitutions corresponding to amino acids starting at positions selected from R36, Q64, I69, F71, P74, E77, G78, Q88, N93, F275, M306, T307, or D309 of SEQ ID NO: 1, wherein Xaa is any amino acid except P.

2. The Factor VII polypeptide according to claim 1, further comprising one or more amino acid substitutions selected from the group consisting of L305V, L305V/M306D/D309S, L3051, L305T, F374P, V158T/M298Q, V158D/E296V/M298Q, K337A, M298Q, V158D/M298Q, L305V/K337A, V158D/E296V/M298Q/L305V, V158D/

E296V/M298Q/K337A, V158D/E296V/M298Q/L305V/K337A, K157A, E296V, E296V/M298Q, V158D/E296V, V158D/M298K, and S336G, L305V/K337A, L305V/V158D, L305V/E296V, L305V/M298Q, L305V/V158T, L305V/K337A/V158T, L305V/K337A/M298Q, L305V/K337A/E296V, L305V/K337A/V158D, L305V/V158D/M298Q, L305V/V158D/E296V, L305V/V158T/M298Q, L305V/V158T/E296V, L305V/E296V/M298Q, L305V/V158D/E296V/M298Q, L305V/V158T/E296V/M298Q, L305V/V158T/K337A/M298Q, L305V/V158T/E296V/K337A, L305V/V158D/K337A/M298Q, L305V/V158D/E296V/K337A, L305V/V158D/E296V/M298Q/K337A, L305V/V158T/E296V/M298Q/K337A, S314E/K316H, S314E/K316Q, S314E/L305V, S314E/K337A, S314E/V158D, S314E/E296V, S314E/M298Q, S314E/V158T, K316H/L305V, K316H/K337A, K316H/V158D, K316H/E296V, K316H/M298Q, K316H/V158T, K316Q/L305V, K316Q/K337A, K316Q/V158D, K316Q/E296V, K316Q/M298Q, K316Q/V158T, S314E/L305V/K337A, S314E/L305V/V158D, S314E/L305V/E296V, S314E/L305V/M298Q, S314E/L305V/V158T, S314E/L305V/K337A/V158T, S314E/L305V/K337A/M298Q, S314E/L305V/K337A/E296V, S314E/L305V/K337A/V158D, S314E/L305V/V158D/M298Q, S314E/L305V/V158D/E296V, S314E/L305V/V158T/M298Q, S314E/L305V/V158T/E296V, S314E/L305V/E296V/M298Q, S314E/L305V/V158D/E296V/M298Q, S314E/L305V/V158T/E296V/M298Q, S314E/L305V/V158T/K337A/M298Q, S314E/L305V/V158T/E296V/K337A, S314E/L305V/V158D/K337A/M298Q, S314E/L305V/V158D/E296V/K337A, S314E/L305V/V158D/E296V/M298Q/K337A, S314E/L305V/V158T/E296V/M298Q/K337A, K316H/L305V/K337A, K316H/L305V/V158D, K316H/L305V/E296V, K316H/L305V/M298Q, K316H/L305V/V158T, K316H/L305V/K337A/V158T, K316H/L305V/K337A/M298Q, K316H/L305V/K337A/E296V, K316H/L305V/K337A/V158D, K316H/L305V/V158D/M298Q, K316H/L305V/V158D/E296V, K316H/L305V/V158T/M298Q, K316H/L305V/V158T/E296V, K316H/L305V/E296V/M298Q, K316H/L305V/V158D/E296V/M298Q, K316H/L305V/V158T/E296V/M298Q, K316H/L305V/V158T/K337A/M298Q, K316H/L305V/V158T/E296V/K337A, K316H/L305V/V158D/K337A/M298Q, K316H/L305V/V158D/E296V/K337A, K316H/L305V/V158D/E296V/M298Q/K337A, K316H/L305V/V158T/E296V/M298Q/K337A, K316Q/L305V/K337A, K316Q/L305V/V158D, K316Q/L305V/E296V, K316Q/L305V/M298Q, K316Q/L305V/V158T, K316Q/L305V/K337AN/158T, K316Q/L305V/K337A/M298Q, K316Q/L305V/K337A/E296V, K316Q/L305V/K337A/V158D, K316Q/L305V/V158D/M298Q, K316Q/L305V/V158D/E296V, K316Q/L305V/V158T/M298Q, K316Q/L305V/V158T/E296V, K316Q/L305V/E296V/M298Q, K316Q/L305V/V158D/E296V/M298Q, K316Q/L305V/V158T/E296V/M298Q, K316Q/L305V/V158T/K337A/M298Q, K316Q/L305V/V158T/E296V/K337A, K316Q/L305V/V158D/K337A/M298Q, K316Q/L305V/V158D/E296V/K337A, K316Q/L305V/V158D/E296V/M298Q/K337A, K316Q/L305V/V158T/E296V/M298Q/K337A, F374Y/K337A, F374Y/V158D, F374Y/E296V, F374Y/M298Q, F374Y/V158T, F374Y/S314E, F374Y/L305V, F374Y/L305V/K337A, F374Y/L305V/V158D, F374Y/L305V/E296V, F374Y/L305V/M298Q, F374Y/L305V/V158T, F374Y/L305V/S314E, F374Y/K337A/S314E, F374Y/K337A/V158T, F374Y/K337A/M298Q, F374Y/K337A/E296V, F374Y/K337A/V158D, F374Y/V158D/S314E, F374Y/V158D/M298Q, F374Y/V158D/E296V, F374Y/V158T/S314E, F374Y/V158T/M298Q, F374Y/V158T/E296V, F374Y/E296V/S314E, F374Y/S314E/M298Q, F374Y/E296V/M298Q, F374Y/L305V/K337A/V158D, F374Y/L305V/K337A/E296V, F374Y/L305V/K337A/M298Q, F374Y/L305V/K337A/V158T, F374Y/L305V/K337A/S314E, F374Y/L305V/V158D/E296V, F374Y/L305V/V158D/M298Q, F374Y/L305V/V158D/S314E, F374Y/L305V/E296V/M298Q, F374Y/L305V/E296V/V158T, F374Y/L305V/E296V/S314E, F374Y/L305V/M298Q/V158T, F374Y/L305V/M298Q/S314E, F374Y/L305V/V158T/S314E, F374Y/K337A/S314E/V158T, F374Y/K337A/S314E/M298Q, F374Y/K337A/S314E/E296V, F374Y/K337A/S314E/V158D, F374Y/K337A/V158T/M298Q, F374Y/K337A/V158T/E296V, F374Y/K337A/M298Q/E296V, F374Y/K337A/M298Q/V158D, F374Y/K337A/E296V/V158D, F374Y/V158D/S314E/M298Q, F374Y/V158D/S314E/E296V, F374Y/V158D/M298Q/E296V, F374Y/V158T/S314E/E296V, F374Y/V158T/S314E/M298Q, F374Y/V158T/M298Q/E296V, F374Y/E296V/S314E/M298Q, F374Y/L305V/M298Q/K337A/S314E, F374Y/L305V/E296V/K337A/S314E, F374Y/E296V/M298Q/K337A/S314E, F374Y/L305V/E296V/M298Q/K337A, F374Y/L305V/E296V/M298Q/S314E, F374Y/V158D/E296V/M298Q/K337A, F374Y/V158D/E296V/M298Q/S314E, F374Y/L305V/V158D/K337A/S314E, F374Y/V158D/M298Q/K337A/S314E, F374Y/L305V/V158D/E296V/K337A/S314E, F374Y/L305V/V158D/E296V/M298Q, F374Y/L305V/V158D/M298Q/K337A, F374Y/L305V/V158D/E296V/K337A, F374Y/L305V/V158D/M298Q/S314E, F374Y/L305V/V158D/E296V/S314E, F374Y/V158T/E296V/M298Q/K337A, F374Y/V158T/E296V/M298Q/S314E, F374Y/L305V/V158T/K337A/S314E, F374Y/V158T/M298Q/K337A/S314E, F374Y/V158T/E296V/K337A/S314E, F374Y/L305V/V158T/E296V/M298Q, F374Y/L305V/V158T/M298Q/K337A, F374Y/L305V/V158T/E296V/K337A, F374Y/L305V/V158T/M298Q/S314E, F374Y/L305V/V158T/E296V/S314E, F374Y/E296V/M298Q/K337A/V158T/S314E, F374Y/V158D/E296V/M298Q/K337A/S314E, F374Y/L305V/V158D/E296V/M298Q/S314E, F374Y/L305V/E296V/M298Q/V158T/S314E, F374Y/L305V/E296V/M298Q/K337A/V158T, F374Y/L305V/E296V/K337A/V158T/S314E, F374Y/L305V/M298Q/K337A/V158T/S314E, F374Y/L305V/V158D/E296V/M298Q/K337A, F374Y/L305V/V158D/E296V/K337A/S314E, F374Y/L305V/V158D/M298Q/K337A/S314E, F374Y/L305V/E296V/M298Q/K337A/V158T/S314E, S52A, S60A; R152E, S344A, P11Q/K33E, T106N, K143N/N145T, V253N, R290N/A292T, G291N, R315N/V317T;

K143N/N145T/R315N/V317T; one or more substitutions, additions or deletions in the amino acid sequence from 233Thr to 240Asn; and one or more substitutions, additions or deletions in the amino acid sequence from 304Arg to 329Cys.

3. The Factor VII polypeptide according to claim 1, wherein the dissociation constant K_d of said Factor VII polypeptide is higher than 5 nM,

4. The Factor VII polypeptide according to claim 3, wherein the dissociation constant K_d of said Factor VII polypeptide is higher than 20 nM.

5. The Factor VII polypeptide according to claim 4, wherein the dissociation constant K_d of said Factor VII polypeptide is dissociation constant K_d of said Factor VII polypeptide is higher than 100 nM.

6. The Factor VII polypeptide according to claim 5, wherein the dissociation constant K_d of said Factor VII polypeptide is dissociation constant K_d of said Factor VII polypeptide is higher than 1 μM.

7. A composition comprising a Factor VII polypeptide according to claim 1.

8. A pharmaceutical composition comprising a Factor VII polypeptide according to claim 1 and a pharmaceutically acceptable carrier or diluent.

9. A polynucleotide construct encoding a Factor VII polypeptide according to claim 1.

10. The polynucleotide construct according to claim 9, wherein said construct is a vector.

11. A eukaryotic host cell comprising the polynucleotide construct according to claim 9.

12. The eukaryotic host cell according to claim 11, wherein said cell is of mammalian origin.

13. The eucaryotic host cell according to claim 12, wherein said cell is selected from the group consisting of CHO cells, BHK cells or HEK cells.

14. A method for producing the Factor VII polypeptide, the method comprising (i) cultivating a eukaryotic host cell according to claim 11 in an appropriate growth medium under conditions allowing protein synthesis from said polynucleotide construct and (ii) recovering said Factor VII polypeptide from the culture medium.

15. A method for the treatment of bleeding episodes or bleeding disorders in a subject or for the enhancement of the normal haemostatic system, the method comprising administering to a subject in need thereof a therapeutically or prophylactically effective amount of a Factor VII polypeptide as defined in claim 1.

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(54) FACTOR VII OR VIIA POLYPEPTIDE VARIANTS

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(57) **ABSTRACT**

The present invention relates to novel polypeptide variants of factor VII (FVII) or factor VIIa (FVIIa) polypeptides, where said variants comprise an amino acid substitution in position 10 and 32 and where said variants further comprise a sugar moiety covalently attached to an introduced in vivo N-glycosylation site located outside of the Gla domain. Such polypeptide variants are useful in therapy, in particular for the treatment of a variety of coagulation-related disorders, such as trauma.

FACTOR VII OR VIIA POLYPEPTIDE VARIANTS

FIELD OF THE INVENTION

[0001] The present invention relates to novel polypeptide variants of factor VII (FVII) or factor VIIa (FVIIa) polypeptides, where said variants comprise an amino acid substitution in position 10 and 32 and where said variants further comprise a sugar moiety covalently attached to an introduced in vivo N-glycosylation site.

[0002] The present invention also relates to use of such polypeptide variants in therapy, in particular for the treatment of a variety of coagulation-related disorders.

BACKGROUND OF THE INVENTION

[0003] Blood coagulation is a process consisting of a complex interaction of various blood components (or factors) that eventually results in a fibrin clot. Generally, the blood components participating in what has been referred to as the "coagulation cascade" are proenzymes or zymogens, i.e. enzymatically inactive proteins that are converted into an active form by the action of an activator. One of these coagulation factors is FVII.

[0004] FVII is a vitamin K-dependent plasma protein synthesized in the liver and secreted into the blood as a single-chain glycoprotein with a molecular weight of 53 kDa (Broze & Majerus, J. Biol. Chem 1980; 255:1242-1247). The FVII zymogen is converted into an activated form (FVIIa) by proteolytic cleavage at a single site, R152-I153, resulting in two chains linked by a single disulfide bridge. FVIIa in complex with tissue factor (FVIIa complex) is able to convert both factor IX and factor X into their activated forms, followed by reactions leading to rapid thrombin production and fibrin formation (Østerud & Rapaport, Proc Natl Acad Sci USA 1977; 74:5260-5264).

[0005] FVII undergoes post-translational modifications, including vitamin K-dependent carboxylation resulting in ten γ-carboxyglutamic acid residues in the N-terminal region of the molecule. Thus, residue number 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35 shown in SEQ ID NO:2 are γ-carboxyglutamic acids residues in the Gla domain important for FVII activity. Other post-translational modifications include sugar moiety attachment at two naturally occurring N-glycosylation sites at position 145 and 322, respectively, and at two naturally occurring O-glycosylation sites at position 52 and 60, respectively.

[0006] The gene coding for human FVII (hFVII) has been mapped to chromosome 13 at q34-qter 9 (de Grouchy et al., Hum Genet 1984; 66:230-233). It contains nine exons and spans 12.8 Kb (O'Hara et al., Proc Natl Acad Sci USA 1987; 84:5158-5162). The gene organisation and protein structure of FVII are similar to those of other vitamin K-dependent procoagulant proteins, with exons 1a and 1b encoding for signal sequence; exon 2 the propeptide and Gla domain; exon 3 a short hydrophobic region; exons 4 and 5 the epidermal growth factor-like domains; and exon 6 through 8 the serine protease catalytic domain (Yoshitake et al., Biochemistry 1985; 24: 3736-3750).

[0007] Reports exist on experimental three-dimensional structures of hFVIIa (Pike et al., PNAS. U.S.A., 1999; 96:8925-30 and Kemball-Cook et al., J. Struct. Biol, 1999; 127:213-223); of hFVIIa in complex with soluble tissue factor using X-ray crystallographic methods (Banner et al., Nature, 1996; 380:41 and Zhang et al., J. Mol. Biol, 1999; 285: 2089); and of smaller fragments of hFVII (Muranyi et al., Biochemistry, 1998; 37:10605 and Kao et al., Biochemistry, 1999; 38:7097).

[0008] Some protein-engineered variants of FVII have been reported. See, e.g., Dickinson & Ruf, J Bio Chem, 1997; 272:19875-19879, Kemball-Cook et al., J Biol Chem, 1998; 273:8516-8521, Bharadwaj et al., J Biol Chem, 1996; 271:30685-30691, Ruf et al., Biochemistry, 1999; 38:1957-1966; WO 99/20767; WO 00/11416; WO 02/22776; WO 02/38162; WO 01/83725; WO 01/58935; U.S. Pat. No. 5,580,560.

[0009] Reports exist on expression of FVII in BHK or other mammalian cells (WO 92/15686, WO 91/11514 and WO 88/10295) and co-expression of FVII and kex2 endoprotease in eukaryotic cells (WO 00/28065).

[0010] Commercial preparations of human recombinant FVIIa are sold as NovoSeven®. NovoSeven® is indicated for the treatment of bleeding episodes in hemophilia A or B patients. NovoSeven® is the only rhFVIIa for effective and reliable treatment of bleeding episodes available on the market.

[0011] An inactive form of FVII in which arginine 152 and/or isoleucine 153 is/are modified has been reported in WO91/1154. These amino acids are located at the activation site. WO 96/12800 describes inactivation of FVIIa by a serine proteinase inhibitor; inactivation by carbamylation of FVIIa at the α-amino acid group I153 has been described by Petersen et al., Eur J Biochem, 1999; 261:124-129. The inactivated form is capable of competing with wild-type FVII or FVIIa for binding to tissue factor and inhibiting clotting activity. The inactivated form of FVIIa is suggested to be used for treatment of patients being in hypercoagulable states, such as patients with sepsis, in risk of myocardial infarction or of thrombotic stroke.

[0012] A circulating rhFVIIa half-life of 2.3 hours was reported in "Summary Basis for Approval for NovoSeven®", FDA reference number 96-0597. Relatively high doses and frequent administration are necessary to reach and sustain the desired therapeutic or prophylactic effect. As a consequence adequate dose regulation is difficult to obtain and the need of frequent intravenous administrations imposes restrictions on the patient's way of living.

[0013] In connection with treatment of uncontrolled bleedings, such as trauma, it is believed that factor VIIa is capable of activating factor X to factor Xa without binding to tissue factor, and this activation reaction is believed to occur primarily on activated blood platelets (Hedner et al. Blood Coagulation & Fibrinolysis, 2000; 11; 107-111). However, hFVIIa or rhFVIIa has a low activity towards factor X in the absence of tissue factor and, consequently, treatment of uncontrolled bleeding, for example in trauma patients, requires relatively high and multiple doses of hFVIIa or rhFVIIa. Therefore, in order to treat uncontrolled bleedings more efficiently (to minimize blood loss) there is need for improved FVIIa molecules, which possess a high activity toward factor X in the absence of tissue factor. Such improved FVIIa molecules will exhibit a lowered clotting time (or faster action) as compared to rhFVIIa when administered in connection with uncontrolled bleedings.

[0014] A molecule with a longer circulation half-life would decrease the number of necessary administrations. Given the association of current the rhFVIIa product with frequent injections, and the potential for obtaining more optimal therapeutic FVIIa levels with concomitant enhanced therapeutic effect, there is a clear need for improved FVII- or FVIIa-like molecules.

[0015] One way to increase the circulation half-life of a protein is to ensure that renal clearance of the protein is reduced. This may be achieved by conjugating the protein to a chemical moiety, which is capable of conferring reduced renal clearance to the protein.

[0016] Furthermore, attachment of a chemical moiety to the protein or substitution of amino acids exposed to proteolysis may effectively block a proteolytic enzyme from contact leading to proteolytic degradation of the protein. Polyethylene glycol (PEG) is one such chemical moiety that has been used in the preparation of therapeutic protein products. WO 98/32466 suggests that FVII, among many other proteins, may be PEGylated but does not contain any further information in this respect. WO 01/58935 discloses a new strategy for developing FVII or FVIIa molecules having inter alia an increased half-life.

[0017] As indicated above, another problem in current rhFVIIa treatment is the relative instability of the molecule with respect to proteolytic degradation. Proteolytic degradation is a major obstacle for obtaining a preparation in solution as opposed to a lyophilized product. The advantage of obtaining a stable soluble preparation lies in easier handling for the patient, and, in the case of emergencies, quicker action, which potentially can become life saving. Attempts to prevent proteolytic degradation by site directed mutagenesis at major proteolytic sites have been disclosed in WO 88/10295.

[0018] One object of the present invention is to provide improved FVII or FVIIa molecules (FVII or FVIIa variants) with a longer circulation half-life (thereby decreasing the number of necessary administrations) and which are capable of activating factor X to factor Xa (without binding to tissue factor) more efficiently than hFVIIa or rhFVIIa (thereby being able to treat uncontrolled bleadings, such as a trauma, more efficiently).

[0019] Another object of the present invention is to provide improved FVII or FVII molecules (FVII or FVIIa variants) with an increased bioavailability (such as an increased Area Under the Curve as compared to rhFVIIa when administered intravenously) and which are capable of activating factor X to factor Xa (without binding to tissue factor) more efficiently than hFVIIa or rhFVIIa (thereby being able to treat uncontrolled bleadings, such as a trauma, more efficiently).

[0020] These objects are met by the FVII or FVIIa variants provided herein.

BRIEF DISCLOSURE OF THE INVENTION

[0021] In its broadest aspect the present invention relates to a FVII or FVIIa polypeptide variant having an amino acid sequence comprising 3-15 amino acid modifications relative to hFVII or hFVIIa having the amino acid sequence shown in SEQ ID NO:2, wherein said amino acid sequence of the variant comprises an amino acid substitution in position 10 and 32 and wherein a sugar moiety is covalently attached to an introduced in vivo N-glycosylation site located outside the Gla domain.

[0022] Another aspect of the invention relates to a nucleotide sequence encoding the polypeptide variant of the invention.

[0023] In a further aspect the invention relates to an expression vector comprising the nucleotide sequence of the invention.

[0024] In a still further aspect the invention relates to a host cell comprising the nucleotide sequence of the invention or the expression vector of the invention.

[0025] In an even further aspect the invention relates to a pharmaceutical composition comprising the polypeptide variant of the invention, and a pharmaceutical acceptable carrier or excipient.

[0026] Still another aspect of the invention relates to the polypeptide variant of the invention, or the pharmaceutical composition of the invention, for use as a medicament.

[0027] Further aspects of the present invention will be apparent from the below description as well as from the appended claims.

DETAILED DISCLOSURE OF THE INVENTION

Definitions

[0028] In the context of the present application and invention the following definitions apply:

[0029] The term "conjugate" (or interchangeably "conjugated polypeptide") is intended to indicate a heterogeneous (in the sense of composite or chimeric) molecule formed by the covalent attachment of one or more polypeptide(s) to one or more non-polypeptide moieties such as polymer molecules, lipophilic compounds, sugar moieties or organic derivatizing agents. Preferably, the conjugate is soluble at relevant concentrations and conditions, i.e. soluble in physiological fluids such as blood. Examples of conjugated polypeptides of the invention include glycosylated and/or PEGylated polypeptides.

[0030] The term "covalent attachment" or "covalently attached" means that the polypeptide variant and the non-polypeptide moiety are either directly covalently joined to one another, or else are indirectly covalently joined to one another through an intervening moiety or moieties, such as a bridge, spacer, or linkage moiety or moieties.

[0031] The term "non-polypeptide moiety" is intended to mean a molecule, different from a peptide polymer composed of amino acid monomers and linked together by peptide bonds, which molecule is capable of conjugating to an attachment group of the polypeptide variant of the invention. Preferred examples of such molecules include polymer molecules, sugar moieties, lipophilic compounds or organic derivatizing agents. When used in the context of a conjugated variant of the invention it will be understood that the non-polypeptide moiety is linked to the polypeptide part of the conjugated variant through an attachment group of the polypeptide. As explained above, the non-polypeptide moiety can be directly covalently joined to the attachment group or it can be indirectly covalently joined to the attachment

group through an intervening moiety or moieties, such as a bridge spacer or linker moiety or moieties.

[0032] A "polymer molecule" is a molecule formed by covalent linkage of two or more monomers, wherein none of the monomers is an amino acid residue, except where the polymer is human albumin or another abundant plasma protein. The term "polymer" may be used interchangeably with the term "polymer molecule". The term is also intended to cover carbohydrate molecules attached by in vitro glycosylation, i.e. a synthetic glycosylation performed in vitro normally involving covalently linking a carbohydrate molecule to an attachment group of the polypeptide variant, optionally using a cross-linking agent. In vitro glycosylation is discussed in detail further below.

[0033] The term "sugar moiety" is intended to indicate a carbohydrate-containing molecule comprising one or more monosaccharide residues, capable of being attached to the polypeptide variant (to produce a polypeptide variant conjugate in the form of a glycosylated polypeptide variant) by way of in vivo glycosylation. The term "in vivo glycosylation" is intended to mean any attachment of a sugar moiety occurring in vivo, i.e. during posttranslational processing in a glycosylating cell used for expression of the polypeptide variant, e.g. by way of N-linked and O-linked glycosylation. The exact oligosaccharide structure depends, to a large extent, on the glycosylating organism in question.

[0034] An "N-glycosylation site" has the sequence N-X-S/T/C, wherein X is any amino acid residue except proline, N is asparagine and S/T/C is either serine, threonine or cysteine, preferably serine or threonine, and most preferably threonine. Preferably, the amino acid residue in position +3 relative to the asparagines residue is not a proline residue.

[0035] An "O-glycosylation site" is the OH-group of a serine or threonine residue.

[0036] The term "attachment group" is intended to indicate a functional group of the polypeptide variant, in particular of an amino acid residue thereof or a carbohydrate moiety, capable of attaching a non-polypeptide moiety such as a polymer molecule, a lipophilic molecule, a sugar moiety or an organic derivatizing agent. Useful attachment groups and their matching non-polypeptide moieties are apparent from the table below.

Attachment group	Amino acid	Examples of non-polypeptide moiety	Conjugation method/-Activated PEG	Reference
—NH_2	N-terminal, Lys	Polymer, e.g. PEG, with amide or imine group	mPEG-SPA Tresylated mPEG	Shearwater Inc. Delgado et al, critical reviews in Therapeutic Drug Carrier Systems 9(3, 4): 249–304 (1992)
—COOH	C-terminal, Asp, Glu	Polymer, e.g. PEG, with ester or amide group	mPEG-Hz	Shearwater Inc.
		Carbohydrate moiety	In vitro coupling	
—SH	Cys	Polymer, e.g. PEG, with disulfide, maleimide or vinylsulfone group	PEG-vinylsulphone PEG-maleimide	Shearwater Inc. Delgado et al, critical reviews in Therapeutic Drug Carrier Systems 9(3, 4): 249–304 (1992)
		Carbohydrate moiety	In vitro coupling	
—OH	Ser, Thr, Lys, OH—	Sugar moiety PEG with ester, ether, carbamate, carbonate	In vivo O-linked glycosylation	
—$CONH_2$	Asn as part of an N-glycosylation site	Sugar moiety Polymer, e.g. PEG	In vivo N-glycosylation	
Aromatic residue	Phe, Tyr, Trp	Carbohydrate moiety	In vitro coupling	
—$CONH_2$	Gln	Carbohydrate moiety	In vitro coupling	Yan and Wold, Biochemistry, 1984, Jul 31; 23(16): 3759–65
Aldehyde Ketone	Oxidized oligosaccharide	Polymer, e.g. PEG, PEG-hydrazide	PEGylation	Andresz et al., 1978, Makromol. Chem. 179: 301, WO 92/16555, WO 00/23114
Guanidino	Arg	Carbohydrate moiety	In vitro coupling	Lundblad and Noyes, Chimical Reagents for Protein Modification, CRC Press Inc., Florida, USA
Imidazole ring	His	Carbohydrate moiety	In vitro coupling	As for guanidine

[0037] For in vivo N-glycosylation, the term "attachment group" is used in an unconventional way to indicate the amino acid residues constituting a N-glycosylation site (with the sequence N-X-S/T/C, wherein X is any amino acid residue except proline, N is asparagine and S/T/C is either serine, threonine or cysteine, preferably serine or threonine, and most preferably threonine). Although the asparagine residue of the N-glycosylation site is the one to which the sugar moiety is attached during glycosylation, such attachment cannot be achieved unless the other amino acid residues of the N-glycosylation site is present.

[0038] Accordingly, when the non-polypeptide moiety is a sugar moiety and the conjugation is to be achieved by in vivo N-glycosylation, the term "amino acid residue comprising an attachment group for a non-polypeptide moiety" as used in connection with alterations of the amino acid sequence of the polypeptide is to be understood as meaning that one or more amino acid residues constituting an in vivo N-glycosylation site are to be altered in such a manner that a functional in vivo N-glycosylation site is introduced into the amino acid sequence.

[0039] In the present application, amino acid names and atom names (e.g. CA, CB, CD, CG, SG, NZ, N, O, C, etc) are used as defined by the Protein DataBank (PDB) (www-.pdb.org) based on the IUPAC nomenclature (IUPAC Nomenclature and Symbolism for Amino Acids and Peptides (residue names, atom names, etc.), *Eur. J. Biochem.,* 138, 9-37 (1984) together with their corrections in *Eur. J. Biochem.,* 152, 1 (1985)).

[0040] The term "amino acid residue" is intended to indicate an amino acid residue contained in the group consisting of alanine (Ala or A), cysteine (Cys or C), aspartic acid (Asp or D), glutamic acid (Glu or E), phenylalanine (Phe or F), glycine (Gly or G), histidine (His or H), isoleucine (Ile or I), lysine (Lys or K), leucine (Leu or L), methionine (Met or M), asparagine (Asn or N), proline (Pro or P), glutamine (Gln or Q), arginine (Arg or R), serine (Ser or S), threonine (Thr or T), valine (Val or V), tryptophan (Trp or W), and tyrosine (Tyr or Y) residues.

[0041] The terminology used for identifying amino acid positions is illustrated as follows: I205 indicates that position 205 is occupied by an isoleucine residue in the amino acid sequence shown in SEQ ID NO:2. I205T indicates that the isoleucine residue of position 205 has been substituted with a threonine residue. Alternative substitutions are indicated with a "/", e.g. I205S/T means an amino acid sequence in which isoleucine in position 205 is substituted with either serine or threonine. Multiple substitutions are indicated with a "+", e.g. K143N+N145T means a substitution of the lysine residue in position 143 with an asparagine residue and a substitution of the asparagine residue in position 145 with a threonine residue. Insertion of an additional amino acid residue is indicated in the following way: Insertion of a tyrosine residue after A3 (i.e. in position 4) is indicated by A3AY (leading to insertion of a tyrosine residue in position 4). A deletion of an amino acid residue is indicated by an asterix. For example, deletion of a valine residue in position 172 is indicated by V172*. Simultaneous insertion and substitution are indicated in the following way: Substitution of an alanine residue in position 175 with a threonine residue followed by insertion of a leucine residue after position 175 is indicated A175TL.

[0042] Unless otherwise indicated, the numbering of amino acid residues made herein is made relative to the amino acid sequence of the hFVII/hFVIIa polypeptide (SEQ ID NO:2).

[0043] The term "differs from" as used in connection with specific mutations is intended to allow for additional differences being present apart from the specified amino acid difference. For instance, in addition to the introduction of in vivo N-glycosylation sites (located outside the Gla domain), the polypeptide may comprise other modifications that are not related to introduction of such amino acid residues. In a similar way, in addition to the modifications performed in the Gla domain aiming at increasing the phospholipid membrane binding affinity, the polypeptide may contain other modifications that are not necessarily related to this effect. Thus, in addition to the amino acid modifications disclosed herein, it will be understood that the amino acid sequence of the polypeptide variant of the invention may, if desired, contain other alterations, i.e. other substitutions, insertions or deletions. These may, for example, include truncation of the N- and/or C-terminus by one or more amino acid residues (e.g. by 1-10 amino acid residues), or addition of one or more extra residues at the N- and/or C-terminus, e.g. addition of a methionine residue at the N-terminus or introduction of a cysteine residue near or at the C-terminus, as well as "conservative amino acid substitutions", i.e. substitutions performed within groups of amino acids with similar characteristics, e.g. small amino acids, acidic amino acids, polar amino acids, basic amino acids, hydrophobic amino acids and aromatic amino acids.

[0044] Examples of such conservative substitutions are shown in the below table.

1	Alanine (A)	Glycine (G)	Serine (S)	Threonine (T)
2	Aspartic acid (D)	Glutamic acid (E)		
3	Asparagine (N)	Glutamine (Q)		
4	Arginine (R)	Histidine (H)	Lysine (K)	
5	Isoleucine (I)	Leucine (L)	Methionine (M)	Valine (V)
6	Phenylalanine (F)	Tyrosine (Y)	Tryptophan (W)	

[0045] Still other examples of additional modifications are disclosed in the section entitled "*Other modifications outside the Gla domain*" below.

[0046] The term "nucleotide sequence" is intended to indicate a consecutive stretch of two or more nucleotide molecules. The nucleotide sequence may be of genomic, cDNA, RNA, semisynthetic, synthetic origin, or any combinations thereof.

[0047] The term "polymerase chain reaction" or "PCR" generally refers to a method for amplification of a desired nucleotide sequence in vitro, as described, for example, in U.S. Pat. No. 4,683,195. In general, the PCR method involves repeated cycles of primer extension synthesis, using oligonucleotide primers capable of hybridising preferentially to a template nucleic acid.

[0048] The term "vector" refers to a plasmid or other nucleotide sequences that are capable of replicating within a host cell or being integrated into the host cell genome, and as such, are useful for performing different functions in

conjunction with compatible host cells (a vector-host system): to facilitate the cloning of the nucleotide sequence, i.e. to produce usable quantities of the sequence, to direct the expression of the gene product encoded by the sequence and to integrate the nucleotide sequence into the genome of the host cell. The vector will contain different components depending upon the function it is to perform.

[0049] "Cell", "host cell", "cell line" and "cell culture" are used interchangeably herein and all such terms should be understood to include progeny resulting from growth or culturing of a cell.

[0050] "Transformation" and "transfection" are used interchangeably to refer to the process of introducing DNA into a cell.

[0051] "Operably linked" refers to the covalent joining of two or more nucleotide sequences, by means of enzymatic ligation or otherwise, in a configuration relative to one another such that the normal function of the sequences can be performed. For example, the nucleotide sequence encoding a presequence or secretory leader is operably linked to a nucleotide sequence coding for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide: a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the nucleotide sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, then synthetic oligonucleotide adaptors or linkers are used, in conjunction with standard recombinant DNA methods.

[0052] In the context of the present invention the terms "modification" or "amino acid modification" is intended to cover replacement of an amino acid side chain, substitution of an amino acid residue, deletion of an amino acid residue and/or insertion of an amino acid residue.

[0053] The terms "mutation" and "substitution" are used interchangeably herein.

[0054] The term "introduce" refers to introduction of an amino acid residue by substitution of an existing amino acid residue or, alternatively, by insertion of an additional amino acid residue.

[0055] The term "remove" refers to removal of an amino acid residue by substitution of the amino acid residue to be removed by another amino acid residue or, alternatively, by deletion (without substitution) of the amino acid residue to be removed

[0056] The term "FVII" or "FVII polypeptide" refers to a FVII molecule provided in single chain form. One example of a FVII polypeptide is wild-type human FVII (hFVII) shown in SEQ ID NO:2. It should be understood, however, that the term "FVII polypeptide" also covers hFVII-like molecules, such as fragments or variants of SEQ ID NO:2, in particular variants where the sequence comprises at least one, such as 1-15, e.g., 1-10, amino acid modifications as compared to SEQ ID NO:2.

[0057] The term "FVIIa" or "FVIIa polypeptide" refers to a FVIIa molecule provided in its activated two-chain form. When the amino acid sequence of SEQ ID NO:2 is used to describe the amino acid sequence of FVIIa it will be understood that the peptide bond between R152 and I153 of the single-chain form has been cleaved, and that one of the chains comprises amino acid residues 1-152, the other chain amino acid residues 153-406.

[0058] The terms "rFVII" and "rFVIIa" refer to FVII and FVIIa polypeptides produced by recombinant techniques.

[0059] The terms "hFVII" and "hFVIIa" refer to human wild-type FVII and FVIIa, respectively, having the amino acid sequence shown in SEQ ID NO:2

[0060] The terms "rhFVII" and "rhFVIIa" refer to human wild-type FVII and FVIIa, having the amino acid sequence shown in SEQ ID NO:2, produced by recombinant means. An example of rhFVIIa is NovoSeven®.

[0061] When used herein, the term "Gla domain" is intended to cover amino acid residues no. 1 to 45 of SEQ ID NO:2.

[0062] Accordingly, the term "located outside the Gla domain" covers amino acid residue no. 46-406 of SEQ ID NO:2.

[0063] The abbreviations "TF" and "TFPI" mean Tissue Factor and Tissue Factor Pathway Inhibitor, respectively.

[0064] The term "protease domain" is used about residues 153-406 counted from the N-terminus.

[0065] The term "catalytic site" is used to mean the catalytic triad consisting of S344, D242 and H193 of the polypeptide variant.

[0066] The term "parent" is intended to indicate the molecule to be modified/improved in accordance with the present invention. Although the parent polypeptide to be modified by the present invention may be any FVII or FVIIa polypeptide, and thus be derived from any origin, e.g. a non-human mammalian origin, it is preferred that the parent polypeptide is hFVII or hFVIIa

[0067] A "variant" is a polypeptide, which differs in one or more amino acid residues from its parent polypeptide, normally in 3-15 amino acid residues (e.g. in 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acid residues), such as in 3-10 amino acid residues, e.g. in 3-8 or 3-5 amino acid residues. In other words, a "variant" typically contains 3-15 amino acid modifications (for example 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acid modifications), such as 3-10 amino acid modifications, e.g. 3-8 or 3-5 amino acid modifications relative to the parent polypeptide. In the present context, the term "modification" encompasses insertions, deletions, substitutions and combinations thereof. It will be understood that a polypeptide variant according to the present invention will be modified in at least three positions, namely in at least position 10 and 32 (located in the Gla domain) and in at least one position located outside the Gla domain, where said at least one modification creates an in vivo N-glycosylation site.

[0068] The term "clotting activity" is used to mean the activity measured in the "Clotting Assay" described herein. In order to exhibit "clotting activity" a variant of the invention, in its activated form, should have at least 10% of the clotting acitivty of rhFVIIa when assayed in the "Clotting Assay" described herein. In a preferred embodiment of

the invention the variant, in its activated form, has at least 20% of the clotting activity of rhFVIIa, such as at least 30%, e.g. at least 40%, more preferably at least 50%, such as at least 60%, e.g. at least 70%, even more preferably at least 80%, such as at least 90% of the clotting activity of rhFVIIa when assayed in the "Clotting Assay" described herein. In an interesting embodiment the variant, in its activated form, has substantially the same clotting activity as rhFVIIs, such as a clotting activity of 75-125% of the clotting acitivity of rhFVIIa.

[0069] The term "amidolytic activity" is used to mean the activity measured in the "Amidolytic Assay" described herein. In order to exhibit "amidolytic activity" a variant of the invention, in its activated form, should have at least 10% of the amidolytic acitivty of rhFVIIa when assayed in the "Amidolytic Assay" described herein. In a preferred embodiment of the invention the variant, in its activated form, has at least 20% of the amidolytic activity of rhFVIIa, such as at least 30%, e.g. at least 40%, more preferably at least 50%, such as at least 60%, e.g. at least 70%, even more preferably at least 80%, such as at least 90% of the amidolytic activity of rhFVIIa when assayed in the "Amidolytic Assay" described herein. In an interesting embodiment the variant, in its activated form, has substantially the same amidolytic activity as rhFVIIs, such as an amidolytic activity of 75-125% of the amidolytic acitivity of rhFVIIa.

[0070] In the present context, the term "activity" is also used in connection with the variants capability of activating FX to FXa. This activity is also denoted "FX activation activity" or "FXa generation activity".

[0071] The term "increased FX activiation activity" or "increased FXa generation activity" is used to indicate that a variant of the invention, in its activated form, has a statistically signifycantly increased capability to activate FX to FXa as compared to rhFVIIa or. To what extent a variant of the invention (in its activated form) has an increased FX activation activity may conveniently be determined in the "TF-independent Factor X Activation Assay" described herein.

[0072] The term "stronger clot" or "increased clot strength" is used to indicate that the strength of the clot generated by the polypeptide variant is statistically significantly increased relative to that generated by rhFVIIa as determined under comparable conditions. This effect may be determined as the Area Under the Curve (AUC_{throm}) generated by the variant of the invention, in its activated form, when assayed in the "Thrombogram Assay" disclosed herein. In a similar way, the term "increased AUC_{throm}" is used to indicate that the Area Under the Curve generated by the variant (in its activated form) is statistically significantly increased relative to that generated by rhFVIIa as determined under comparable conditions and when measured in the "Thrombogram Assay" described herein

[0073] The term "T_{max}" is used about the time it takes to obtain the maximum thrombin activity level in the "Thrombogram Assay".

[0074] The term "immunogenicity" as used in connection with a given substance is intended to indicate the ability of the substance to induce a response from the immune system. The immune response may be a cell or antibody mediated response (see, e.g., Roitt: Essential Immunology (8th Edition, Blackwell) for further definition of immunogenicity). Normally, reduced antibody reactivity will be an indication of reduced immunogenicity. The reduced immunogenicity may be determined by use of any suitable method known in the art, e.g. in vivo or in vitro.

[0075] The term "functional in vivo half-life" is used in its normal meaning, i.e. the time at which 50% of the biological activity of the polypeptide is still present in the body/target organ, or the time at which the activity of the polypeptide is 50% of the initial value.

[0076] As an alternative to determining functional in vivo half-life, "serum half-life" may be determined, i.e. the time at which 50% of the polypeptide circulates in the plasma or bloodstream prior to being cleared. Determination of serum half-life is often more simple than determining the functional in vivo half-life and the magnitude of serum half-life is usually a good indication of the magnitude of functional in vivo half-life. Alternatively terms to serum half-life include "plasma half-life", "circulating half-life", "serum clearance", "plasma clearance" and "clearance half-life". The polypeptide is cleared by the action of one or more of the reticuloendothelial systems (RES), kidney, spleen or liver, by tissue factor, SEC receptor or other receptor mediated elimination, or by specific or unspecific proteolysis. Normally, clearance depends on size (relative to the cutoff for glomerular filtration), charge, attached carbohydrate chains, and the presence of cellular receptors for the protein. The functionality to be retained is normally selected from procoagulant, proteolytic or receptor binding activity. The functional in vivo half-life and the serum half-life may be determined by any suitable method known in the art.

[0077] The term "increased" as used about the functional in vivo half-life or serum half-life is used to indicate that the relevant half-life of the polypeptide variant is statistically significantly increased relative to that of rhFVIIa as determined under comparable conditions (typically determined in an experimental animal, such as rats, rabbits, pigs or monkeys).

[0078] The term "AUC_{iv}" or "Area Under the Curve when administered intravenously" is used in its normal meaning, i.e. as the area under the activity in serum-time curve, where the polypeptide variant has been administered intravenously, in particular when administered intravenously in rats. Typically, the activity measured is the "clotting activity" as defined hereinbefore. Once the experimental activity-time points have been determined, the AUC_{iv} may conveniently be calculated by a computer program, such as GraphPad Prism 3.01.

[0079] It will be understood that in order to make a direct comparison between the AUC_{iv}-values of different molecules (e.g. between the variants of the invention and a reference molecule such as rhFVIIa) the same amount of activity should be administered. Consequently, the AUC_{iv}-values are typically normalized (i.e. corrected for differences in the injected dose) and expressed as AUC_{iv}/dose administered.

[0080] The term "reduced sensitivity to proteolytic degradation" is primarily intended to mean that the polypeptide variant has reduced sensitivity to proteolytic degradation in comparison to hFVIIa or rhFVIIa as determined under comparable conditions. Preferably, the proteolytic degrada-

tion is reduced by at least 10% (e.g. by 10-25% or by 10-50%), such as at least 25% (e.g. by 25-50%, by 25-75% or by 25-100%), more preferably by at least 35%, such as at least 50%, (e.g. by 50-75% or by 50-100%) even more preferably by at least 60%, such as by at least 75% (e.g. by 75-100%) or even at least 90%. Most preferably, the proteolytic degradation is reduced by at least 100%.

[0081] The term "renal clearance" is used in its normal meaning to indicate any clearance taking place by the kidneys, e.g. by glomerular filtration, tubular excretion or degradation in the tubular cells. Renal clearance depends on physical characteristics of the polypeptide, including size (diameter), hydrodynamic volume, symmetry, shape/rigidity, and charge. Normally, a molecular weight of about 67 kDa is considered to be a cut-off-value for renal clearance. Renal clearance may be established by any suitable assay, e.g. an established in vivo assay. Typically, renal clearance is determined by administering a labelled (e.g. radiolabelled or fluorescence labelled) polypeptide to a patient and measuring the label activity in urine collected from the patient. Reduced renal clearance is determined relative to a corresponding reference polypeptide, e.g. rhFVIIa, under comparable conditions. Preferably, the renal clearance rate of the polypeptide variant is reduced by at least 50%, preferably by at least 75%, and most preferably by at least 90% compared to rhFVIIa.

[0082] The terms "at least 25% of its side chain exposed to the surface of the molecule" and "at least 50% of its side chain exposed to the surface of the molecule" are defined with reference to Example 1, where the calculations, etc. are described in detail.

[0083] It should be noted that when the terms "at least 25% of its side chain exposed to the surface of the molecule" and "at least 50% of its side chain exposed to the surface of the molecule" are used in connection with introduction of an in vivo N-glycosylation site these terms refer to the surface accessibility of the amino acid side chain in the position where the sugar moiety is actually attached. In many cases it will be necessary to introduce a serine or a threonine residue in position +2 relative to the asparagine residue to which the sugar moiety is actually attached (unless, of course, this position is already occupied by a serine or a threonine residue) and these positions, where the serine or threonine residues are introduced, are allowed to be buried, i.e. to have less than 25% or 50% of their side chains exposed to the surface of the molecule.

[0084] The terms "tissue factor binding site", "active site region" and "ridge of the active site binding cleft" are defined with reference to Example 1 herein, wherein the above-mentioned sites/regions are determined.

[0085] The term "hydrophobic amino acid residue" includes the following amino acid residues: isoleucine (I), leucine (L), methionine (M), valine (V), phenylalanine (F), tyrosine (Y) and tryptophan (W).

[0086] The term "negatively charged amino acid residue" includes the following amino acid residues: Aspartic acid (D) and glutamic acid (E).

[0087] The term "positively charged amino acid residue" includes the following amino acid residues: Lysine (K), arginine (R) and histidine (H).

Variants of the Invention

[0088] In its broadest aspect the present invention relates to a FVII or FVIIa polypeptide variant having an amino acid sequence comprising 3-15 amino acid modifications relative to hFVII or hFVIIa having the amino acid sequence shown in SEQ ID NO:2, wherein said amino acid sequence of the variant comprises an amino acid substitution in position 10 and 32 and wherein a sugar moiety is covalently attached to an introduced in vivo N-glycosylation site located outside the Gla domain.

[0089] The modifications perfomed in the two above-indicated regions of the parent FVII polypeptide serve the following purposes:

[0090] The modifications performed in positions located outside the Gla domain (introduction of in vivo N-glycosylation site(s)) are preferably of such nature that the AUC_{iv}, the functional in vivo half-life and/or the serum half-life of the resulting variant is increased as compared to rhFVIIa.

[0091] The modifications perfomed in the Gla domain of the parent polypeptide are preferably of such nature that an increased phospholipid membrane binding affinity of the resulting molecule is achieved and/or of such nature the resulting molecule has an improved capability to activate FX to FXa and/or of such nature that a stronger clot is formed.

[0092] Without being limited by any particular theory, it is presently believed that the enhanced membrane affinity results in a higher local concentration of the activated polypeptide variants in close proximity to the other coagulation factors, particularly FX. Thus, the rate of activation of FX to FXa will be higher, simply due to a higher molar ratio of the activated FVII variant to FX. The increased activation rate of FX then results in a higher amount of active thrombin, and thus a higher rate of cross-linking of fibrin.

[0093] Thus, in preferred embodiments of the invention the parent FVII or FVIIa polypeptide has been modified so that the resulting activated polypeptide variant has (as compared to rhFVIIa):

[0094] i) an increased bioavailability (AUC_{iv}) and an increased phospholipid membrane binding affinity;

[0095] ii) an increased bioavailability (AUC_{iv}) and an increased capability to activate FX to FXa;

[0096] iii) an increased bioavailability (AUC_{iv}) and is capable of generating a stronger clot (increased AUC_{throm});

[0097] iv) an increased bioavailability (AUC_{iv}) and a reduced T_{max};

[0098] v) an increased functional in vivo half-life and an increased phospholipid membrane binding affinity;

[0099] vi) an increased functional in vivo half-life and an increased capability to activate FX to FXa;

[0100] vii) an increased functional in vivo half-life and is capable of generating a stronger clot (increased AUC_{throm});

[0101] viii) an increased functional in vivo half-life and a reduced T_{max};

[0102] ix) an increased serum half-life and an increased phospholipid membrane binding affinity;

[0103] x) an increased serun half-life and an increased capability to activate FX to FXa;

[0104] xi) an increased serum half-life and is capable of generating a stronger clot (increased AUC_{throm}); and/or

[0105] xii) an increased serum half-life and a reduced T_{max}.

[0106] Consequently, medical treatment with a polypeptide variant according to the invention offers a number of advantages over the currently available rhFVIIa compound (NovoSeven®), such as administration of lower dosage, longer duration between injections, increased clot strength and/or faster action.

[0107] Thus, preferred variants of the invention are such variants which, in their activated forms and when compared to rhFVIIa, generates in increased Area Under the Curve when administered intravenously (AUC_{iv}), in particular when administered intravenously in rats. More particularly, variants of the present invention, which are preferred, are such variants where the ratio between the AUC_{iv} of said variant, in its actvated form, and the AUC_{iv} of rhFVIIa is at least 1.25, such as at least 1.5, e.g. at least 1.75, more preferably at least 2, such as at least 3, even more preferably at least 4, such as at least 5, in particular when administered (intravenously) in rats.

[0108] This effect may in turn (but do not necessarily do so) correspond to an increased functional in vivo half-life and/or an increased serum half-life as compared to rhFVIIa. Accordingly, in another preferred embodiment of the invention, the ratio between the functional in vivo half-life or the serum half-life for the variant, in its activated form, and the functional in vivo half-life or the serum half-life for rhFVIIa is at least 1.25. More preferably, the ratio between the relevant half-life for the variant, in its activated form, and the relevant half-life for hFVIIa or rhFVIIa is at least 1.5, such as at least 1.75, e.g. at least 2, even more preferably at least 3, such as at least 4, e.g. at least 5.

[0109] As will be understood, the variants of the invention also possess, in addition to the above-mentioned functionality (i.e. increased AUC_{iv}, increased functional in vivo half-life and/or increased serum half-life), an increased phospholipid membrane binding affinity as compared to rhFVII, an an increased capability to activate FX to Fxa, a capability of generating a stronger clot (increased AUC_{throm}) and/or a reduced T_{max}.

[0110] Thus, in one preferred embodiment of the invention, the polypeptide variant has (in addition to an increased AUC_{iv}, increased functional in vivo half-life and/or increased serum half-life) an increased phospholipid membrane binding affinity relative to rhFVIIa. Membrane binding affinity may be measured by methods known in the art, such as by the Biacore® assay described in K. Nagata and H. Handa (Ads.), Real-Time Analysis of Biomolecular Interactions, Springer-Verlag, Tokyo, 2000, Chapter 6 entitled "Lipid-Protein Interactions". Alternatively, the membrane binding affinity may be measured as described in Example 1 in WO 99/20767.

[0111] In another preferred embodiment of the invention, the polypeptide variant (in addition to an increased AUC_{iv}, increased functional in vivo half-life and/or increased serum half-life), has an increased FX activation activity as compared to rhFVIIa, in particular when assayed in a TF-independent assay, such as the "TF-independent Factor X Activation Assay" disclosed herein. More particularly, it is preferred that the ratio between the FX activation activity of the polypeptide variant, in its activated form, and the FX activation activity of rhFVIIa is at least 1.25 when assayed in the "TF-independent Factor X Activation Assay" disclosed herein. More preferably, the ratio between the FX activation activity of the variant, in its activated form, and the FX activation activity of rhFVIIa is at least 1.5, such as at least 1.75, e.g. at least 2, even more preferably at least 3, such as at least 4, e.g. at least 5, still more preferably at least 6, such as at least 7, e.g. at least 8, most preferably at least 9, such as at least 10, when assayed in the "TF-independent Factor X Activation Assay" described herein.

[0112] In still another preferred embodiment of the invention, the polypeptide variant is (in addition to an increased AUC_{iv}, increased functional in vivo half-life and/or increased serum half-life), in its activated form, capable of generating a stronger clot as compared to rhFVIIa. This effect may be determined in the "Thrombogram Assay" described herein as an increase in the Area Under the Curve (AUC_{throm}). The AUC_{throm} is also sometimes denoted "total thrombin work" and constitutes a measure for the strength of the clot formed. More particularly, it is preferred that the ratio between the AUC_{throm}, generated by the variant in its activated form, and the AUC_{throm} generated by rhFVIIa is at least 1.15 when assayed in the "Thrombogram Assay" described herein. More preferably, the ratio is at least 1.2, such as at least 1.25, e.g. at least 1.3, even more preferably at least 1.4, such as at least 1.5, e.g. at least 1.6, most preferably at least 1.7, such as at least 1.8, e.g. at least 1.9 or at least 2.

[0113] In even another preferred embodiment of the invention the polypeptide variant has (in addition to an increased AUC_{iv}, increased functional in vivo half-life and/or increased serum half-life), in its activated form, a faster action. This effect may be determined in the "Thrombogram Assay" described herein as a reduction in the time needed to reach maximum thrombin level (T_{max}). Accordingly, preferred variants are such variants where the ratio between T_{max} for the variant, in its activated form, and T_{max} for rhFVIIa is at the most 0.95 when assayed in the "Thrombogram Assay" described herein. Preferably, the ratio is at the most 0.9, such as at the most 0.8, e.g. at the most 0.7, more preferably at the most 0.6, such as at the most 0.5.

Introduction of In Vivo N-glycosylation Sites Located Outside the Gla Domain

[0114] A number of suitable modifications leading to an increase in AUC_{iv}, functional in vivo half-life and/or serum half-life is disclosed in WO 01/58935. The variants disclosed in WO 01/58935 are the result of a generally new strategy for developing improved FVII or FVIIa molecules, which may also be used for the parent FVII or FVIIa polypeptide of the present invention.

[0115] The position to be modified is preferably selected from a part of the FVII or FVIIa molecule that is located outside the tissue factor binding site, and/or outside the active site region, and/or outside the ridge of the active site binding cleft. These sites/regions are identified in Example 1 herein. It should be emphasized, however, that in certain situations, e.g. in case an inactivated polypeptide variant is

desired, it may be advantageous to perform modifications in or close to such regions. For example, it is contemplated that one or more in vivo N-glycosylation sites may advantageously be introduced in the active site region or at the ridge of the active site binding cleft of the FVII or FVIIa molecule. The active site region, the tissue factor binding site and the ridge of the active site binding cleft are defined in Example 1 herein and are constituted by the following residues:

[0116] I153, Q167, V168, L169, L170, L171, Q176, L177, C178, G179, G180, T181, V188, V189, S190, A191, A192, H193, C194, F195, D196, K197, I198, W201, V228, I229, I230, P231, S232, T233, Y234, V235, P236, G237, T238, T239, N240, H241, D242, I243, A244, L245, L246, V281, S282, G283, W284, G285, Q286, T293, T324, E325, Y326, M327, F328, D338, S339, C340, K341, G342, D343, S344, G345, G346, P347, H348, L358, T359, G360, 1361, V362, S363, W364, G365, C368, V376, Y377, T378, R379, V380, Q382, Y383, W386, L387, L400 and F405 (active site region);

[0117] L13, K18, F31, E35, R36, L39, F40, I42, S43, S60, K62, D63, Q64, L65, I69, C70, F71, C72, L73, P74, F76, E77, G78, R79, E82, K85, Q88, I90, V92, N93, E94, R271, A274, F275, V276, R277, F278, R304, L305, M306, T307, Q308, D309, Q312, Q313, E325 and R379 (tissue factor binding site); and

[0118] N173, A175, K199, N200, N203, D289, R290, G291, A292, P321 and T370 (the ridge of the active site binding cleft).

[0119] The total number of amino acid residues to be modified outside the Gla domain in the parent FVII or FVIIa polypeptide (as compared to the amino acid sequence shown in SEQ ID NO:2) will typically not exceed 10. Preferably, the FVII or FVIIa variant comprises an amino acid sequence which differs in 1-10 amino acid residues from amino acid residues 46-406 shown in SEQ ID NO:2, typically in 1-8 or in 2-8 amino acid residues, e.g. in 1-5 or in 2-5 amino acid residues, such as in 1-4 or in 1-3 amino acid residues, e.g. in 1, 2 or 3 amino acid residues from amino acid residues 46-406 shown in SEQ ID NO:2.

[0120] Thus, the polypeptide variant of the invention may contain 1-10 (additional or introduced) in vivo N-glycosylation sites, typically 1-8 or 2-8 (additional or introduced) in vivo N-glycosylation sites, preferably 1-5 or 2-5 (additional or introduced) in vivo N-glycosylation sites, such as 1-4 or 1-3 (additional or introduced) in vivo N-glycosylation sites, e.g. 1, 2 or 3 (additional or introduced) in vivo N-glycosylation sites. Analogously, the polypeptide variant of the invention may contain 1-10 (additional or introduced) sugar moieties, typically 1-8 or 2-8 (additional or introduced) sugar moieties, preferably 1-5 or 2-5 (additional or introduced) sugar moieties, such as 1-4 or 1-3 (additional or introduced) sugar moieties, e.g. 1, 2 or 3 (additional or introduced) sugar moieites. It will be understood that the introduced sugar moiety/moieties will be covalently attached to the introduced in vivo N-glycosylation site(s)

[0121] When used in the present context, the term "naturally occurring glycosylation site" covers the glycosylation sites at postions N145, N322, S52 and S60. In a similar way, the term "naturally occurring in vivo O-glycosylation site" includes the positions S52 and S60, whereas the term "naturally occurring in vivo N-glycosylation site" includes positions N145 and N322.

[0122] It will be understood that in order to prepare a polypeptide variant, wherein the polypeptide variant comprises one or more sugar moieties covalently attached to one or more in vivo N-glycosylation sites, the polypeptide variant must be expressed in a host cell capable of attaching sugar (oligosaccharide) moieties at the glycosylation site(s) or alternatively subjected to in vitro glycosylation. Examples of glycosylating host cells are given in the section further below entitled "Coupling to a sugar moiety".

[0123] Examples of positions, wherein the in vivo N-glycosylation sites may be introduced include, but is not limited to, positions comprising an amino acid residue having an amino acid residue having at least 25% of its side chain exposed to the surface (as defined in Example 1 herein), such as in a position comprising an amino acid residue having at least 50% of its side chain exposed to the surface (as defined in Example 1 herein). In general, it is preferred that the in vivo N-glycosylation site is introduced by substitution, although insertion is also contemplated. The position is preferably selected from a part of the molecule that is located outside the tissue factor binding site and/or the active site region and/or outside the ridge of the active site cleft. These sites/regions are identified in Example 1 herein. It should be understood that when the term "at least 25% (or at least 50%) of its side chain exposed to the surface" is used in connection with introduction of an in vivo N-glycosylation site this term refers to the surface accessibility of the amino acid side chain in the position where the sugar moiety is actually attached. In many cases it will be necessary to introduce a serine or a threonine residue in position +2 relative to the asparagine residue to which the sugar moiety is actually attached (unless, of course, this position is already occupied by a serine or a threonine residue) and these positions, where the serine or threonine residues are introduced, are allowed to be buried, i.e. to have less than 25% of their side chains exposed to the surface.

[0124] Specific and preferred examples of such substitutions creating an in vivo N-glycosylation site include a substitution selected from the group consisting of A51N, G58N, T106N, K109N, G124N, K143N+N145T, A175T, I205S, I205T, V253N, T267N, T267N+S269T, S314N+K316S, S314N+K316T, R315N+V317S, R315N+V317T, K316N+G318S, K316N+G318T, G318N, D334N and combinations thereof. More preferably, the in vivo N-glycosylation site is introduced by a substitution selected from the group consisting of A51N, G58N, T106N, K109N, G124N, K143N+N145T, A175T, I205T, V253N, T267N+S269T, S314N+K316T, R315N+V317T, K316N+G318T, G318N, D334N and combinations thereof. Even more preferably, the in vivo N-glycosylation site is introduced by a substitution selected from the group consisting of T106N, A175T, I205T, V253N, T267N+S269T and combinations thereof, in particular I205T.

[0125] In one embodiment, only one in vivo N-glycosylation site has been introduced by substitution. In another embodiment, two or more (such as two) in vivo N-glycosylation sites have been introduced by substitution. Examples of preferred substitutions creating two in vivo N-glycosylation sites include substitutions selected from the group consisting of A51N+G58N, A51N+T106N, A51N+K109N, A51N+G124N, A51N+K143N+N145T, A51N+A175T, A51N+I205T, A51N+V253N, A51N+T267N+S269T, A51N+S314N+K316T, A51N+R315N+V317T,

A51N+K316N+G318T, A51N+G318N, A51N+D334N, G58N+T106N, G58N+K109N, G58N+G124N, G58N+K143N+N145T, G58N+A175T, G58N+I205T, G58N+V253N, G58N+T267N+S269T, G58N+S314N+K316T, G58N+R315N+V317T, G58N+K316N+G318T, G58N+G318N, G58N+D334N, T106N+K109N, T106N+G124N, T106N+K143N+N145T, T106N+A175T, T106N+I205T, T106N+V253N, T106N+T267N+S269T, T106N+S314N+K316T, T106N+R315N+V317T, T106N+K316N+G318T, T106N+G318N, T106N+D334N, K109N+G124N, K109N+K143N+N145T, K109N+A175T, K109N+I205T, K109N+V253N, K109N+T267N+S269T, K109N+S314N+K316T, K109N+R315N+V317T, K109N+K316N+G318T, K109N+G318N, K109N+D334N, G124N+K143N+N145T, G124N+A175T, G124N+I205T, G124N+V253N, G124N+T267N+S269T, G124N+S314N+K316T, G124N+R315N+V317T, G124N+K316N+G318T, G124N+G318N, G124N+D334N, K143N+N145T+A175T, K143N+N145T+I205T, K143N+N145T+V253N, K143N+N145T+T267N+S269T, K143N+N145T+S314N+K316T, K143N+N145T+R315N+V317T, K143N+N145T+K316N+G318T, K143N+N145T+G318N, K143N+N145T+D334N, A175T+I205T, A175T+V253N, A175T+T267N+S269T, A175T+S314N+K316T, A175T+R315N+V317T, A175T+K316N+G318T, A175T+G318N, A175T+D334N, I205T+V253N, I205T+T267N+S269T, I205T+S314N+K316T, I205T+R315N+V317T, I205T+K316N+G318T, I205T+G318N, I205T+D334N, V253N+T267N+S269T, V253N+S314N+K316T, V253N+R315N+V317T, V253N+K316N+G318T, V253N+G318N, V253N+D334N, T267N+S269T+S314N+K316T, T267N+S269T+R315N+V317T, T267N+S269T+K316N+G318T, T267N+S269T+G318N, T267N+S269T+D334N, S314N+K316T+R315N+V317T, S314N+K316T+G318N, S314N+K316T+D334N, R315N+V317T+K316N+G318T, R315N+V317T+G318N, R315N+V317T+D334N and G318N+D334N. More preferably, the substitutions are selected from the group consisiting of T106N+A175T, T106N+I205T, T106N+V253N, T106N+T267N+S269T, A175T+I205T, A175T+V253N, A175T+T267N+S269T, I205T+V253N, I205T+T267N+S269T and V253N+T267N+S269T, even more preferably from the group consisiting of T106N+I205T, T106N+V253N and I205T+T267N+S269T.

[0126] In an even further embodiment, three or more (such as three) in vivo N-glycosylation sites have been introduced by substitution. Examples of preferred substitutions creating three in vivo N-glycosylation sites include substitutions selected from the group consisiting of I205T+V253N+T267N+S269T and T106N+I205T+V253N.

[0127] As discussed above, it is preferred that the in vivo N-glycosylation site is introduced in a position which does neither form part of the tissue factor binding site nor form part of the active site region and the ridge of the active site binding cleft as defined herein. It is envisaged that such glycosylation variants will primarily belong to the class of active polypeptide variants as defined hereinbefore.

[0128] It will be understood that as an alternative to introduction of in vivo N-glycosylation sites in the above-discussed positions one may introduce (either by substitution or by insertion) cysteine residues in the same positions, where the introduced cysteine residue is then subsequently covalently attached to a non-polypeptide moiety, such as PEG, in particular mPEG. Thus, examples of positions, wherein a cysteine residue may be introduced include, but is not limited to, positions comprising an amino acid residue having an amino acid residue having at least 25% of its side chain exposed to the surface (as defined in Example 1 herein), such as in a position comprising an amino acid residue having at least 50% of its side chain exposed to the surface (as defined in Example 1 herein). The position is preferably selected from a part of the molecule that is located outside the tissue factor binding site and/or the active site region and/or outside the ridge of the active site cleft. These sites/regions are identified in Example 1 herein. Thus, the above disclosure concerning introduction of in vivo N-glycosylation sites applies mutatis mutandis to introduction of cysteine residues.

[0129] It will be understood that the modifications in positions located outside the Gla domain discussed in the above section should be combined with one or more modifications in the Gla domain (see the section entitled "Modifications in the Gla domain" below).

Modifications in the Gla Domain

[0130] As will be understood the variants of the present invention comprises, in addition to at least one introduced in vivo N-glycosylation site located outside the Gla domain (cf. above), at least two substitutions in the Gla domain, namely a substitution in position 10 and position 32.

[0131] A number of suitable modifications leading to increased phospholipid membrane binding affinity is disclosed in WO 99/20767 and WO 00/66753.

[0132] In a preferred embodiment of the invention the substitution in position 10 is P10Q. In another preferred embodiment of the invention the substitution in position 32 is K32E. In a particularly preferred embodiment of the invention the variant comprises the following substitutions P10Q+K32E.

[0133] In an interesting embodiment of the invention the variant comprises, in addition to the substitutions in position 10 and 32, such as in addition to the substitutions P10Q+K32E, at least one further modification in the Gla domain.

[0134] In one preferred embodiment of the invention the further modification in the Gla domain comprises an amino acid substitution in position 33. Preferably, a hydrophobic amino acid residue is introduced by substitution in position 33, such as D33I, D33L, D33M, D33V, D33F, D33Y or D33W, in particular D33F. Accordingly, in one very interesting embodiment of the invention, the variant comprises the following substitutions P10Q+K32+D33F.

[0135] In another preferred embodiment of the invention the further modification in the Gla domain comprises an insertion of at least one (such as one) amino acid residue between position 3 and 4. It is preferred that the inserted amino acid residue is a hydrophobic amino acid residue. Most preferably the insertion is A3AY. Accordingly, in another very interesting embodiment of the invention, the variant comprises the following modifications A3AY+P10Q+K32E or A3AY+P10Q+K32E+D33F.

[0136] In still another preferred embodiment of the invention the further modification in the Gla domain comprises a substitution in position 34. It is preferred that a negatively charged amino acid residue is introduced by substitution in position 34. Most preferably the substitution is A34E. Accordingly, in still another very interesting embodiment of

the invention, the variant comprises the following modifications P10Q+K32E+A34E, P10Q+K32E+D33F+A34E, A3AY+P10Q+K32E+A34E or A3AY+P10Q+K32E+D33F+A34E.

[0137] The Gla domain may also contain modifications in other positions, in particular in positions 8, 11 and 28, such as R28F or R28E. On the other hand it should be understood that the Gla domain should not be modified to such an extent that the membrane binding properties are impaired. Accordingly, it is preferred that no modifications are made in the residues that become 7-carboxylated, i.e. it is preferred that no modifications are made in residues 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35. In a similar way, it is in general not preferred that non-polypeptide moieties, such as sugar moieties and/or PEG groups, are introduced in the Gla domain. Consequently, it is preferred that no modifications are made in the Gla domain that creates an in vivo N-glycosylation site.

[0138] Finally, it will be understood that the modifications in the Gla domain discussed in this section must be combined with one or more of the modifications disclosed in the section entitled "Introduction of in vivo N-glycosylation sites located outside the Gla domain" above.

[0139] Specific examples of such "combined" variants are given below.

[0140] In one embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+T106N.

[0141] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A175T.

[0142] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+I205T.

[0143] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+V253N.

[0144] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+T267+S269T.

[0145] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+T106N+I205T.

[0146] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+T106N+V253N.

[0147] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+I205T+T267N+S269T. In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+T106N.

[0148] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A175T.

[0149] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+I205T.

[0150] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+V253N.

[0151] In a further preferred embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+T267+S269T.

[0152] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+T106N+I205T.

[0153] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+T106N+V253N.

[0154] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+I205T+T267N+S269T.

[0155] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+T106N.

[0156] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A175T.

[0157] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+I205T.

[0158] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+V253N.

[0159] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+T267+S269T.

[0160] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+T106N+I205T.

[0161] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+T106N+V253N.

[0162] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+I205T+T267N+S269T.

[0163] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+T106N.

[0164] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A175T.

[0165] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+T205T.

[0166] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+V253N.

[0167] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+T267+S269T.

[0168] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+T106N+I205T.

[0169] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+T106N+V253N.

[0170] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+I205T+T267N+S269T.

[0171] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+T106N.

[0172] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+A175T.

[0173] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+I205T.

[0174] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+V253N.

[0175] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+T267+S269T.

[0176] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+T106N+I205T.

[0177] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+T106N+V253N.

[0178] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+I205T+T267N+S269T.

[0179] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E+T106N.

[0180] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E A175T.

[0181] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E I205T.

[0182] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E V253N.

[0183] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E T267+S269T.

[0184] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E T106N+I205T.

[0185] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E T106N+V253N.

[0186] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E I205T+T267N+S269T.

[0187] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E+T106N.

[0188] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E A175T.

[0189] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E I205T.

[0190] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E V253N.

[0191] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E T267+S269T.

[0192] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E T106N+I205T.

[0193] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E T106N+V253N.

[0194] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E I205T+T267N+S269T.

[0195] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E+T106N.

[0196] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E A175T.

[0197] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E I205T.

[0198] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E V253N.

[0199] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E T267+S269T.

[0200] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E T106N+I205T.

[0201] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E T106N+V253N.

[0202] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E I205T+T267N+S269T.

Other Modifications Outside the Gla Domain

[0203] In a further embodiment of the present invention, the FVII or FVIIa variant may, in addition to the modifications described in the sections above, also contain mutations,

which are already known to increase the intrinsic activity of the polypeptide, e.g. such as those described in WO 02/22776

[0204] Examples of preferred substitutions include substitutions selected from the group consisting of V158D, E296D, M298Q, L305V and K337A. More preferably, said substitutions are selected from the group consisting of V158D+E296D+M298Q+L305V+K337A, V158D+E296D+M298Q+K337A, V158D+E296D+M298Q+L305V, V158D+E296D+M298Q, M298Q, L305V+K337A, L305V and K337A.

[0205] In a further embodiment of the present invention, the FVII or FVIIa variant may, in addition to the modifications described in the sections above, also contain mutations, which are already known to cause a decreased inhibition by TFPI. One example includes the substitution K341Q disclosed by Neuenschwander et al, Biochemistry, 1995; 34:8701-8707.

[0206] Moreover, the variant may contain modifications which are belived to increase the TF binding affinity. Examples of such modifications include substitutions selected from the group consisting of L39E, L39Q, L39H, I42K, I42R, S43H, S43Q, K62E, K62R, L65Q, L65S, F71D, F71Y, F71E, F71Q, F71N, E82Q, E82N, E82K and F275H

[0207] As already indicated above, the variant may also contain conservative amino acid substitutions.

The Non-Polypeptide Moiety

[0208] Based on the present disclosure the skilled person will be aware that amino acid residues comprising other attachment groups may be introduced by substitution into the parent polypeptide, using the same approach as that illustrated above with in vivo N-glycosylation sites. For instance, one or more amino acid residues comprising an acid group (glutamic acid or aspartic acid), tyrosine or lysine may be introduced into the positions discussed above. In particular, one or more cysteine residues may be introduced in the positions discussed above.

[0209] As indicated further above the non-polypeptide moiety of the conjugated variant is preferably selected from the group consisting of a polymer molecule, a lipophilic compound, a sugar moiety (by way of in vivo glycosylation) and an organic derivatizing agent. All of these agents may confer desirable properties to the variant polypeptide, in particular increased AUC_{iv}, increased functional in vivo half-life and/or increased plasma half-life. The variant polypeptide is normally conjugated to only one type of non-polypeptide moiety, but may also be conjugated to two or more different types of non-polypeptide moieties, e.g. to a polymer molecule and a sugar moiety, to a lipophilic group and a sugar moiety, to an organic derivatizing agent and a sugar moiety, to a lipophilic group and a polymer molecule, etc. The conjugation to two or more different non-polypeptide moieties may be done simultaneous or sequentially.

Methods of Preparing a Conjugated Variant of the Invention

[0210] In the following sections "Conjugation to a lipophilic compound", "Conjugation to a polymer molecule", "Conjugation to a sugar moiety" and "Conjugation to an organic derivatizing agent" conjugation to specific types of non-polypeptide moieties is described. In general, a conjugated variant according to the invention may be produced by culturing an appropriate host cell under conditions conducive for the expression of the variant polypeptide, and recovering the variant polypeptide, wherein a) the variant polypeptide comprises at least one N- or O-glycosylation site and the host cell is an eukaryotic host cell capable of in vivo glycosylation, and/or b) the variant polypeptide is subjected to conjugation to a non-polypeptide moiety in vitro.

[0211] It will be understood that the conjugation should be designed so as to produce the optimal molecule with respect to the number of non-polypeptide moieties attached, the size and form of such molecules (e.g. whether they are linear or branched), and the attachment site(s) in the polypeptide. The molecular weight of the non-polypeptide moiety to be used may, e.g., be chosen on the basis of the desired effect to be achieved. For instance, if the primary purpose of the conjugation is to achieve a conjugated variant having a high molecular weight (e.g. to reduce renal clearance) it is usually desirable to conjugate as few high molecular weight non-polypeptide moieties as possible to obtain the desired molecular weight. When a high degree of shielding is desirable this may be obtained by use of a sufficiently high number of low molecular weight non-polypeptide moieties (e.g. with a molecular weight of from about 300 Da to about 5 kDa, such as a molecular weight of from 300 Da to 2 kDa).

Conjugation to a Polymer Molecule

[0212] The polymer molecule to be coupled to the variant polypeptide may be any suitable polymer molecule, such as a natural or synthetic homo-polymer or hetero-polymer, typically with a molecular weight in the range of about 300-100,000 Da, such as about 500-20,000 Da, more preferably in the range of about 500-15,000 Da, even more preferably in the range of about 2-12 kDa, such as in the range of about 3-10 kDa. When the term "about" is used herein in connection with a certain molecular weight, the word "about" indicates an approximate average molecular weight and reflects the fact that there will normally be a certain molecular weight distribution in a given polymer preparation.

[0213] Examples of homo-polymers include a polyol (i.e. poly-OH), a polyamine (i.e. poly-NH_2) and a polycarboxylic acid (i.e. poly-COOH). A hetero-polymer is a polymer comprising different coupling groups, such as a hydroxyl group and an amine group.

[0214] Examples of suitable polymer molecules include polymer molecules selected from the group consisting of polyalkylene oxide (PAO), including polyalkylene glycol (PAG), such as polyethylene glycol (PEG) and polypropylene glycol (PPG), branched PEGs, poly-vinyl alcohol (PVA), poly-carboxylate, poly-(vinylpyrolidone), polyethylene-co-maleic acid anhydride, polystyrene-co-maleic acid anhydride, dextran, including carboxymethyl-dextran, or any other biopolymer suitable for reducing immunogenicity and/or increasing functional in vivo half-life and/or serum half-life. Another example of a polymer molecule is human albumin or another abundant plasma protein. Generally, polyalkylene glycol-derived polymers are biocompatible, non-toxic, non-antigenic, non-immunogenic, have various water solubility properties, and are easily excreted from living organisms.

[0215] PEG is the preferred polymer molecule, since it has only few reactive groups capable of cross-linking compared

to, e.g., polysaccharides such as dextran. In particular, mono-functional PEG, e.g. methoxypolyethylene glycol (mPEG), is of interest since its coupling chemistry is relatively simple (only one reactive group is available for conjugating with attachment groups on the polypeptide). Consequently, as the risk of cross-linking is eliminated, the resulting conjugated variants are more homogeneous and the reaction of the polymer molecules with the variant polypeptide is easier to control.

[0216] To effect covalent attachment of the polymer molecule(s) to the variant polypeptide, the hydroxyl end groups of the polymer molecule must be provided in activated form, i.e. with reactive functional groups (examples of which include primary amino groups, hydrazide (HZ), thiol, succinate (SUC), succinimidyl succinate (SS), succinimidyl succinamide (SSA), succinimidyl propionate (SPA), succinimidyl butyrate (SBA), succinimidy carboxymethylate (SCM), benzotriazole carbonate (BTC), N-hydroxysuccinimide (NHS), aldehyde, nitrophenylcarbonate (NPC), and tresylate (TRES)). Suitable activated polymer molecules are commercially available, e.g. from Shearwater Polymers, Inc., Huntsville, Ala., USA, or from PolyMASC Pharmaceuticals plc, UK.

[0217] Alternatively, the polymer molecules can be activated by conventional methods known in the art, e.g. as disclosed in WO 90/13540. Specific examples of activated linear or branched polymer molecules for use in the present invention are described in the Shearwater Polymers, Inc. 1997 and 2000 Catalogs (Functionalized Biocompatible Polymers for Research and pharmaceuticals, Polyethylene Glycol and Derivatives, incorporated herein by reference).

[0218] Specific examples of activated PEG polymers include the following linear PEGs: NHS-PEG (e.g. SPA-PEG, SSPA-PEG, SBA-PEG, SS-PEG, SSA-PEG, SC-PEG, SG-PEG, and SCM-PEG), and NOR-PEG, BTC-PEG, EPOX-PEG, NCO-PEG, NPC-PEG, CDI-PEG, ALD-PEG, TRES-PEG, VS-PEG, IODO-PEG, and MAL-PEG, and branched PEGs such as PEG2-NHS and those disclosed in U.S. Pat. No. 5,932,462 and U.S. Pat. No. 5,643,575, both of which are incorporated herein by reference. Furthermore, the following publications, incorporated herein by reference, disclose useful polymer molecules and/or PEGylation chemistries: U.S. Pat. No. 5,824,778, U.S. Pat. No. 5,476,653, WO 97/32607, EP 229,108, EP 402,378, U.S. Pat. No. 4,902,502, U.S. Pat. No. 5,281,698, U.S. Pat. No. 5,122,614, U.S. Pat. No. 5,219,564, WO 92/16555, WO 94/04193, WO 94/14758, WO 94/17039, WO 94/18247, WO 94/28024, WO 95/00162, WO 95/11924, WO95/13090, WO 95/33490, WO 96/00080, WO 97/18832, WO 98/41562, WO 98/48837, WO 99/32134, WO 99/32139, WO 99/32140, WO 96/40791, WO 98/32466, WO 95/06058, EP 439 508, WO 97/03106, WO 96/21469, WO 95/13312, EP 921 131, U.S. Pat. No. 5,736,625, WO 98/05363, EP 809 996, U.S. Pat. No. 5,629,384, WO 96/41813, WO 96/07670, U.S. Pat. No. 5,473,034, U.S. Pat. No. 5,516,673, EP 605 963, U.S. Pat. No. 5,382,657, EP 510 356, EP 400 472, EP 183 503 and EP 154 316.

[0219] Specific examples of activated PEG polymers particularly preferred for coupling to cysteine residues, include the following linear PEGs: vinylsulfone-PEG (VS-PEG), preferably vinylsulfone-mPEG (VS-mPEG); maleimide-PEG (MAL-PEG), preferably maleimide-mPEG (MAL-mPEG) and orthopyridyl-disulfide-PEG (OPSS-PEG), preferably orthopyridyl-disulfide-mPEG (OPSS-mPEG). Typically, such PEG or mPEG polymers will have a size of about 5 kDa, about 10 kD, about 12 kDa or about 20 kDa.

[0220] The conjugation of the polypeptide variant and the activated polymer molecules is conducted by use of any conventional method, e.g. as described in the following references (which also describe suitable methods for activation of polymer molecules): Harris and Zalipsky, eds., Poly(ethylene glycol) Chemistry and Biological Applications, AZC, Washington; R. F. Taylor, (1991), "Protein immobilisation. Fundamental and applications", Marcel Dekker, N.Y.; S. S. Wong, (1992), "Chemistry of Protein Conjugation and Crosslinking", CRC Press, Boca Raton; G. T. Hermanson et al., (1993), "Immobilized Affinity Ligand Techniques", Academic Press, N.Y.).

[0221] The skilled person will be aware that the activation method and/or conjugation chemistry to be used depends on the attachment group(s) of the variant polypeptide (examples of which are given further above), as well as the functional groups of the polymer (e.g. being amine, hydroxyl, carboxyl, aldehyde, sulfydryl, succinimidyl, maleimide, vinysulfone or haloacetate). The PEGylation may be directed towards conjugation to all available attachment groups on the variant polypeptide (i.e. such attachment groups that are exposed at the surface of the polypeptide) or may be directed towards one or more specific attachment groups, e.g. the N-terminal amino group as described in U.S. Pat. No. 5,985,265 or to cysteine residues. Furthermore, the conjugation may be achieved in one step or in a stepwise manner (e.g. as described in WO 99/55377).

[0222] For PEGylation to cysteine residues (see above) the FVII or FVIIa variant is usually treated with a reducing agent, such as dithiothreitol (DDT) prior to PEGylation. The reducing agent is subsequently removed by any conventional method, such as by desalting. Conjugation of PEG to a cysteine residue typically takes place in a suitable buffer at pH 6-9 at temperatures varying from 4° C. to 25° C. for periods up to 16 hours.

[0223] It will be understood that the PEGylation is designed so as to produce the optimal molecule with respect to the number of PEG molecules attached, the size and form of such molecules (e.g. whether they are linear or branched), and the attachment site(s) in the variant polypeptide. The molecular weight of the polymer to be used may e.g. be chosen on the basis of the desired effect to be achieved.

[0224] In connection with conjugation to only a single attachment group on the protein (e.g. the N-terminal amino group), it may be advantageous that the polymer molecule, which may be linear or branched, has a high molecular weight, preferably about 10-25 kDa, such as about 15-25 kDa, e.g. about 20 kDa.

[0225] Normally, the polymer conjugation is performed under conditions aimed at reacting as many of the available polymer attachment groups with polymer molecules. This is achieved by means of a suitable molar excess of the polymer relative to the polypeptide. Typically, the molar ratios of activated polymer molecules to polypeptide are up to about 1000-1, such as up to about 200-1, or up to about 100-1. In some cases the ration may be somewhat lower, however, such as up to about 50-1, 10-1, 5-1, 2-1 or 1-1 in order to obtain optimal reaction.

[0226] It is also contemplated according to the invention to couple the polymer molecules to the polypeptide through a linker. Suitable linkers are well known to the skilled person. A preferred example is cyanuric chloride (Abuchowski et al., (1977), J. Biol. Chem., 252, 3578-3581; U.S. Pat. No. 4,179,337; Shafer et al., (1986), J. Polym. Sci. Polym. Chem. Ed., 24, 375-378).

[0227] Subsequent to the conjugation, residual activated polymer molecules are blocked according to methods known in the art, e.g. by addition of primary amine to the reaction mixture, and the resulting inactivated polymer molecules are removed by a suitable method.

[0228] It will be understood that depending on the circumstances, e.g. the amino acid sequence of the variant polypeptide, the nature of the activated PEG compound being used and the specific PEGylation conditions, including the molar ratio of PEG to polypeptide, varying degrees of PEGylation may be obtained, with a higher degree of PEGylation generally being obtained with a higher ratio of PEG to variant polypeptide. The PEGylated variant polypeptides resulting from any given PEGylation process will, however, normally comprise a stochastic distribution of conjugated polypeptide variants having slightly different degrees of PEGylation.

Coupling to a Sugar Moiety

[0229] In order to achieve in vivo glycosylation of a FVII molecule comprising one or more glycosylation sites the nucleotide sequence encoding the variant polypeptide must be inserted in a glycosylating, eucaryotic expression host. The expression host cell may be selected from fungal (filamentous fungal or yeast), insect or animal cells or from transgenic plant cells. In one embodiment the host cell is a mammalian cell, such as a CHO cell, BHK or HEK, e.g. HEK 293, cell, or an insect cell, such as an SF9 cell, or a yeast cell, e.g. *S. cerevisiae* or *Pichia pastoris*, or any of the host cells mentioned hereinafter.

[0230] Covalent in vitro coupling of sugar moieties (such as dextran) to amino acid residues of the variant polypeptide may also be used, e.g. as described, for example in WO 87/05330 and in Aplin etl al., CRC Crit Rev. Biochem, pp. 259-306,1981. The in vitro coupling of sugar moieties or PEG to protein- and peptide-bound Gln-residues can be carried out by transglutaminases (TGases). Transglutaminases catalyse the transfer of donor amine-groups to protein- and peptide-bound Gln-residues in a so-called cross-linking reaction. The donor-amine groups can be protein- or peptide-bound, such as the s-amino-group in Lys-residues or it can be part of a small or large organic molecule. An example of a small organic molecule functioning as amino-donor in TGase-catalysed cross-linking is putrescine (1,4-diaminobutane). An example of a larger organic molecule functioning as amino-donor in TGase-catalysed cross-linking is an amine-containing PEG (Sato et al., 1996, Biochemistry 35, 13072-13080).

[0231] TGases, in general, are highly specific enzymes, and not every Gln-residues exposed on the surface of a protein is accessible to TGase-catalysed cross-linking to amino-containing substances. On the contrary, only few Gln-residues are naturally functioning as TGase substrates but the exact parameters governing which Gln-residues are good TGase substrates remain unknown. Thus, in order to render a protein susceptible to TGase-catalysed cross-linking reactions it is often a prerequisite at convenient positions to add stretches of amino acid sequence known to function very well as TGase substrates. Several amino acid sequences are known to be or to contain excellent natural TGase substrates e.g. substance P, elafin, fibrinogen, fibronectin, α_2-plasmin inhibitor, α-caseins, and β-caseins.

Conjugation to an Organic Derivatizing Agent

[0232] Covalent modification of the variant polypeptide may be performed by reacting one or more attachment groups of the variant polypeptide with an organic derivatizing agent. Suitable derivatizing agents and methods are well known in the art. For example, cysteinyl residues most commonly are reacted with α-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, α-bromo-β-(4-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole. Histidyl residues are derivatized by reaction with diethylpyrocarbonateat pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful. The reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0. Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing α-amino-containing residues include imidoesters such as methyl picolinimidate, pyridoxal phosphate, pyridoxal, chloroborohydride, trinitrobenzenesulfonic acid, O-methylisourea, 2,4-pentanedione and transaminase-catalyzed reaction with glyoxylate. Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pKa of the guanidine functional group.

[0233] Furthermore, these reagents may react with the groups of lysine as well as the arginine guanidino group. Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R—N═C═N—R'), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl)carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Conjugation to a Lipophilic Compound

[0234] The variant polypeptide and the lipophilic compound may be conjugated to each other, either directly or by use of a linker. The lipophilic compound may be a natural compound such as a saturated or unsaturated fatty acid, a fatty acid diketone, a terpene, a prostaglandin, a vitamine, a carotenoide or steroide, or a synthetic compound such as a carbon acid, an alcohol, an amine and sulphonic acid with one or more alkyl-, aryl-, alkenyl- or other multiple unsaturated compounds. The conjugation between the variant polypeptide and the lipophilic compound, optionally

through a linker may be done according to methods known in the art, e.g. as described by Bodanszky in Peptide Synthesis, John Wiley, New York, 1976 and in WO 96/12505.

Methods of Preparing a Polypeptide Variant of the Invention

[0235] The polypeptide variant of the present invention may be produced by any suitable method known in the art. Such methods include constructing a nucleotide sequence encoding the polypeptide variant and expressing the sequence in a suitable transformed or transfected host. Preferably, the host cell is a gammacarboxylating host cell such as a mammalian cell. However, polypeptide variants of the invention may be produced, albeit less efficiently, by chemical synthesis or a combination of chemical synthesis or a combination of chemical synthesis and recombinant DNA technology.

[0236] A nucleotide sequence encoding a polypeptide of the invention may be constructed by isolating or synthesizing a nucleotide sequence encoding the parent FVII, such as hFVII with the amino acid sequence shown in SEQ ID NO:2 and then changing the nucleotide sequence so as to effect introduction (i.e. insertion or substitution) or removal (i.e. deletion or substitution) of the relevant amino acid residue(s).

[0237] The nucleotide sequence is conveniently modified by site-directed mutagenesis in accordance with conventional methods. Alternatively, the nucleotide sequence is prepared by chemical synthesis, e.g. by using an oligonucleotide synthesizer, wherein oligonucleotides are designed based on the amino acid sequence of the desired polypeptide, and preferably selecting those codons that are favored in the host cell in which the recombinant polypeptide will be produced. For example, several small oligonucleotides coding for portions of the desired polypeptide may be synthesized and assembled by PCR, ligation or ligation chain reaction (LCR) (Barany, PNAS 88:189-193, 1991). The individual oligonucleotides typically contain 5' or 3' overhangs for complementary assembly.

[0238] Alternative nucleotide sequence modification methods are available for producing polypeptide variants for high throughput screening, for instance methods which involve homologous cross-over such as disclosed in U.S. Pat. No. 5,093,257, and methods which involve gene shuffling, i.e. recombination between two or more homologous nucleotide sequences resulting in new nucleotide sequences having a number of nucleotide alterations when compared to the starting nucleotide sequences. Gene shuffling (also known as DNA shuffling) involves one or more cycles of random fragmentation and reassembly of the nucleotide sequences, followed by screening to select nucleotide sequences encoding polypeptides with desired properties. In order for homology-based nucleic acid shuffling to take place, the relevant parts of the nucleotide sequences are preferably at least 50% identical, such as at least 60% identical, more preferably at least 70% identical, such as at least 80% identical. The recombination can be performed in vitro or in vivo.

[0239] Examples of suitable in vitro gene shuffling methods are disclosed by Stemmer et al. (1994), Proc. Natl. Acad. Sci. USA; vol. 91, pp. 10747-10751; Stemmer (1994), Nature, vol. 370, pp. 389-391; Smith (1994), Nature vol. 370, pp. 32^4-325; Zhao et al., Nat. Biotechnol. 1998, Mar; 16(3): 258-61; Zhao H. and Arnold, F B, Nucleic Acids Research, 1997, Vol. 25. No. 6 pp. 1307-1308; Shao et al., Nucleic Acids Research 1998, Jan 15; 26(2): pp. 681-83; and WO 95/17413.

[0240] An example of a suitable in vivo shuffling method is disclosed in WO 97/07205. Other techniques for mutagenesis of nucleic acid sequences by in vitro or in vivo recombination are disclosed e.g. in WO 97/20078 and U.S. Pat. No. 5,837,458. Examples of specific shuffling techniques include "family shuffling", "synthetic shuffling" and "in silico shuffling".

[0241] Family shuffling involves subjecting a family of homologous genes from different species to one or more cycles of shuffling and subsequent screening or selection. Family shuffling techniques are disclosed e.g. by Crameri et al. (1998), Nature, vol. 391, pp. 288-291; Christians et al. (1999), Nature Biotechnology, vol. 17, pp. 259-264; Chang et al. (1999), Nature Biotechnology, vol. 17, pp. 793-797; and Ness et al. (1999), Nature Biotechnology, vol. 17, 893-896.

[0242] Synthetic shuffling involves providing libraries of overlapping synthetic oligonucleotides based e.g. on a sequence alignment of homologous genes of interest. The synthetically generated oligonucleotides are recombined, and the resulting recombinant nucleic acid sequences are screened and if desired used for further shuffling cycles. Synthetic shuffling techniques are disclosed in WO 00/42561.

[0243] In silico shuffling refers to a DNA shuffling procedure, which is performed or modelled using a computer system, thereby partly or entirely avoiding the need for physically manipulating nucleic acids. Techniques for in silico shuffling are disclosed in WO 00/42560.

[0244] Once assembled (by synthesis, site-directed mutagenesis or another method), the nucleotide sequence encoding the polypeptide is inserted into a recombinant vector and operably linked to control sequences necessary for expression of the FVII in the desired transformed host cell.

[0245] It should of course be understood that not all vectors and expression control sequences function equally well to express the nucleotide sequence encoding the polypeptide variants described herein. Neither will all hosts function equally well with the same expression system. However, one of skill in the art may make a selection among these vectors, expression control sequences and hosts without undue experimentation. For example, in selecting a vector, the host must be considered because the vector must replicate in it or be able to integrate into the chromosome. The vector's copy number, the ability to control that copy number, and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered. In selecting an expression control sequence, a variety of factors should also be considered. These include, for example, the relative strength of the sequence, its controllability, and its compatibility with the nucleotide sequence encoding the polypeptide, particularly as regards potential secondary structures. Hosts should be selected by consideration of their compatibility with the chosen vector, the toxicity of the product coded for by the nucleotide

sequence, their secretion characteristics, their ability to fold the polypeptide variant correctly, their fermentation or culture requirements, and the ease of purification of the products coded for by the nucleotide sequence.

[0246] The recombinant vector may be an autonomously replicating vector, i.e. a vector, which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector is one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

[0247] The vector is preferably an expression vector, in which the nucleotide sequence encoding the polypeptide variant of the invention is operably linked to additional segments required for transcription of the nucleotide sequence. The vector is typically derived from plasmid or viral DNA. A number of suitable expression vectors for expression in the host cells mentioned herein are commercially available or described in the literature. Useful expression vectors for eukaryotic hosts, include, for example, vectors comprising expression control sequences from SV40, bovine papilloma virus, adenovirus and cytomegalovirus. Specific vectors are, e.g., pCDNA3.1(+)\Hyg (Invitrogen, Carlsbad, Calif., USA) and pCI-neo (Stratagene, La Jola, Calif., USA). Useful expression vectors for yeast cells include the 2μ plasmid and derivatives thereof, the POT1 vector (U.S. Pat. No. 4,931,373), the pJSO37 vector described in Okkels, Ann. New York Acad. Sci. 782, 202-207, 1996, and pPICZ A, B or C (Invitrogen). Useful vectors for insect cells include pVL941, pBG311 (Cate et al., "Isolation of the Bovine and Human Genes for Mullerian Inhibiting Substance And Expression of the Human Gene In Animal Cells", Cell, 45, pp. 685-98 (1986), pBluebac 4.5 and pMelbac (both available from Invitrogen). Useful expression vectors for bacterial hosts include known bacterial plasmids, such as plasmids from *E. coli*, including pBR322, pET3a and pET12a (both from Novagen Inc., WI, USA), wider host range plasmids, such as RP4, phage DNAs, e.g., the numerous derivatives of phage lambda, e.g., NM989, and other DNA phages, such as M13 and filamentous single stranded DNA phages.

[0248] Other vectors for use in this invention include those that allow the nucleotide sequence encoding the polypeptide variant to be amplified in copy number. Such amplifiable vectors are well known in the art. They include, for example, vectors able to be amplified by DHFR amplification (see, e.g., Kaufman, U.S. Pat. No. 4,470,461, Kaufman and Sharp, "Construction Of A Modular Dihydrafolate Reductase cDNA Gene: Analysis Of Signals Utilized For Efficient Expression", Mol. Cell. Biol., 2, pp. 1304-19 (1982)) and glutamine synthetase ("GS") amplification (see, e.g., U.S. Pat. No. 5,122,464 and EP 338,841).

[0249] The recombinant vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. An example of such a sequence (when the host cell is a mammalian cell) is the SV40 origin of replication. When the host cell is a yeast cell, suitable sequences enabling the vector to replicate are the yeast plasmid 2μ replication genes REP 1-3 and origin of replication.

[0250] The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or the *Schizosaccharomyces pombe* TPI gene (described by P. R. Russell, Gene 40, 1985, pp. 125-130), or one which confers resistance to a drug, e.g. ampicillin, kanamycin, tetracyclin, chloramphenicol, neomycin, hygromycin or methotrexate. For *Saccharomyces cerevisiae*, selectable markers include ura3 and leu2. For filamentous fungi, selectable markers include amdS, pyrG, arcB, niaD and sC.

[0251] The term "control sequences" is defined herein to include all components, which are necessary or advantageous for the expression of the polypeptide variant of the invention. Each control sequence may be native or foreign to the nucleic acid sequence encoding the polypeptide variant. Such control sequences include, but are not limited to, a leader sequence, polyadenylation sequence, propeptide sequence, promoter, enhancer or upstream activating sequence, signal peptide sequence, and transcription terminator. At a minimum, the control sequences include a promoter.

[0252] A wide variety of expression control sequences may be used in the present invention. Such useful expression control sequences include the expression control sequences associated with structural genes of the foregoing expression vectors as well as any sequence known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof.

[0253] Examples of suitable control sequences for directing transcription in mammalian cells include the early and late promoters of SV40 and adenovirus, e.g. the adenovirus 2 major late promoter, the MT-1 (metallothionein gene) promoter, the human cytomegalovirus immediate-early gene promoter (CMV), the human elongation factor 1α (EF-1α) promoter, the *Drosophila* minimal heat shock protein 70 promoter, the Rous Sarcoma Virus (RSV) promoter, the human ubiquitin C (UbC) promoter, the human growth hormone terminator, SV40 or adenovirus E1b region polyadenylation signals and the Kozak consensus sequence (Kozak, M. *J Mol Biol* 1987 Aug. 20; 196(4):947-50).

[0254] In order to improve expression in mammalian cells a synthetic intron may be inserted in the 5' untranslated region of the nucleotide sequence encoding the polypeptide. An example of a synthetic intron is the synthetic intron from the plasmid pCI-Neo (available from Promega Corporation, WI, USA).

[0255] Examples of suitable control sequences for directing transcription in insect cells include the polyhedrin promoter, the P10 promoter, the *Autographa californica* polyhedrosis virus basic protein promoter, the baculovirus immediate early gene 1 promoter and the baculovirus 39K delayed-early gene promoter, and the SV40 polyadenylation sequence. Examples of suitable control sequences for use in yeast host cells include the promoters of the yeast α-mating system, the yeast triose phosphate isomerase (TPI) promoter, promoters from yeast glycolytic genes or alcohol dehydrogenase genes, the ADH2-4c promoter, and the inducible GAL promoter. Examples of suitable control sequences for use in filamentous fungal host cells include the ADH3 promoter and terminator, a promoter derived from the genes encoding *Aspergillus oryzae* TAKA amylase triose phosphate isomerase or alkaline protease, an *A. niger* α-amylase, *A. niger* or *A. nidulans* glucoamylase, *A. nidu-*

lans acetamidase, *Rhizomucor miehei* aspartic proteinase or lipase, the TPI1 terminator and the ADH3 terminator. Examples of suitable control sequences for use in bacterial host cells include promoters of the lac system, the trp system, the TAC or TRC system, and the major promoter regions of phage lambda.

[0256] The presence or absence of a signal peptide will, e.g., depend on the expression host cell used for the production of the polypeptide variant to be expressed (whether it is an intracellular or extracellular polypeptide) and whether it is desirable to obtain secretion. For use in filamentous fungi, the signal peptide may conveniently be derived from a gene encoding an *Aspergillus* sp. amylase or glucoamylase, a gene encoding a *Rhizomucor miehei* lipase or protease or a *Humicola lanuginosa* lipase. The signal peptide is preferably derived from a gene encoding *A. oryzae* TAKA amylase, *A. niger* neutral α-amylase, *A. niger* acid-stable amylase, or *A. niger* glucoamylase. For use in insect cells, the signal peptide may conveniently be derived from an insect gene (cf. WO 90/05783), such as the Lepidopteran *manduca sexta* adipokinetic hormone precursor, (cf. U.S. Pat. No. 5,023,328), the honeybee melittin (Invitrogen), ecdysteroid UDPglucosyltransferase (egt) (Murphy et al., Protein Expression and Purification 4, 349-357 (1993) or human pancreatic lipase (hpl) (Methods in Enzymology 284, pp. 262-272, 1997). A preferred signal peptide for use in mammalian cells is that of hFVII or the murine Ig kappa light chain signal peptide (Coloma, M (1992) J. Imm. Methods 152:89-104). For use in yeast cells suitable signal peptides have been found to be the α-factor signal peptide from *S. cereviciae* (cf. U.S. Pat. No. 4,870,008), a modified carboxypeptidase signal peptide (cf. L. A. Valls et al., Cell 48, 1987, pp. 887-897), the yeast BAR1 signal peptide (cf. WO 87/02670), the yeast aspartic protease 3 (YAP3) signal peptide (cf. M. Egel-Mitani et al., Yeast 6, 1990, pp. 127-137), and the synthetic leader sequence TA57 (WO98/32867). For use in *E. coli* cells a suitable signal peptide have been found to be the signal peptide ompA (EP581821).

[0257] The nucleotide sequence of the invention encoding a polypeptide variant, whether prepared by site-directed mutagenesis, synthesis, PCR or other methods, may optionally include a nucleotide sequence that encode a signal peptide. The signal peptide is present when the polypeptide variant is to be secreted from the cells in which it is expressed. Such signal peptide, if present, should be one recognized by the cell chosen for expression of the polypeptide variant. The signal peptide may, e.g. be that normally associated with hFVII) or, alternatively, the signal peptide may be from another source than hFVII, such as any of those normally associated with other human wild-type vitamin K-dependent polypeptides. Furthermore, the signal peptide may be a signal peptide normally expressed from the host cell or one which is not normally expressed from the host cell. Accordingly, the signal peptide may be prokaryotic, e.g. derived from a bacterium such as *E. coli*, or eukaryotic, e.g. derived from a mammalian, or insect or yeast cell.

[0258] Any suitable host may be used to produce the polypeptide variant, including bacteria (although not particularly preferred), fungi (including yeasts), plant, insect, mammal, or other appropriate animal cells or cell lines, as well as transgenic animals or plants. Examples of bacterial host cells include grampositive bacteria such as strains of *Bacillus*, e.g. *B. brevis* or *B. subtilis, Pseudomonas* or *Streptomyces*, or gramnegative bacteria, such as strains of *E. coli*. The introduction of a vector into a bacterial host cell may, for instance, be effected by protoplast transformation (see, e.g., Chang and Cohen, 1979, *Molecular General Genetics* 168: 111-115), using competent cells (see, e.g., Young and Spizizin, 1961, *Journal of Bacteriology* 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, *Journal of Molecular Biology* 56: 209-221), electroporation (see, e.g., Shigekawa and Dower, 1988, *Biotechniques* 6: 742-751), or conjugation (see, e.g., Koehler and Thorne, 1987, *Journal of Bacteriology* 169: 5771-5278). Examples of suitable filamentous fungal host cells include strains of *Aspergillus*, e.g. *A. oryzae, A. niger*, or *A. nidulans, Fusarium* or *Trichoderma*. Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known per se. Suitable procedures for transformation of *Aspergillus* host cells are described in EP 238 023 and U.S. Pat. No. 5,679,543. Suitable methods for transforming *Fusarium* species are described by Malardier et al., 1989, *Gene* 78: 147-156 and WO 96/00787. Examples of suitable yeast host cells include strains of *Saccharomyces*, e.g. *S. cerevisiae, Schizosaccharomyces, Klyveroinyces, Pichia*, such as *P. pastoris* or *P. methzanolica, Hansenula*, such as *H. Polymorpha* or *Yarrowia*. Yeast may be transformed using the procedures described by Becker and Guarente, In Abelson, J. N. and Simon, M. I., editors, *Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology*, Volume 194, pp 182-187, Academic Press, Inc., New York; Ito et al., 1983, *Journal of Bacteriology* 153: 163; Hinnen et al., 1978, *Proceedings of the National Academy of Sciences USA* 75: 1920: and as disclosed by Clontech Laboratories, Inc, Palo Alto, Calif., USA (in the product protocol for the Yeastmaker™ Yeast Transformation System Kit). Examples of suitable insect host cells include a Lepidoptora cell line, such as *Spodoptera frugiperda* (Sf9 or Sf21) or *Trichoplusioa ni* cells (High Five) (U.S. Pat. No. 5,077,214). Transformation of insect cells and production of heterologous polypeptides therein may be performed as described by Invitrogen. Examples of suitable mammalian host cells include Chinese hamster ovary (CHO) cell lines, (e.g. CHO-K1; ATCC CCL-61), Green Monkey cell lines (COS) (e.g. COS 1 (ATCC CRL-1650), COS 7 (ATCC CRL-1651)); mouse cells (e.g. NS/O), Baby Hamster Kidney (BHK) cell lines (e.g. ATCC CRL-1632 or ATCC CCL-10), and human cells (e.g. HEK 293 (ATCC CRL-1573)), as well as plant cells in tissue culture. Additional suitable cell lines are known in the art and available from public depositories such as the American Type Culture Collection, Rockville, Md. Also, the mammalian cell, such as a CHO cell, may be modified to express sialyltransferase, e.g. 1,6-sialyltransferase, e.g. as described in U.S. Pat. No. 5,047,335, in order to provide improved glycosylation of the polypeptide variant.

[0259] In order to increase secretion it may be of particular interest to produce the polypeptide variant of the invention together with an endoprotease, in particular a PACE (paired basic amino acid converting enzyme) (e.g. as described in U.S. Pat. No. 5,986,079), such as a Kex2 endoprotease (e.g. as described in WO 00/28065).

[0260] Methods for introducing exogeneous DNA into mammalian host cells include calcium phosphate-mediated transfection, electroporation, DEAE-dextran mediated transfection, liposome-mediated transfection, viral vectors

and the transfection method described by Life Technologies Ltd, Paisley, UK using Lipofectamin 2000. These methods are well known in the art and e.g. described by Ausbel et al. (eds.), 1996, Current Protocols in Molecular Biology, John Wiley & Sons, New York, USA. The cultivation of mammalian cells are conducted according to established methods, e.g. as disclosed in (Animal Cell Biotechnology, Methods and Protocols, Edited by Nigel Jenkins, 1999, Human Press Inc, Totowa, N.J., USA and Harrison M A and Rae I F, General Techniques of Cell Culture, Cambridge University Press 1997).

[0261] In the production methods of the present invention, the cells are cultivated in a nutrient medium suitable for production of the polypeptide variant using methods known in the art. For example, the cell may be cultivated by shake flask cultivation, small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermenters performed in a suitable medium and under conditions allowing the polypeptide to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or may be prepared according to published compositions (e.g., in catalogues of the American Type Culture Collection). If the polypeptide variant is secreted into the nutrient medium, the polypeptide can be recovered directly from the medium. If the polypeptide variant is not secreted, it can be recovered from cell lysates.

[0262] The resulting polypeptide variant may be recovered by methods known in the art. For example, the polypeptide variant may be recovered from the nutrient medium by conventional procedures including, but not limited to, centrifugation, filtration, extraction, spray drying, evaporation, or precipitation.

[0263] The polypeptides may be purified by a variety of procedures known in the art including, but not limited to, chromatography (e.g., ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (e.g., preparative isoelectric focusing), differential solubility (e.g., ammonium sulfate precipitation), HPLC, or extraction (see, e.g., *Protein Purification*, J.-C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989).

[0264] Single chain polypeptide variants of the invention can be purified and activated to two-chain polypeptide variants by a number of methods as described in the literature (Broze and Majerus, 1980, J. Biol. Chem. 255:1242-47 and Hedner and Kisiel, 1983, J. Clin. Invest. 71:1836-41). Another method whereby single chain polypeptide variant can be purified is by incorporation of Zn ions during purification as described in U.S. Pat. No. 5,700,914. In a preferred embodiment the polypeptide variant is purified as a single chain polypeptide variant. The single chain polypeptide variant is activated by either use of an immobilized enzyme (e.g. factors IIa, IXa, Xa and XIIa) or by autoactivation using a positively charged ion exchange matrix or the like.

[0265] It is advantageous to first purify the polypeptide variant in its single chain form, then PEGylate (if desired) and last activate by one of the methods described above or by autoactivation as described by Pedersen et al, 1989, Biochemistry 28: 9331-36. The advantage of carrying out PEGylation before activation is that PEGylation of the new aminoterminal formed by cleavage of R152-I153 is avoided. PEGylation of this new amino terminal would render the molecule inactive since the formation of a hydrogen bond between D242 and the amino terminal of I153 is necessary for activity.

Pharmaceutical Composition of the Invention and its Use

[0266] In a further aspect, the present invention relates to a composition, in particular to a pharmaceutical composition, comprising a polypeptide variant of the invention and a pharmaceutically acceptable carrier or excipient.

[0267] The polypeptide variant or the pharmaceutical composition according to the invention may be used as a medicament.

[0268] Due to the improved properties mentioned hereinbefore, the polypeptide variants of the invention, or the pharmaceutical composition of the invention, are particular useful for the treatment of uncontrollable bleeding events in trauma patients, thrombocytopenic patients, patients in anticoagulant treatment, and cirrhosis patients with variceal bleeds, or other upper gastrointestinal bleedings, and in patients undergoing orthotopic liver transplantation, or liver resection (allowing for transfusion free surgery).

[0269] Trauma is defined as an injury to living tissue caused by an extrinsic agent. It is the 4th leading cause of death in the US and places a large financial burden on the economy.

[0270] Trauma is classified as either blunt or penetrative. Blunt trauma results in internal compression, organ damage and internal haemorrhage whereas penetrative trauma (as the consequence of an agent penetrating the body and destroying tissue, vessels and organs) results in external haemorrhage.

[0271] Haemorrhage, as a result of trauma, can start a cascade of problems. For example physiological compensation mechanisms are initiated with initial peripheral and mesenteric vasoconstriction to shunt blood to the central circulation. If circulation is not restored, hypovolemia shock (multiple organ failure due to inadequate perfusion) ensues. Since tissues throughout the body become starved for oxygen, anaerobic metabolism begins. However, the concomitant lactic acid leads the blood pH to drop and metabolic acidosis develops. If acidosis is severe and uncorrected, the patient may develop multisystem failure and die.

[0272] Although the majority of trauma patients are hypothermic on arrival in the emergency room due to the environmental conditions at the scene, inadequate protection, intravenous fluid administration and ongoing blood loss worsen the hypothermic state. Deficiencies in coagulation factors can result from blood loss or transfusions. Meanwhile, acidosis and hypothermia interfere with blood clotting mechanisms. Thus coagulopathy develops, which in turn, may mask surgical bleeding sites and hamper the control of mechanical bleeding.

[0273] Hypothermia, coagulopathy and acidosis are often characterised as the "trauma triad of death"

[0274] Trauma may be caused by several events. For example, road traffic accidents result in many different types of trauma. Whilst some road traffic accidents are likely to

result in penetrative trauma, many road traffic accidents are likely to inflict blunt trauma to both head and body. However, these various types of trauma can all result in coagulopathy in the patient. Road traffic accidents are the leading cause of accidental death in the US. There are over 42,000 deaths from them in the US each year. Many trauma patients die at the location of the accident either whilst being treated by the paramedics, before they arrive or in transit to the ER.

[0275] Another example includes gunshot wounds. Gunshot wounds are traumas that can result in massive bleeding. They are penetrative and destroy tissue as the bullet passes through the body, whether it be in the torso or a limb. In the US about 40,000 people a year die from gunshot wounds

[0276] A further example includes falls. Falls result in a similar profile of trauma type to road traffic accidents. By falling onto a solid object or the ground from height can cause both penetrative and decelerative blunt trauma. In the US, falls are a common cause of accidental death, numbering about 13,000.

[0277] A still further example includes machinery accidents. A smaller number of people die in the US from machinery accident related deaths, whether struck by, or entangled in machinery. The figures are small but significant—around 2,000.

[0278] A still further example includes stab wounds. Stab wounds are penetrative injuries that can also cause massive bleeding. The organs most likely to be damaged in a stab wound are the liver, small intestine and the colon.

[0279] Cirrhosis of the liver is the terminal sequel of prolonged repeated injury to the hepatic parenchyma. The end result is the formation of broad bands of fibrous tissue separating regenerative nodules that do not maintain the normal organization of liver lobules and thus cause deteriorated liver function. Patients have prolonged prothrombin times as a result of the depletion of vitamin K-dependent coagulation factors. Pathogenetically, liver cirrhosis should be regarded as the final common pathway of chronic liver injury, which can result from any form of intense repeated prolonged liver cell injury. Cirrhosis of the liver may be caused by direct liver injury, including chronic alcoholism, chronic viral hepatitis (types B, C, and D), and auto immune hepatitis as well as by indirect injury by way of bile duct damage, including primary biliary cirrhosis, primary sclerosing cholangitis and biliary atresia. Less common causes of cirrhosis include direct liver injury from inherited disease such as cystic fibrosis, alpha-1-antitrypsin deficiency, hemochromatosis, Wilson's disease, galactosernia, and glycogen storage disease.

[0280] Transplantation is primarily reserved for late stage cirrhotic patients, where it is the key intervention for treating the disease. To be eligible for transplantation, a patient must be classified as Child's B or C, as well as meet additional criteria for selection. Last year, in the US alone, 4,954 transplants were performed.

[0281] It has been estimated that there are 6,000 bleeding episodes associated with patients undergoing resection each year. This correlates with the reserved position of this procedure although seems slightly high in comparison with transplantation numbers.

[0282] Accurate data on the incidence of variceal bleeding is hard to obtain. The key facts known are that at the time of diagnosis, varices are present in about 60% of decompensated and 30% of compensated patients and that about 30% of these patients with varices will experience a bleed and that each episode of variceal bleeding is associated with a 30% risk of mortality.

[0283] Thus, in a further aspect the present invention relates to a polypeptide variant of the invention for the manufacture of a medicament for the treatment of diseases or disorder wherein clot formation is desirable. A still further aspect of the present invention relates to a method for treating a mammal having a disease or disorder wherein clot formation is desirable, comprising administering to a mammal in need thereof an effective amount of the polypeptide variant or the pharmaceutical composition of the invention.

[0284] Thrombocytopenia is caused by one of three mechanisms-decreased bone marrow production, increased splenic sequestration, or accelerated destruction of platelets. Thronmbocytopenia is a risk factor for hemorrhage, and platelet transfusion reduces the incidence of bleeding. The threshold for prophylactic platelet transfusion is 10,000/μl. In patients without fever or infections, a threshold of 5000/μl may be sufficient to prevent spontaneous hemorrhage. For invasive procedures, 50,000/μl platelets is the usual target level. In patients who develop antibodies to platelets following repeated transfusions, bleeding can be extremely difficult to control.

[0285] Examples of diseases/disorders wherein increased clot formation is desirable include, but is not limited to, hemorrhages, including brain hemorrhages, as well as patient with severe uncontrolled bleedings, such as trauma. Further examples include patients undergoing living transplantations, patients undergoing resection and patients with variceal bleedings.

[0286] The polypeptide variants of the invention is administered to patients in a therapeutically effective dose, normally one approximately paralleling that employed in therapy with rFVII such as NovoSeven®, or at lower dosage. By "therapeutically effective dose" herein is meant a dose that is sufficient to produce the desired effects in relation to the condition for which it is administered. The exact dose will depend on the circumstances, and will be ascertainable by one skilled in the art using known techniques. Normally, the dose should be capable of preventing or lessening the severity or spread of the condition or indication being treated. It will be apparent to those of skill in the art that an effective amount of a polypeptide variant or composition of the invention depends, inter alia, upon the disease, the dose, the administration schedule, whether the polypeptide variant or composition is administered alone or in conjunction with other therapeutic agents, the plasma half-life of the compositions, and the general health of the patient. Preferably, the polypeptide variant or composition of the invention is administered in an effective dose, in particular a dose which is sufficient to normalize the coagulation disorder.

[0287] The polypeptide variant of the invention is preferably administered in a composition including a pharmaceutically acceptable carrier or excipient. "Pharmaceutically acceptable" means a carrier or excipient that does not cause any untoward effects in patients to whom it is administered. Such pharmaceutically acceptable carriers and excipients are well known in the art (see, for example, Remington's

Pharmaceutical Sciences, 18th edition, A. R. Gennaro, Ed., Mack Publishing Company [1990]; Pharmaceutical Formulation Development of Peptides and Proteins, S. Frokjaer and L. Hovgaard, Eds., Taylor & Francis [2000]; and Handbook of Pharmaceutical Excipients, 3rd edition, A. Kibbe, Ed., Pharmaceutical Press [2000]).

[0288] The polypeptide variant of the invention can be formulated into pharmaceutical compositions by well-known methods. Suitable formulations are described by Remington's Pharmaceutical Sciences by E. W. Martin (Mark Publ. Co., 16th Ed., 1980).

[0289] The polypeptide variant of the invention can be used "as is" and/or in a salt form thereof. Suitable salts include, but are not limited to, salts with alkali metals or alkaline earth metals, such as sodium, potassium, calcium and magnesium, as well as e.g. zinc salts. These salts or complexes may by present as a crystalline and/or amorphous structure.

[0290] The pharmaceutical composition of the invention may be administered alone or in conjunction with other therapeutic agents. These agents may be incorporated as part of the same pharmaceutical composition or may be administered separately from the polypeptide variant of the invention, either concurrently or in accordance with another treatment schedule. In addition, the polypeptide variant or pharmaceutical composition of the invention may be used as an adjuvant to other therapies.

[0291] A "patient" for the purposes of the present invention includes both humans and other mammals. Thus, the methods are applicable to both human therapy and veterinary applications. The pharmaceutical composition comprising the polypeptide variant of the invention may be formulated in a variety of forms, e.g. as a liquid, gel, lyophilized, or as a compressed solid. The preferred form will depend upon the particular indication being treated and will be apparent to one skilled in the art.

[0292] In particular, the pharmaceutical composition comprising the polypeptide variant of the invention may be formulated in lyophilised or stable soluble form. The polypeptide variant may be lyophilised by a variety of procedures known in the art. The polypeptide variant may be in a stable soluble form by the removal or shielding of proteolytic degradation sites as described herein. The advantage of obtaining a stable soluble preparation lies in easier handling for the patient and, in the case of emergencies, quicker action, which potentially can become life saving. The preferred form will depend upon the particular indication being treated and will be apparent to one of skill in the art.

[0293] The administration of the formulations of the present invention can be performed in a variety of ways, including, but not limited to, orally, subcutaneously, intravenously, intracerebrally, intranasally, transdermally, intraperitoneally, intramuscularly, intrapulmonary, vaginally, rectally, intraocularly, or in any other acceptable manner. The formulations can be administered continuously by infusion, although bolus injection is acceptable, using techniques well known in the art, such as pumps or implantation. In some instances the formulations may be directly applied as a solution or spray.

Parentals

[0294] A preferred example of a pharmaceutical composition is a solution designed for parenteral administration. Although in many cases pharmaceutical solution formulations are provided in liquid form, appropriate for immediate use, such parenteral formulations may also be provided in frozen or in lyophilized form. In the former case, the composition must be thawed prior to use. The latter form is often used to enhance the stability of the active compound contained in the composition under a wider variety of storage conditions, as it is recognized by those skilled in the art that lyophilized preparations are generally more stable than their liquid counterparts. Such lyophilized preparations are reconstituted prior to use by the addition of one or more suitable pharmaceutically acceptable diluents such as sterile water for injection or sterile physiological saline solution.

[0295] In case of parenterals, they are prepared for storage as lyophilized formulations or aqueous solutions by mixing, as appropriate, the polypeptide variant having the desired degree of purity with one or more pharmaceutically acceptable carriers, excipients or stabilizers typically employed in the art (all of which are termed "excipients"), for example buffering agents, stabilizing agents, preservatives, isotonifiers, non-ionic surfactants or detergents, antioxidants and/or other miscellaneous additives.

[0296] Buffering agents help to maintain the pH in the range which approximates physiological conditions. They are typically present at a concentration ranging from about 2 mM to about 50 mM. Suitable buffering agents for use in the present invention include both organic and inorganic acids and salts thereof such as citrate buffers (e.g., monosodium citrate-disodium citrate mixture, citric acid-trisodium citrate mixture, citric acid-monosodium citrate mixture, etc.), succinate buffers (e.g., succinic acid-monosodium succinate mixture, succinic acid-sodium hydroxide mixture, succinic acid-disodium succinate mixture, etc.), tartrate buffers (e.g., tartaric acid-sodium tartrate mixture, tartaric acid-potassium tartrate mixture, tartaric acid-sodium hydroxide mixture, etc.), fumarate buffers (e.g., fumaric acid-monosodium fumarate mixture, fumaric acid-disodium fumarate mixture, monosodium fumarate-disodium fumarate mixture, etc.), gluconate buffers (e.g., gluconic acid-sodium glyconate mixture, gluconic acid-sodium hydroxide mixture, gluconic acid-potassium glyuconate mixture, etc.), oxalate buffer (e.g., oxalic acid-sodium oxalate mixture, oxalic acid-sodium hydroxide mixture, oxalic acid-potassium oxalate mixture, etc.), lactate buffers (e.g., lactic acid-sodium lactate mixture, lactic acid-sodium hydroxide mixture, lactic acid-potassium lactate mixture, etc.) and acetate buffers (e.g., acetic acid-sodium acetate mixture, acetic acid-sodium hydroxide mixture, etc.). Additional possibilities are phosphate buffers, histidine buffers and trimethylamine salts such as Tris.

[0297] Stabilizers refer to a broad category of excipients, which can range in function from a bulking agent to an additive which solubilizes the therapeutic agent or helps to prevent denaturation or adherence to the container wall. Typical stabilizers can be polyhydric sugar alcohols (enumerated above); amino acids such as arginine, lysine, glycine, glutamine, asparagine, histidine, alanine, ornithine, L-leucine, 2-phenylalanine, glutamic acid, threonine, etc., organic sugars or sugar alcohols, such as lactose, trehalose,

stachyose, mannitol, sorbitol, xylitol, ribitol, myoinisitol, galactitol, glycerol and the like, including cyclitols such as inositol; polyethylene glycol; amino acid polymers; sulfur-containing reducing agents, such as urea, glutathione, thioctic acid, sodium thioglycolate, thioglycerol, α-monothioglycerol and sodium thiosulfate; low molecular weight polypeptides (i.e. <10 residues); proteins such as human serum albumin, bovine serum albumin, gelatin or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; monosaccharides such as xylose, mannose, fructose and glucose; disaccharides such as lactose, maltose and sucrose; trisaccharides such as raffinose, and polysaccharides such as dextran. Stabilizers are typically present in the range of from 0.1 to 10,000 parts by weight based on the active protein weight.

[0298] Preservatives are added to retard microbial growth, and are typically added in amounts of about 0.2%-1% (w/v). Suitable preservatives for use with the present invention include phenol, benzyl alcohol, meta-cresol, methyl paraben, propyl paraben, octadecyldimethylbenzyl ammonium chloride, benzalkonium halides (e.g. benzalkonium chloride, bromide or iodide), hexamethonium chloride, alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol and 3-pentanol.

[0299] Isotonicifiers are added to ensure isotonicity of liquid compositions and include polyhydric sugar alcohols, preferably trihydric or higher sugar alcohols, such as glycerin, erythritol, arabitol, xylitol, sorbitol and mannitol. Polyhydric alcohols can be present in an amount between 0.1% and 25% by weight, typically 1% to 5%, taking into account the relative amounts of the other ingredients.

[0300] Non-ionic surfactants or detergents (also known as "wetting agents") may be present to help solubilizing the therapeutic agent as well as to protect the therapeutic polypeptide against agitation-induced aggregation, which also permits the formulation to be exposed to shear surface stress without causing denaturation of the polypeptide. Suitable non-ionic surfactants include polysorbates (20, 80, etc.), polyoxamers (184, 188 etc.), Pluronic® polyols, polyoxyethylene sorbitan monoethers (Tween®-20, Tween®-80, etc.). Additional miscellaneous excipients include bulking agents or fillers (e.g. starch), chelating agents (e.g. EDTA), antioxidants (e.g., ascorbic acid, methionine, vitamin E) and cosolvents.

[0301] The active ingredient may also be entrapped in microcapsules prepared, for example, by coascervation techniques or by interfacial polymerization, for example hydroxymethylcellulose, gelatin or poly-(methylmethacylate) microcapsules, in colloidal drug delivery systems (for example liposomes, albumin micro spheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, supra.

[0302] Parenteral formulations to be used for in vivo administration must be sterile. This is readily accomplished, for example, by filtration through sterile filtration membranes.

Sustained Release Preparations

[0303] Examples of sustained-release preparations include semi-permeable matrices of solid hydrophobic polymers containing the polypeptide variant, the matrices having a suitable form such as a film or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate) or poly(vinylalcohol)), polylactides, copolymers of L-glutamic acid and ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the ProLease® technology or Lupron Depot® (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(−)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for long periods such as up to or over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated polypeptides remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37° C., resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization is depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S—S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

[0304] The invention is further described in the following non-limiting examples.

Materials and Methods

Accessible Surface Area (ASA)

[0305] The computer program Access (B. Lee and F. M. Richards, J. Mol. Biol. 55: 379-400 (1971)) version 2 (© 1983 Yale University) is used to compute the accessible surface area (ASA) of the individual atoms in the structure. This method typically uses a probe-size of 1.4 Å and defines the Accessible Surface Area (ASA) as the area formed by the center of the probe. Prior to this calculation all water molecules and all hydrogen atoms should be removed from the coordinate set, as should other atoms not directly related to the protein.

Fractional ASA of Side Chain

[0306] The fractional ASA of the side chain atoms is computed by division of the sum of the ASA of the atoms in the side chain with a value representing the ASA of the side chain atoms of that residue type in an extended Ala-x-Ala tripeptide (See Hubbard, Campbell & Thornton (1991) J. Mol. Biol. 220,507-530). For this example the CA atom is regarded as a part of the side chain of Glycine residues but not for the remaining residues. The following table is used as standard 100% ASA for the side chain:

Ala	69.23 Å^2
Arg	200.35 Å^2
Asn	106.25 Å^2
Asp	102.06 Å^2
Cys	96.69 Å^2
Gln	140.58 Å^2
Glu	134.61 Å^2
Gly	32.28 Å^2
His	147.00 Å^2
Ile	137.91 Å^2
Leu	140.76 Å^2

-continued

Lys	162.50 Å^2
Met	156.08 Å^2
Phe	163.90 Å^2
Pro	119.65 Å^2
Ser	78.16 Å^2
Thr	101.67 Å^2
Trp	210.89 Å^2
Tyr	176.61 Å^2
Val	114.14 Å^2

[0307] Residues not detected in the structure are defined as having 100% exposure as they are thought to reside in flexible regions. The gamma-carboxy glutamic acids at positions 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35 are all defined as being 100% exposed.

Determining Distances Between Atoms

[0308] The distance between atoms is most easily determined using molecular graphics software e.g. InsightII® v. 98.0, MSI INC.

Active Site Region

[0309] The active site region is defined as any residues having at least one atom within 10 Å of any atom in the catalytic triad (residues H193, D242, S344).

Determination of Tissue Factor Binding Site

[0310] The TF binding site is defined as comprising of all residues having their accessible surface area changed upon TF binding. This is determined by at least two ASA calculations; one on the isolated ligand(s) in the ligand(s)/receptor(s) complex and one on the complete ligand(s)/receptor(s) complex.

Measurement of Reduced Sensitivity to Proteolytic Degradation

[0311] Proteolytic degradation can be measured using the assay described in U.S. Pat. No. 5,580,560, Example 5, where proteolysis is autoproteolysis.

[0312] Furthermore, reduced proteolysis can be tested in an in vivo model using radiolabelled samples and comparing proteolysis of rhFVIIa and the polypeptide variant of the invention by withdrawing blood samples and subjecting these to SDS-PAGE and autoradiography.

[0313] Irrespectively of the assay used for determining proteolytic degradation, "reduced proteolytic degradation" is intended to mean a measurable reduction in cleavage compared to is that obtained by rhFVIIa as measured by gel scanning of Coomassie stained SDS-PAGE gels, HPLC or as measured by conserved catalytic activity in comparison to wild type using the tissue factor independent activity assay decribed below.

Determination of the Molecular Weight of Polypeptide Variants

[0314] The molecular weight of polypeptide variants is determined by either SDS-PAGE, get filtration, Western Blots, matrix assisted laser desorption mass spectrometry or equilibrium centrifugation, e.g. SDS-PAGE according to Laemmli, U.K., Nature Vol 227 (1970), pp. 680-85.

TF-Independent Factor X Activation Assay

[0315] This assay has been described in detail on page 39826 in Nelsestuen et al., J Biol Chem, 2001; 276:39825-39831.

[0316] Briefly, the molecule to be assayed (either hFVIIa, rhFVIIa or the polypeptide variant of the invention in its activated form) is mixed with a source of phospholipid (phosphatidylcholine and phosphatidylserine in a ratio of 8:2 or phosphatidyleholine, phosphatidylserine and phosphatidylethanol in a ratio of 4:2:4) and Factor X in Tris buffer containing BSA. After a specified incubation time the reaction is stopped by addition of excess EDTA. The concentration of factor Xa is then measured from absorbance change at 405 nm after addition of a chromogenic substrate (S-2222, Chromogenix). After correction from background the tissue factor independent activity of rhFVIIa (a_{wt}) is determined as the absorbance change after 10 minutes and the tissue factor independent activity of the polypeptide variant of the invention ($a_{variant}$) is also determined as the absorbance change after 10 minutes. The ratio between the activity of the polypeptide variant, in its activated form, and the activity of rhFVIIa is defined as $a_{variant}/a_{wt}$.

Clotting Assay

[0317] Clotting activity is measured in one-stage assays and clotting times are recorded on a Thrombotrack IV coagulometer (MEDINOR). FVII depleted human plasma (American Diagnostica) is reconstituted and equilibrated at room temperature for 15-20 minutes. 50 μl of plasma is then transferred to the coagulometer cups.

[0318] hFVIIa, rhFVIIa or variants are diluted in Glyoxaline Buffer (5.7 mM barbiturate, 4.3 mM sodium citrate, 117 mM NaCl, 1 mg/mL BSA, pH 7.35). The samples are added to the cup in 50 μl and incubated at 37° C. for 2 minutes.

[0319] Thromboplastin (MEDINOR) is reconstituted with water and $CaCl_2$ is added. The reaction is initiated by adding 0.1 ml thromboplastin containing 4.5 mM $CaCl_2$.

[0320] Data are analysed using PRISM software.

TF-Independent Clotting Assay

[0321] This assay is performed as described above under "Clotting Assay" but without addition of thromboplastin.

Amidolytic Assay

[0322] The ability of the variants to cleave small peptide substrates can be measured using the chromogenic substrate S-2288 (D-Ile-Pro-Arg-p-nitroanilide). The amidolytic activity may be measured both in the presence and absence of antithrombin III (ATIII).

[0323] HFVIIa, rhFVIIa or variant is diluted to 90 nM in assay buffer (50 mM Na-Hepes pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 0.1% BSA, 1U/ml Heparin). Furthermore, soluble TF (sTF) is diluted to 450 nM in assay buffer. ATIII is diluted to 900 nM in assay buffer. 120 μl of assay buffer is mixed with 20 μl of the FVIIa sample, 20 μl sTF and 20 μl ATIII or assay buffer. The final concentrations of FVIIa, sTF and ATIII are 10, 50 and 100 nM, respectively. After 5 min incubation at room temperature with gentle shaking, followed by 10 min incubation at 37° C., the reaction is started by addition of the S-2288 substrate to 1 mM and the absorption at 405 nm is determined at several time points.

Thrombogram Assay

[0324] The effect of hFVIIa, rhFVIIa or variant on thrombin generation in human plasma is tested in a modified version of the assay described on page 589 in Hemker et al. in Thromb Haemost 2000; 83: 589-91. Briefly, the molecule to be assayed (either hFVIIa, rhFVIIa or variant) is mixed with normal platelet rich plasma (PRP), normal platelet poor plasma (PPP) or FVII depleted PPP with or without the addition of recombinant human tissue factor (rTF), relipidated rTF or another source of TF (such as thromboplastin). A source of phospholipid (phosphatidylcholine and phosphatidylserine in a ratio of 8:2 or phosphatidylcholine, phosphatidylserine and phosphatidylethanol in a ratio of 4:2:4) can be added.

[0325] The reaction is started by addition of a fluoregenic thrombin substrate and calcium chloride. The fluorescence is measured continuously and the thrombin amidolytic activity is calculated by calculating the slope of the fluorescence curve (the increase in fluorescence over time). In this way the time until maximum thrombin amidolytic activity is obtained (T_{max}) and total thrombin work (area under the curve (AUC)) can be calculated.

[0326] The following procedure is used: PRP is obtained by centrifuging freshly drawn blood at 250 g, 15° C. for 10 min. Blood coagulation is inhibited either by using citrate (13 mM tri-sodium citrate), corn trypsin inhibitor (50-100 μg/ml blood) or a combination of citrate and corn trypsin inhibitor. The platelet count is adjusted to 3×10^8/mL using buffer or autologous platelet poor plasma (PPP). PPP is obtained by double centrifugation of PRP at 1000 g, 15° C. for 10 min. FVII depletion is done by incubating PPP with a FVII specific monoclonal antibody coupled to a solid phase.

[0327] Per well of a 96-well microtiter plate 80 μl PRP is added and 20 μL buffer containing rhFVII or variant to be tested in final concentrations between 0.1 and 100 nM. rTF is added in 5 μL assay buffer to a final concentration of 1 pM. The assay buffer consists of 20 mM Hepes, 150 mM NaCl and 60 mg/ml BSA in distilled water. The reaction is started by adding 20 μL of the substrate solution containing 0.1 M calcium chloride. The assay plate and reagents are prewarmed to 37° C. and the reaction takes place at this temperature. The fluorimeter used is a BMG Fluormeter with a excitation filter at 390 nm and an emission filter at 460 nm. The fluorescence is measured in each well of 96-well clear bottom plates in 20-40 second interval over 30-180 minutes. Data are analyzed using PRISM Software.

ELISA Assay

[0328] FVII/FVIIa (or variant) concentrations are determined by ELISA. Wells of a microtiter late are coated with an antibody directed against the protease domain using a solution of 2 μg/ml in PBS (100 μl per well). After 2 hours coating at R.T., the wells are washed 4 times with THT buffer (100 mM NaCl, 50 mM Tris-HCl pH 7.2 0.05% Tween-20). Subsequently, 200 μl of 1% Casein (diluted from 2.5% stock using 100 mM NaCl, 50 mM Tris-HCl pH 7.2) is added per well for blocking. After 1 hr incubation at R.T., the wells are emptied, and 100 μl of sample (optionally diluted in dilution buffer (THT+0.1% Casein)) is added. After another incubation of 1 hr at room temperature, the wells are washed 4 times with THT buffer, and 100 μl of a biotin-labelled antibody directed against the EGF-like domain (1 μg/ml) is added. After another 1 hr incubation at R.T., followed by 4 more washes with THT buffer, 100 μl of streptavidin-horse radish peroxidase (DAKO A/S, Glostrup, Denmark, 1/10000 diluted) is added. After another 1 hr incubation at R.T., followed by 4 more washes with THT buffer, 100 μl of TMB (3,3',5,5'-tetramethylbenzidine, Kemen-Tech A/S, Denmark) is added. After 30 min incubation at R.T. in the dark, 100 μl of 1 M H_2SO_4 is added and OD_{450nm} is determined. A standard curve is prepared using rhFVIIa (NovoSeven®).

[0329] Alternatively, FVII/FVIIa or variants may be quantified through the Gla domain rather than through the protease domain. In this ELISA set-up, wells are coated overnight with an antibody directed against the EGF-like domain and for detection, a calcium-dependent biotin-labelled monoclonal anti-Gla domain antibody is used (2 μg/ml, 100 μl per well). In this set-up, 5 mM $CaCl_2$ is added to the THT and dilution buffers.

Whole Blood Assay

[0330] The whole blood clotting assay is performed as described by Elg et al. Thrombosis Res. 2001, 101(3):159-170.

Reconstituted Coagulation Assay

[0331] The reconstituted coagulation assay is performed as described by Van't Veer et al. Blood 2000, 95(4), 1330-1335.

EXAMPLES

Example 1

[0332] The X-ray structure of hFVIIa in complex with soluble tissue factor by Banner et. al., J Mol Biol, 1996; 285:2089 is used for this example. It is noted that the numbering of residues in the reference does not follow the sequence. Here we have used the sequential numbering according to SEQ ID NO:2. The gamma-carboxy glutamic acids at positions 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35 are all here named GLU (three letter abbreviation) or E (one letter abbreviation). Residues 143-152 are not present in the structure.

Surface Exposure

[0333] Performing fractional ASA calculations on FVII fragments alone combined with the definition of accessibilities of non standard and/or missing residues described in the methods resulted in the following residues having more than 25% of their side chain exposed to the surface: A1, N2, A3, F4, L5, E6, E7, L8, R9, P10, S12, L13, E14, E16, K18, E19, E20, Q21, S23, F24, E25, E26, R8, E29, F31, K32, D33, A34, E35, R36, K38, L39, W41, I42, S43, S45, G47, D48, Q49, A51, S52, S53, Q56, G58, S60, K62, D63, Q64, L65, Q66, S67, I69, F71, L73, P74, A75, E77, G78, R79, E82, T83, H84, K85, D86, D87, Q88, L89, I90, V92, N93, E94, G97, E99, S103, D104, H105, T106, G107, T108, K109, S111, R113, E116, G117, S119, L120, L121, A122, D123, G124, V125, S126, T128, P129, T130, V131, E132, 1140, L141, E142, K143, R144, N145, A146, S147, K148, P149, Q150, G151, R152, G155, K157, V158, P160, K161, E163, L171, N173, G174, A175, N184, T185, 1186, H193, K197, K199, N200, R202, N203, 1205, S214, E215, H216, D217, G218, D219, S222, R224, S232, T233, V235, P236, G237, T238, T239, N240, H249, Q250, P251, V253, T255, D256,

E265, R266, T267, E270, R271, F275, V276, R277, F278, L280, L287, L288, D289, R290, G291, A292, T293, L295, E296, N301, M306, T307, Q308, D309, L311, Q312, Q313, R315, K316, V317, G318, D319, S320, P321, N322, T324, E325, Y326, Y332, S333, D334, S336, K337, K341, G342, H351, R353, G354, Q366, G367, T370, V371, G372, R379, E385, Q388, K389, R392, S393, E394, P395, R396, P397, G398, V399, L400, L401, R402, P404 and P406 (A1-S45 are located in the Gla domain, the remaining positions are located outside the Gla domain).

[0334] The following residues had more than 50% of their side chain exposed to the surface: A1, A3, F4, L5, E6, E7, L8, R9, P10, E14, E16, K18, E19, E20, Q21, S23, E25, E26, E29, K32, A34, E35, R36, K38, L39, I42, S43, G47, D48, A51, S52, S53, Q56, G58, S60, K62, L65, Q66, S67, I69, F71, L73, P74, A75, E77, G78, R79, E82, H84, K85, D86, D87, Q88, L89, I90, V92, N93, E94, G97, T106, G107, T108, K109, S111, E116, S119, L121, A122, D123, G124, V131, E132, L141, E142, K143, R144, N145, A146, S147, K148, P149, Q150, G151, R152, G155, K157, P160, N173, G174, A175, K197, K199, N200, R202, S214, E215, H216, G218, R224, V235, P236, G237, T238, H249, Q250, V253, D256, T267, F275, R277, F278, L288, D289, R290, G291, A292, T293, L295, N301, M306, Q308, D309, L311, Q312, Q313, R315, K316, G318, D319, N322, E325, D334, K341, G354, G367, V371, E385, K389, R392, E394, R396, P397, G398, R402, P404 and P406 (A1-S43 are located in the Gla domain, the remaining positions are located outside the Gla domain).

Tissue Factor Binding Site

[0335] Performing ASA calculations the following residues in human FVII change their ASA in the complex. These residues were defined as constituting the receptor binding site: L13, K18, F31, E35, R36, L39, F40, I42, S43, S60, K62, D63, Q64, L65, I69, C70, F71, C72, L73, P74, F76, E77, G78, R79, E82, K85, Q88, I90, V92, N93, E94, R271, A274, F275, V276, R277, F278, R304, L305, M306, T307, Q308, D309, Q312, Q313, E325 and R379.

Active Site Region

[0336] The active site region is defined as any residue having at least one atom within a distance of 10 Å from any atom in the catalytic triad (residues H193, D242, S344): I153, Q167, V168, L169, L170, L171, Q176, L177, C178, G179, G180, T181, V188, V189, S190, A191, A192, H193, C194, F195, D196, K197, 1198, W201, V228, I229, I230, P231, S232, T233, Y234, V235, P236, G237, T238, T239, N240, H241, D242, I243, A244, L245, L246, V281, S282, G283, W284, G285, Q286, T293, T324, E325, Y326, M327, F328, D338, S339, C340, K341, G342, D343, S344, G345, G346, P347, H348, L358, T359, G360, I361, V362, S363, W364, G365, C368, V376, Y377, T378, R379, V380, Q382, Y383, W386, L387, L400 and F405.

The Ridge of the Active Site Binding Cleft

[0337] The ridge of the active site binding cleft region was defined by visual inspection of the FVIIa structure 1FAK-.pdb as: N173, A175, K199, N200, N203, D289, R290, G291, A292, P321 and T370.

Example 2

Design of an Expression Cassette for Expression of rhFVII in Mammalian Cells

[0338] The DNA sequence shown in SEQ ID NO:1, encompassing the short form of the full length cDNA encoding hFVII with its native short signal peptide (Hagen et al., 1986. PNAS 83:2412), was synthesized in order to facilitate high expression in mammalian cells. First the ATG start codon context was modified according to the Kozak consensus sequence (Kozak, M. J Mol Biol 1987 Aug. 20; 196(4):947-50), so that there is a perfect match to the consensus sequence upstream of the ATG start codon. Secondly the open reading frame of the native cDNA was modified by making a bias in the codon usage towards the codons frequently used in highly expressed human genes. Further, two translational stop codons were inserted at the end of the open reading frame in order to facilitate efficient translational stop. The fully synthetic and expression optimized hFVII gene was assembled from 70-mer DNA oligonucleotides and finally amplified using end primers inserting BamHI and HindIII sites at the 5' and 3' ends respectively using standard PCR techniques, which resulted in the following sequence:

```
ggatcccgccaccatggtcagccaggccctccgcctcctgtgcctgctcc
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gcggagcgaaccccggcccggggtgctcctgcgggccccttttcccttgat
aaaagctt
```

[0339] A vector for the cloning of the generated PCR product encompassing the expression cassette for hFVII was prepared by cloning the intron from pCINeo (Promega). The synthetic intron from pCI-Neo was amplified using standard PCR conditions and the primers:

```
CBProFpr174: 5'-AGCTGGCTAGCCACTGGGCAGGTAAGTATCA-3'
and

CBProFpr175: 5'-TGGCGGGATCCTTAAGAGCTGTAATTGAACT-3'
```

resulting in a 332 bp PCR fragment. The fragment was cut with NheI and BamHI before cloning into pCDNA3.1/HygR (obtained from Invitrogen) resulting in PF#34.

[0340] The expression cassette for hFVII was cloned between the BamHI and HindIII sites of PF434, resulting in plasmid PF#226.

Example 3

Expression of Polypeptide Variants in CHO K1 Cells

[0341] The cell line CHO K1 (ATCC # CCL-61) is seeded at 50% confluence in T-25 flasks using MEMα, 10% FCS (Gibco/BRL Cat # 10091), P/S and 5 μg/ml phylloquinone and allowed to grow until confluent. The confluent mono cell layer is transfected with 5 μg of the relevant plasmid described above using the Lipofectamine 2000 transfection agent (Life technologies) according to the manufacturer's instructions. Twenty four hours post transfection a sample is drawn and quantified using e.g. an ELISA recognizing the EGF1 domain of hFVII. At this time point relevant selection (e.g. Hygromycin B) may be applied to the cells with the purpose of generating a pool of stable transfectants. When using CHO K1 cells and the Hygromycin B resistance gene as selectable marker on the plasmid, this is usually achieved within one week.

Example 4

Generation of CHO K1 Cells Stably Expressing Polypeptide Variants.

[0342] A vial of CHO-K1 transfectant pool is thawed and the cells seeded in a 175 cm^2 tissue flask containing 25 ml of MEMα, 10% FCS, phylloquinone (5 μg/ml), 100 U/l penicillin, 100 μg/l streptomycin and grown for 24 hours. The cells are harvested, diluted and plated in 96 well microtiter plates at a cell density of ½-1 cell/well. After a week of growth, colonies of 20-100 cells are present in the wells and those wells containing only one colony are labelled. After a further two weeks, the media in all wells containing only one colony is substituted with 200 μl fresh medium. After 24 hours, a medium sample is withdrawn and analysed by e.g. ELISA. High producing clones are selected and used to produce FVII or variant on large scale.

Example 5

Purification of Polypeptide Variants and Subsequent Activation

[0343] FVII and FVII variants are purified as follows: The procedure is performed at 4° C. The harvested culture media from large-scale production is ultrafiltered using a Millipore TFF system with 30 KDa cut-off Pellicon membranes. After concentration of the medium, citrate is added to 5 mM and the pH is adjusted to 8.6. If necessary, the conductivity is lowered to below 10 mS/cm. Subsequently, the sample is applied to a Q-sepharose FF column, equilibrated with 50 mM NaCl, 10 mM Tris pH 8.6. After washing the column with 100 mM NaCl, 10 mM Tris pH 8.6, followed by 150 mM NaCl, 10 mM Tris pH 8.6, FVII is eluted using 10 mM Tris, 25 mM NaCl, 35 mM $CaCl_2$, pH 8.6.

[0344] For the second chromatographic step, an affinity column is prepared by coupling of a monoclonal Calcium-dependent antiGla-domain antibody to CNBr-activated Sepharose FF. About 5.5 mg antibody is coupled per ml resin. The column is equilibrated with 10 mM Tris, 100 mM NaCl, 35 mM $CaCl_2$, pH 7.5. NaCl is added to the sample to a concentration of 100 mM NaCl and the pH is adjusted to 7.4-7.6. After O/N application of the sample, the column is washed with 100 mM NaCl, 35 mM $CaCl_2$, 10 mM Tris pH 7.5, and the FVII protein is eluted with 100 mM NaCl, 50 mM citrate, 75 mM Tris pH 7.5.

[0345] For the third chromatographic, the conductivity of the sample is lowered to below 10 mS/cm, if necessary, and the pH is adjusted to 8.6. The sample is then applied to a Q-sepharose column (equilibrated with 50 mM NaCl, 10 mM Tris pH 8.6) at a density around 3-5 mg protein per ml gel to obtain efficient activation. After application, the column is washed with 50 mM NaCl, 10 mM Tris pH 8.6 for about 4 hours with a flow of 3-4 column volumes (cv) per hour. The FVII protein is eluted using a gradient of 0-100% of 500 mM NaCl, 10 mM Tris pH 8.6 over 40 cv. FVII containing fractions are pooled.

[0346] For the final chromatographic step, the conductivity is lowered to below 10 mS/cm. Subsequently, the sample is applied to a Q-sepharose column (equilibrated with 140 mM NaCl, 10 mM glycylglycine pH 8.6) at a concentration of 3-5 mg protein per ml gel. The column is then washed with 140 mM NaCl, 10 mM glycylglycine pH 8.6 and FVII is eluted with 140 mM NaCl, 15 mM $CaCl_2$, 10 mM glycylglycine pH 8.6. The eluate is diluted to 10 mM $CaCl_2$ and the pH is adjusted 6.8-7.2. Finally, Tween-80 is added to 0.01% and the pH is adjusted to 5.5 for storage at −80° C.

SEQUENCE LISTING

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<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
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<221> NAME/KEY: CDS
<222> LOCATION: (115)..(1335)

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Ala
1

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Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu Cys
             5                  10                  15

aaa gag gaa cag tgc agc ttt gag gaa gcc cgg gag att ttc aaa gac      213
Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys Asp
         20                  25                  30

gct gag cgg acc aaa ctg ttt tgg att agc tat agc gat ggc gat cag      261
Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp Gln
     35                  40                  45

tgc gcc tcc agc cct tgc cag aac ggg ggc tcc tgc aaa gac cag ctg      309
Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln Leu
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cag agc tat atc tgc ttc tgc ctg cct gcc ttt gag ggg cgc aat tgc      357
Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn Cys
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gaa acc cat aag gat gac cag ctg att tgc gtc aac gaa aac ggg ggc      405
Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly Gly
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tgc gag cag tac tgc agc gat cac acg ggc acg aag cgg agc tgc cgc      453
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    115                 120                 125

acg gtg gaa tac cct tgc ggg aag att ccc att cta gaa aag cgg aac      549
Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg Asn
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Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro Lys
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210                 215                 220                 225

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Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp His
            245                 250                 255

gtc gtg cct ctg tgc ctg cct gag cgg acc ttt agc gaa cgc acg ctg      933
Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr Leu
        260                 265                 270

gct ttc gtc cgc ttt agc ctc gtg tcc ggc tgg ggc cag ctg ctc gac      981
Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu Asp
    275                 280                 285

cgg ggc gct acc gct ctc gag ctg atg gtg ctc aac gtc ccc cgg ctg     1029
Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg Leu
290                 295                 300                 305
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atg acc cag gac tgc ctg cag cag tcc cgc aaa gtg ggg gac tcc ccc     1077
Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser Pro
               310                 315                 320

aat atc acg gag tat atg ttt tgc gct ggc tat agc gat ggc tcc aag     1125
Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser Lys
           325                 330                 335

gat agc tgc aag ggg gac tcc ggc ggg ccc cat gcc acg cac tat cgc     1173
Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr Arg
       340                 345                 350

ggg acc tgg tac ctc acc ggg atc gtc agc tgg ggc cag ggc tgc gcc     1221
Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys Ala
    355                 360                 365

acg gtg ggg cac ttt ggc gtc tac acg cgc gtc agc cag tac att gag     1269
Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile Glu
370                 375                 380                 385

tgg ctg cag aag ctc atg cgg agc gaa ccc cgg ccc ggg gtg ctc ctg     1317
Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu Leu
               390                 395                 400

cgg gcc cct ttc cct tga taa                                        1338
Arg Ala Pro Phe Pro
           405
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<210> SEQ ID NO 2
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  1               5                  10                  15

Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys
             20                  25                  30

Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp
         35                  40                  45

Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln
     50                  55                  60

Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn
 65                  70                  75                  80

Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly
                 85                  90                  95

Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys
            100                 105                 110

Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr
        115                 120                 125

Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg
    130                 135                 140

Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro
145                 150                 155                 160

Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln
                165                 170                 175

Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala
            180                 185                 190

His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu
        195                 200                 205

Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg
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-continued

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    210                 215                 220

Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn
225                 230                 235                 240

His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp
                245                 250                 255

His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr
            260                 265                 270

Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu
        275                 280                 285

Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg
    290                 295                 300

Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser
305                 310                 315                 320

Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser
                325                 330                 335

Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr
            340                 345                 350

Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys
        355                 360                 365

Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile
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Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu
385                 390                 395                 400

Leu Arg Ala Pro Phe Pro
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tggcgggatc cttaagagct gtaattgaac t                                  31
```

1. A Factor VII (FVII) or Factor VIIa (FVIIa) polypeptide variant having an amino acid sequence comprising 3-15 amino acid modifications relative to human Factor VII (hFVII) or human Factor VIIa (hFVIIa) having the amino acid sequence shown in SEQ ID NO:2, wherein said amino acid sequence of the variant comprises an amino acid substitution in position 10 and 32 and wherein a sugar moiety is covalently attached to an introduced in vivo N-glycosylation site located outside the Gla domain.

2. The variant according to claim 1, wherein said substitution in position 10 is P10Q.

3. The variant according to claim 1, wherein said substitution in position 32 is K32E.

4. The variant according to claim 1, wherein said substitution in position 10 is P10Q and said substitution in position 32 is K32E.

5. The variant according to claim 1, wherein said variant comprises at least one further amino acid modification in the Gla domain.

6-15. (canceled)

16. The variant according to claim 5, wherein said further modification in the Gla domain comprises an amino acid substitution in position 34.

17. The variant according to claim 16, wherein a negatively charged amino acid residue is introduced by substitution in position 34.

18. The variant according to claim 17, wherein said substitution is A34E.

19. The variant according to claim 18, wherein said variant comprises the substitutions P10Q+K32E+A34E.

20-25. (canceled)

26. The variant according to claim 1, wherein said in vivo N-glycosylation site is introduced by substitution.

27-28. (canceled)

29. The variant according to claim 26, wherein said in vivo N-glycosylation site is introduced by a substitution selected from the group consisting of A51N, G58N, T106N, K109N, G124N, K143N+N145T, A175T, I205S, I205T, V253N, T267N, T267N+S269T, S314N+K316S, S314N+K316T, R315N+V317S, R315N+V317T, K316N+G318S, K316N+G318T, G318N, D334N and combinations thereof.

30. The variant according to claim 29, wherein said in vivo N-glycosylation site is introduced by a substitution selected from the group consisting of A51N, G58N, T106N, K109N, G124N, K143N+N145T, A175T, I205T, V253N, T267N+S269T, S314N+K316T, R315N+V317T, K316N+G318T, G318N, D334N and combinations thereof.

31. The variant according to claim 30, wherein said in vivo N-glycosylation site is introduced by a substitution selected from the group consisting of T106N, A175T, I205T, V253N, T267N+S269T and combinations thereof.

32. The variant according to claim 16, wherein one in vivo N-glycosylation site has been introduced by substitution.

33. The variant according to claim 16, wherein two or more in vivo N-glycosylation sites have been introduced by substitution.

34-40. (canceled)

41. The variant according to claim 1, wherein said variant is in its activated form.

42. The variant according to claim 1, wherein said variant, in its activated form, has at least 10% of the amidolytic activity of rhFVIIa when assayed in the "Amidolytic Assay" described herein.

43. The variant according to claim 1, wherein said variant, in its activated form, has at least 10% of the clotting activity of rhFVIIa when assayed in the "Clotting Assay" described herein.

44. A nucleotide sequence encoding the variant of claim 1.

45. An expression vector comprising the nucleotide sequence of claim 44.

46. A host cell comprising the nucleotide sequence of claim 44.

47. The host cell according to claim 46, wherein said host cell is a gammacarboxylating cell capable of in vivo glycosylation.

48. A pharmaceutical composition comprising the variant of claim 1, and a pharmaceutical acceptable carrier or excipient.

49.-54. (canceled)

55. A method for treating a mammal having a disease or a disorder wherein clot formation is desirable, comprising administering to a mammal in need thereof an effective amount of the pharmaceutical composition of claim 48.

56. The method according to claim 55, wherein said disease or disorder is selected from the group consisting of hemorrhage, including brain hemorrhage, severe uncontrolled bleeding, such as trauma, bleeding in patients undergoing living transplantations, bleeding in patients undergoing resection and variceal bleedings.

57-59. (canceled)

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(54) **FACTOR VII OR VIIA POLYPEPTIDE VARIANTS**

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C12P 21/04	(2006.01)

(52) **U.S. Cl.** **435/69.6**; 435/320.1; 435/325; 530/383; 514/12; 536/23.5

(57) **ABSTRACT**

The present invention relates to novel polypeptide variants of factor VII (FVII) or factor VIIa (FVIIa) polypeptides, where said variants comprise an amino acid substitution in position 10 and 32 and where said variants further comprise a sugar moiety covalently attached to an introduced in vivo N-glycosylation site located outside of the Gla domain. Such polypeptide variants are useful in therapy, in particular for the treatment of a variety of coagulation-related disorders, such as trauma.

FACTOR VII OR VIIA POLYPEPTIDE VARIANTS

FIELD OF THE INVENTION

[0001] The present invention relates to novel polypeptide variants of factor VII (FVII) or factor VIIa (FVIIa) polypeptides, where said variants comprise an amino acid substitution in position 10 and 32 and where said variants further comprise a sugar moiety covalently attached to an introduced in vivo N-glycosylation site.

[0002] The present invention also relates to use of such polypeptide variants in therapy, in particular for the treatment of a variety of coagulation-related disorders.

BACKGROUND OF THE INVENTION

[0003] Blood coagulation is a process consisting of a complex interaction of various blood components (or factors) that eventually results in a fibrin clot. Generally, the blood components participating in what has been referred to as the "coagulation cascade" are proenzymes or zymogens, i.e. enzymatically inactive proteins that are converted into an active form by the action of an activator. One of these coagulation factors is FVII.

[0004] FVII is a vitamin K-dependent plasma protein synthesized in the liver and secreted into the blood as a single-chain glycoprotein with a molecular weight of 53 kDa (Broze & Majerus, J. Biol. Chem 1980; 255:1242-1247). The FVII zymogen is converted into an activated form (FVIIa) by proteolytic cleavage at a single site, R152-I153, resulting in two chains linked by a single disulfide bridge. FVIIa in complex with tissue factor (FVIIa complex) is able to convert both factor IX and factor X into their activated forms, followed by reactions leading to rapid thrombin production and fibrin formation (Østerud & Rapaport, Proc Natl Acad Sci USA 1977; 74:5260-5264).

[0005] FVII undergoes post-translational modifications, including vitamin K-dependent carboxylation resulting in ten γ-carboxyglutamic acid residues in the N-terminal region of the molecule. Thus, residue number 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35 shown in SEQ ID NO:2 are γ-carboxyglutamic acids residues in the Gla domain important for FVII activity. Other post-translational modifications include sugar moiety attachment at two naturally occurring N-glycosylation sites at position 145 and 322, respectively, and at two naturally occurring O-glycosylation sites at position 52 and 60, respectively.

[0006] The gene coding for human FVII (hFVII) has been mapped to chromosome 13 at q34-qter 9 (de Grouchy et al., Hum Genet 1984; 66:230-233). It contains nine exons and spans 12.8 Kb (O'Hara et al., Proc Natl Acad Sci USA 1987; 84:5158-5162). The gene organisation and protein structure of FVII are similar to those of other vitamin K-dependent procoagulant proteins, with exons 1a and 1b encoding for signal sequence; exon 2 the propeptide and Gla domain; exon 3 a short hydrophobic region; exons 4 and 5 the epidermal growth factor-like domains; and exon 6 through 8 the serine protease catalytic domain (Yoshitake et al., Biochemistry 1985; 24: 3736-3750).

[0007] Reports exist on experimental three-dimensional structures of hFVIIa (Pike et al., PNAS. U.S.A., 1999; 96:8925-30 and Kemball-Cook et al., J. Struct. Biol, 1999; 127:213-223); of hFVIIa in complex with soluble tissue factor using X-ray crystallographic methods (Banner et al., Nature, 1996; 380:41 and Zhang et al., J. Mol. Biol, 1999; 285: 2089); and of smaller fragments of hFVII (Muranyi et al., Biochemistry, 1998; 37:10605 and Kao et al., Biochemistry, 1999; 38:7097).

[0008] Some protein-engineered variants of FVII have been reported. See, e.g., Dickinson & Ruf, J Bio Chem, 1997;272:19875-19879, Kemball-Cook et al., J Biol Chem, 1998; 273:8516-8521, Bharadwaj et al., J Biol Chem, 1996; 271:30685-30691, Ruf et al., Biochemistry, 1999; 38:1957-1966; WO 99/20767; WO 00/11416; WO 02/22776; WO 02/38162; WO 01/83725; WO 01/58935; U.S. Pat. No. 5,580,560.

[0009] Reports exist on expression of FVII in BHK or other mammalian cells (WO 92/15686, WO 91/11514 and WO 88/10295) and co-expression of FVII and kex2 endoprotease in eukaryotic cells (WO 00/28065).

[0010] Commercial preparations of human recombinant FVIIa are sold as NovoSeven®. NovoSeven® is indicated for the treatment of bleeding episodes in hemophilia A or B patients. NovoSeven® is the only rhFVIIa for effective and reliable treatment of bleeding episodes available on the market.

[0011] An inactive form of FVII in which arginine 152 and/or isoleucine 153 is/are modified has been reported in WO91/1154. These amino acids are located at the activation site. WO 96/12800 describes inactivation of FVIIa by a serine proteinase inhibitor; inactivation by carbamylation of FVIIa at the α-amino acid group I153 has been described by Petersen et al., Eur J Biochem, 1999;261:124-129. The inactivated form is capable of competing with wild-type FVII or FVIIa for binding to tissue factor and inhibiting clotting activity. The inactivated form of FVIIa is suggested to be used for treatment of patients being in hypercoagulable states, such as patients with sepsis, in risk of myocardial infarction or of thrombotic stroke.

[0012] A circulating rhFVIIa half-life of 2.3 hours was reported in "Summary Basis for Approval for NovoSeven®", FDA reference number 96-0597. Relatively high doses and frequent administration are necessary to reach and sustain the desired therapeutic or prophylactic effect. As a consequence adequate dose regulation is difficult to obtain and the need of frequent intravenous administrations imposes restrictions on the patient's way of living.

[0013] In connection with treatment of uncontrolled bleedings, such as trauma, it is believed that factor VIIa is capable of activating factor X to factor Xa without binding to tissue factor, and this activation reaction is believed to occur primarily on activated blood platelets (Hedner et al. Blood Coagulation & Fibrinolysis, 2000;11;107-111). However, hFVIIa or rhFVIIa has a low activity towards factor X in the absence of tissue factor and, consequently, treatment of uncontrolled bleeding, for example in trauma patients, requires relatively high and multiple doses of hFVIIa or rhFVIIa. Therefore, in order to treat uncontrolled bleedings more efficiently (to minimize blood loss) there is need for improved FVIIa molecules, which possess a high activity toward factor X in the absence of tissue factor. Such improved FVIIa molecules will exhibit a lowered clotting time (or faster action) as compared to rhFVIIa when administered in connection with uncontrolled bleedings.

[0014] A molecule with a longer circulation half-life would decrease the number of necessary administrations. Given the association of current the rhFVIIa product with frequent injections, and the potential for obtaining more optimal therapeutic FVIIa levels with concomitant enhanced therapeutic effect, there is a clear need for improved FVII- or FVIIa-like molecules.

[0015] One way to increase the circulation half-life of a protein is to ensure that renal clearance of the protein is reduced. This may be achieved by conjugating the protein to a chemical moiety, which is capable of conferring reduced renal clearance to the protein.

[0016] Furthermore, attachment of a chemical moiety to the protein or substitution of amino acids exposed to proteolysis may effectively block a proteolytic enzyme from contact leading to proteolytic degradation of the protein. Polyethylene glycol (PEG) is one such chemical moiety that has been used in the preparation of therapeutic protein products. WO 98/32466 suggests that FVII, among many other proteins, may be PEGylated but does not contain any further information in this respect. WO 01/58935 discloses a new strategy for developing FVII or FVIIa molecules having inter alia an increased half-life.

[0017] As indicated above, another problem in current rhFVIIa treatment is the relative instability of the molecule with respect to proteolytic degradation. Proteolytic degradation is a major obstacle for obtaining a preparation in solution as opposed to a lyophilized product. The advantage of obtaining a stable soluble preparation lies in easier handling for the patient, and, in the case of emergencies, quicker action, which potentially can become life saving. Attempts to prevent proteolytic degradation by site directed mutagenesis at major proteolytic sites have been disclosed in WO 88/10295.

[0018] One object of the present invention is to provide improved FVII or FVIIa molecules (FVII or FVIIa variants) with a longer circulation half-life (thereby decreasing the number of necessary administrations) and which are capable of activating factor X to factor Xa (without binding to tissue factor) more efficiently than hFVIIa or rhFVIIa (thereby being able to treat uncontrolled bleadings, such as a trauma, more efficiently).

[0019] Another object of the present invention is to provide improved FVII or FVII molecules (FVII or FVIIa variants) with an increased bioavailability (such as an increased Area Under the Curve as compared to rhFVIIa when administered intravenously) and which are capable of activating factor X to factor Xa (without binding to tissue factor) more efficiently than hFVIIa or rhFVIIa (thereby being able to treat uncontrolled bleadings, such as a trauma, more efficiently).

[0020] These objects are met by the FVII or FVIIa variants provided herein.

BRIEF DISCLOSURE OF THE INVENTION

[0021] In its broadest aspect the present invention relates to a FVII or FVIIa polypeptide variant having an amino acid sequence comprising 3-15 amino acid modifications relative to hFVII or hFVIIa having the amino acid sequence shown in SEQ ID NO:2, wherein said amino acid sequence of the variant comprises an amino acid substitution in position 10 and 32 and wherein a sugar moiety is covalently attached to an introduced in vivo N-glycosylation site located outside the Gla domain.

[0022] Another aspect of the invention relates to a nucleotide sequence encoding the polypeptide variant of the invention.

[0023] In a further aspect the invention relates to an expression vector comprising the nucleotide sequence of the invention.

[0024] In a still further aspect the invention relates to a host cell comprising the nucleotide sequence of the invention or the expression vector of the invention.

[0025] In an even further aspect the invention relates to a pharmaceutical composition comprising the polypeptide variant of the invention, and a pharmaceutical acceptable carrier or excipient.

[0026] Still another aspect of the invention relates to the polypeptide variant of the invention, or the pharmaceutical composition of the invention, for use as a medicament.

[0027] Further aspects of the present invention will be apparent from the below description as well as from the appended claims.

DETAILED DISCLOSURE OF THE INVENTION

Definitions

[0028] In the context of the present application and invention the following definitions apply:

[0029] The term "conjugate" (or interchangeably "conjugated polypeptide") is intended to indicate a heterogeneous (in the sense of composite or chimeric) molecule formed by the covalent attachment of one or more polypeptide(s) to one or more non-polypeptide moieties such as polymer molecules, lipophilic compounds, sugar moieties or organic derivatizing agents. Preferably, the conjugate is soluble at relevant concentrations and conditions, i.e. soluble in physiological fluids such as blood. Examples of conjugated polypeptides of the invention include glycosylated and/or PEGylated polypeptides.

[0030] The term "covalent attachment" or "covalently attached" means that the polypeptide variant and the non-polypeptide moiety are either directly covalently joined to one another, or else are indirectly covalently joined to one another through an intervening moiety or moieties, such as a bridge, spacer, or linkage moiety or moieties.

[0031] The term "non-polypeptide moiety" is intended to mean a molecule, different from a peptide polymer composed of amino acid monomers and linked together by peptide bonds, which molecule is capable of conjugating to an attachment group of the polypeptide variant of the invention. Preferred examples of such molecules include polymer molecules, sugar moieties, lipophilic compounds or organic derivatizing agents. When used in the context of a conjugated variant of the invention it will be understood that the non-polypeptide moiety is linked to the polypeptide part of the conjugated variant through an attachment group of the polypeptide. As explained above, the non-polypeptide moiety can be directly covalently joined to the attachment group or it can be indirectly covalently joined to the attachment

group through an intervening moiety or moieties, such as a bridge spacer or linker moiety or moieties.

[0032] A "polymer molecule" is a molecule formed by covalent linkage of two or more monomers, wherein none of the monomers is an amino acid residue, except where the polymer is human albumin or another abundant plasma protein. The term "polymer" may be used interchangeably with the term "polymer molecule". The term is also intended to cover carbohydrate molecules attached by in vitro glycosylation, i.e. a synthetic glycosylation performed in vitro normally involving covalently linking a carbohydrate molecule to an attachment group of the polypeptide variant, optionally using a cross-linking agent. In vitro glycosylation is discussed in detail further below.

[0033] The term "sugar moiety" is intended to indicate a carbohydrate-containing molecule comprising one or more monosaccharide residues, capable of being attached to the polypeptide variant (to produce a polypeptide variant conjugate in the form of a glycosylated polypeptide variant) by way of in vivo glycosylation. The term "in vivo glycosylation" is intended to mean any attachment of a sugar moiety occurring in vivo, i.e. during posttranslational processing in a glycosylating cell used for expression of the polypeptide variant, e.g. by way of N-linked and O-linked glycosylation. The exact oligosaccharide structure depends, to a large extent, on the glycosylating organism in question.

[0034] An "N-glycosylation site" has the sequence N-X-S/T/C, wherein X is any amino acid residue except proline, N is asparagine and S/T/C is either serine, threonine or cysteine, preferably serine or threonine, and most preferably threonine. Preferably, the amino acid residue in position +3 relative to the asparagines residue is not a proline residue.

[0035] An "O-glycosylation site" is the OH-group of a serine or threonine residue.

[0036] The term "attachment group" is intended to indicate a functional group of the polypeptide variant, in particular of an amino acid residue thereof or a carbohydrate moiety, capable of attaching a non-polypeptide moiety such as a polymer molecule, a lipophilic molecule, a sugar moiety or an organic derivatizing agent. Useful attachment groups and their matching non-polypeptide moieties are apparent from the table below.

Attachment group	Amino acid	Examples of non-polypeptide moiety	Conjugation method/-Activated PEG	Reference
—NH_2	N-terminal, Lys	Polymer, e.g. PEG, with amide or imine group	mPEG-SPA Tresylated mPEG	Shearwater Inc. Delgado et al, critical reviews in Therapeutic Drug Carrier Systems 9(3, 4): 249–304 (1992)
—COOH	C-terminal, Asp, Glu	Polymer, e.g. PEG, with ester or amide group	mPEG-Hz	Shearwater Inc.
		Carbohydrate moiety	In vitro coupling	
—SH	Cys	Polymer, e.g. PEG, with disulfide, maleimide or vinylsulfone group	PEG-vinylsulphone PEG-maleimide	Shearwater Inc. Delgado et al, critical reviews in Therapeutic Drug Carrier Systems 9(3, 4): 249–304 (1992)
		Carbohydrate moiety	In vitro coupling	
—OH	Ser, Thr, Lys, OH—	Sugar moiety PEG with ester, ether, carbamate, carbonate	In vivo O-linked glycosylation	
—$CONH_2$	Asn as part of an N-glycosylation site	Sugar moiety Polymer, e.g. PEG	In vivo N-glycosylation	
Aromatic residue	Phe, Tyr, Trp	Carbohydrate moiety	In vitro coupling	
—$CONH_2$	Gln	Carbohydrate moiety	In vitro coupling	Yan and Wold, Biochemistry, 1984, Jul 31; 23(16): 3759–65
Aldehyde Ketone	Oxidized oligosaccharide	Polymer, e.g. PEG, PEG-hydrazide	PEGylation	Andresz et al., 1978, Makromol. Chem. 179: 301, WO 92/16555, WO 00/23114
Guanidino	Arg	Carbohydrate moiety	In vitro coupling	Lundblad and Noyes, Chimical Reagents for Protein Modification, CRC Press Inc., Florida, USA
Imidazole ring	His	Carbohydrate moiety	In vitro coupling	As for guanidine

[0037] For in vivo N-glycosylation, the term "attachment group" is used in an unconventional way to indicate the amino acid residues constituting a N-glycosylation site (with the sequence N-X-S/T/C, wherein X is any amino acid residue except proline, N is asparagine and S/T/C is either serine, threonine or cysteine, preferably serine or threonine, and most preferably threonine). Although the asparagine residue of the N-glycosylation site is the one to which the sugar moiety is attached during glycosylation, such attachment cannot be achieved unless the other amino acid residues of the N-glycosylation site is present.

[0038] Accordingly, when the non-polypeptide moiety is a sugar moiety and the conjugation is to be achieved by in vivo N-glycosylation, the term "amino acid residue comprising an attachment group for a non-polypeptide moiety" as used in connection with alterations of the amino acid sequence of the polypeptide is to be understood as meaning that one or more amino acid residues constituting an in vivo N-glycosylation site are to be altered in such a manner that a functional in vivo N-glycosylation site is introduced into the amino acid sequence.

[0039] In the present application, amino acid names and atom names (e.g. CA, CB, CD, CG, SG, NZ, N, O, C, etc) are used as defined by the Protein DataBank (PDB) (www-.pdb.org) based on the IUPAC nomenclature (IUPAC Nomenclature and Symbolism for Amino Acids and Peptides (residue names, atom names, etc.), *Eur. J. Biochem.,* 138, 9-37 (1984) together with their corrections in *Eur. J. Biochem.,* 152, 1 (1985)).

[0040] The term "amino acid residue" is intended to indicate an amino acid residue contained in the group consisting of alanine (Ala or A), cysteine (Cys or C), aspartic acid (Asp or D), glutamic acid (Glu or E), phenylalanine (Phe or F), glycine (Gly or G), histidine (His or H), isoleucine (Ile or I), lysine (Lys or K), leucine (Leu or L), methionine (Met or M), asparagine (Asn or N), proline (Pro or P), glutamine (Gln or Q), arginine (Arg or R), serine (Ser or S), threonine (Thr or T), valine (Val or V), tryptophan (Trp or W), and tyrosine (Tyr or Y) residues.

[0041] The terminology used for identifying amino acid positions is illustrated as follows: I205 indicates that position 205 is occupied by an isoleucine residue in the amino acid sequence shown in SEQ ID NO:2. I205T indicates that the isoleucine residue of position 205 has been substituted with a threonine residue. Alternative substitutions are indicated with a "/", e.g. I205S/T means an amino acid sequence in which isoleucine in position 205 is substituted with either serine or threonine. Multiple substitutions are indicated with a "+", e.g. K143N+N145T means a substitution of the lysine residue in position 143 with an asparagine residue and a substitution of the asparagine residue in position 145 with a threonine residue. Insertion of an additional amino acid residue is indicated in the following way: Insertion of a tyrosine residue after A3 (i.e. in position 4) is indicated by A3AY (leading to insertion of a tyrosine residue in position 4). A deletion of an amino acid residue is indicated by an asterix. For example, deletion of a valine residue in position 172 is indicated by V172*. Simultaneous insertion and substitution are indicated in the following way: Substitution of an alanine residue in position 175 with a threonine residue followed by insertion of a leucine residue after position 175 is indicated A175TL.

[0042] Unless otherwise indicated, the numbering of amino acid residues made herein is made relative to the amino acid sequence of the hFVII/hFVIIa polypeptide (SEQ ID NO:2).

[0043] The term "differs from" as used in connection with specific mutations is intended to allow for additional differences being present apart from the specified amino acid difference. For instance, in addition to the introduction of in vivo N-glycosylation sites (located outside the Gla domain), the polypeptide may comprise other modifications that are not related to introduction of such amino acid residues. In a similar way, in addition to the modifications performed in the Gla domain aiming at increasing the phospholipid membrane binding affinity, the polypeptide may contain other modifications that are not necessarily related to this effect.

[0044] Thus, in addition to the amino acid modifications disclosed herein, it will be understood that the amino acid sequence of the polypeptide variant of the invention may, if desired, contain other alterations, i.e. other substitutions, insertions or deletions. These may, for example, include truncation of the N- and/or C-terminus by one or more amino acid residues (e.g. by 1-10 amino acid residues), or addition of one or more extra residues at the N- and/or C-terminus, e.g. addition of a methionine residue at the N-terminus or introduction of a cysteine residue near or at the C-terminus, as well as "conservative amino acid substitutions", i.e. substitutions performed within groups of amino acids with similar characteristics, e.g. small amino acids, acidic amino acids, polar amino acids, basic amino acids, hydrophobic amino acids and aromatic amino acids.

[0045] Examples of such conservative substitutions are shown in the below table.

1	Alanine (A)	Glycine (G)	Serine (S)	Threonine (T)
2	Aspartic acid (D)	Glutamic acid (E)		
3	Asparagine (N)	Glutamine (Q)		
4	Arginine (R)	Histidine (H)	Lysine (K)	
5	Isoleucine (I)	Leucine (L)	Methionine (M)	Valine (V)
6	Phenyl-alanine (F)	Tyrosine (Y)	Tryptophan (W)	

[0046] Still other examples of additional modifications are disclosed in the section entitled "Other modifications outside the Gla domain" below.

[0047] The term "nucleotide sequence" is intended to indicate a consecutive stretch of two or more nucleotide molecules. The nucleotide sequence may be of genomic, cDNA, RNA, semisynthetic, synthetic origin, or any combinations thereof.

[0048] The term "polymerase chain reaction" or "PCR" generally refers to a method for amplification of a desired nucleotide sequence in vitro, as described, for example, in U.S. Pat. No. 4,683,195. In general, the PCR method involves repeated cycles of primer extension synthesis, using oligonucleotide primers capable of hybridising preferentially to a template nucleic acid.

[0049] The term "vector" refers to a plasmid or other nucleotide sequences that are capable of replicating within a

host cell or being integrated into the host cell genome, and as such, are useful for performing different functions in conjunction with compatible host cells (a vector-host system): to facilitate the cloning of the nucleotide sequence, i.e. to produce usable quantities of the sequence, to direct the expression of the gene product encoded by the sequence and to integrate the nucleotide sequence into the genome of the host cell. The vector will contain different components depending upon the function it is to perform.

[0050] "Cell", "host cell", "cell line" and "cell culture" are used interchangeably herein and all such terms should be understood to include progeny resulting from growth or culturing of a cell.

[0051] "Transformation" and "transfection" are used interchangeably to refer to the process of introducing DNA into a cell.

[0052] "Operably linked" refers to the covalent joining of two or more nucleotide sequences, by means of enzymatic ligation or otherwise, in a configuration relative to one another such that the normal function of the sequences can be performed. For example, the nucleotide sequence encoding a presequence or secretory leader is operably linked to a nucleotide sequence coding for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide: a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the nucleotide sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, then synthetic oligonucleotide adaptors or linkers are used, in conjunction with standard recombinant DNA methods.

[0053] In the context of the present invention the terms "modification" or "amino acid modification" is intended to cover replacement of an amino acid side chain, substitution of an amino acid residue, deletion of an amino acid residue and/or insertion of an amino acid residue.

[0054] The terms "mutation" and "substitution" are used interchangeably herein.

[0055] The term "introduce" refers to introduction of an amino acid residue by substitution of an existing amino acid residue or, alternatively, by insertion of an additional amino acid residue.

[0056] The term "remove" refers to removal of an amino acid residue by substitution of the amino acid residue to be removed by another amino acid residue or, alternatively, by deletion (without substitution) of the amino acid residue to be removed

[0057] The term "FVII" or "FVII polypeptide" refers to a FVII molecule provided in single chain form. One example of a FVII polypeptide is wild-type human FVII (hFVII) shown in SEQ ID NO:2. It should be understood, however, that the term "FVII polypeptide" also covers hFVII-like molecules, such as fragments or variants of SEQ ID NO:2, in particular variants where the sequence comprises at least one, such as 1-15, e.g., 1-10, amino acid modifications as compared to SEQ ID NO:2.

[0058] The term "FVIIa" or "FVIIa polypeptide" refers to a FVIIa molecule provided in its activated two-chain form. When the amino acid sequence of SEQ ID NO:2 is used to describe the amino acid sequence of FVIIa it will be understood that the peptide bond between R152 and I153 of the single-chain form has been cleaved, and that one of the chains comprises amino acid residues 1-152, the other chain amino acid residues 153-406.

[0059] The terms "rFVII" and "rFVIIa" refer to FVII and FVIIa polypeptides produced by recombinant techniques.

[0060] The terms "hFVII" and "hFVIIa" refer to human wild-type FVII and FVIIa, respectively, having the amino acid sequence shown in SEQ ID NO:2

[0061] The terms "rhFVII" and "rhFVIIa" refer to human wild-type FVII and FVIIa, having the amino acid sequence shown in SEQ ID NO:2, produced by recombinant means. An example of rhFVIIa is NovoSeven®.

[0062] When used herein, the term "Gla domain" is intended to cover amino acid residues no. 1 to 45 of SEQ ID NO:2.

[0063] Accordingly, the term "located outside the Gla domain" covers amino acid residue no. 46-406 of SEQ ID NO:2.

[0064] The abbreviations "TF" and "TFPI" mean Tissue Factor and Tissue Factor Pathway Inhibitor, respectively.

[0065] The term "protease domain" is used about residues 153-406 counted from the N-terminus.

[0066] The term "catalytic site" is used to mean the catalytic triad consisting of S344, D242 and H193 of the polypeptide variant.

[0067] The term "parent" is intended to indicate the molecule to be modified/improved in accordance with the present invention. Although the parent polypeptide to be modified by the present invention may be any FVII or FVIIa polypeptide, and thus be derived from any origin, e.g. a non-human mammalian origin, it is preferred that the parent polypeptide is hFVII or hFVIIa

[0068] A "variant" is a polypeptide, which differs in one or more amino acid residues from its parent polypeptide, normally in 3-15 amino acid residues (e.g. in 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acid residues), such as in 3-10 amino acid residues, e.g. in 3-8 or 3-5 amino acid residues. In other words, a "variant" typically contains 3-15 amino acid modifications (for example 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acid modifications), such as 3-10 amino acid modifications, e.g. 3-8 or 3-5 amino acid modifications relative to the parent polypeptide. In the present context, the term "modification" encompasses insertions, deletions, substitutions and combinations thereof. It will be understood that a polypeptide variant according to the present invention will be modified in at least three positions, namely in at least position 10 and 32 (located in the Gla domain) and in at least one position located outside the Gla domain, where said at least one modification creates an in vivo N-glycosylation site.

[0069] The term "clotting activity" is used to mean the activity measured in the "Clotting Assay" described herein. In order to exhibit "clotting activity" a variant of the invention, in its activated form, should have at least 10% of

the clotting acitivty of rhFVIIa when assayed in the "Clotting Assay" described herein. In a preferred embodiment of the invention the variant, in its activated form, has at least 20% of the clotting activity of rhFVIIa, such as at least 30%, e.g. at least 40%, more preferably at least 50%, such as at least 60%, e.g. at least 70%, even more preferably at least 80%, such as at least 90% of the clotting activity of rhFVIIa when assayed in the "Clotting Assay" described herein. In an interesting embodiment the variant, in its activated form, has substantially the same clotting activity as rhFVIIs, such as a clotting activity of 75-125% of the clotting acitivity of rhFVIIa.

[0070] The term "amidolytic activity" is used to mean the activity measured in the "Amidolytic Assay" described herein. In order to exhibit "amidolytic activity" a variant of the invention, in its activated form, should have at least 10% of the amidolytic acitivty of rhFVIIa when assayed in the "Amidolytic Assay" described herein. In a preferred embodiment of the invention the variant, in its activated form, has at least 20% of the amidolytic activity of rhFVIIa, such as at least 30%, e.g. at least 40%, more preferably at least 50%, such as at least 60%, e.g. at least 70%, even more preferably at least 80%, such as at least 90% of the amidolytic activity of rhFVIIa when assayed in the "Amidolytic Assay" described herein. In an interesting embodiment the variant, in its activated form, has substantially the same amidolytic activity as rhFVIIs, such as an amidolytic activity of 75-125% of the amidolytic acitivity of rhFVIIa.

[0071] In the present context, the term "activity" is also used in connection with the variants capability of activating FX to FXa. This activity is also denoted "FX activation activity" or "FXa generation activity".

[0072] The term "increased FX activiation activity" or "increased FXa generation activity" is used to indicate that a variant of the invention, in its activated form, has a statistically signifycantly increased capability to activate FX to FXa as compared to rhFVIIa or. To what extent a variant of the invention (in its activated form) has an increased FX activation activity may conveniently be determined in the "TF-independent Factor X Activation Assay" described herein.

[0073] The term "stronger clot" or "increased clot strength" is used to indicate that the strength of the clot generated by the polypeptide variant is statistically significantly increased relative to that generated by rhFVIIa as determined under comparable conditions. This effect may be determined as the Area Under the Curve (AUC_{throm}) generated by the variant of the invention, in its activated form, when assayed in the "Thrombogram Assay" disclosed herein. In a similar way, the term "increased AUC_{throm}" is used to indicate that the Area Under the Curve generated by the variant (in its activated form) is statistically significantly increased relative to that generated by rhFVIIa as determined under comparable conditions and when measured in the "Thrombogram Assay" described herein

[0074] The term "T_{max}" is used about the time it takes to obtain the maximum thrombin activity level in the "Thrombogram Assay".

[0075] The term "immunogenicity" as used in connection with a given substance is intended to indicate the ability of the substance to induce a response from the immune system. The immune response may be a cell or antibody mediated response (see, e.g., Roitt: Essential Immunology (8th Edition, Blackwell) for further definition of immunogenicity). Normally, reduced antibody reactivity will be an indication of reduced immunogenicity. The reduced immunogenicity may be determined by use of any suitable method known in the art, e.g. in vivo or in vitro.

[0076] The term "functional in vivo half-life" is used in its normal meaning, i.e. the time at which 50% of the biological activity of the polypeptide is still present in the body/target organ, or the time at which the activity of the polypeptide is 50% of the initial value.

[0077] As an alternative to determining functional in vivo half-life, "serum half-life" may be determined, i.e. the time at which 50% of the polypeptide circulates in the plasma or bloodstream prior to being cleared. Determination of serum half-life is often more simple than determining the functional in vivo half-life and the magnitude of serum half-life is usually a good indication of the magnitude of functional in vivo half-life. Alternatively terms to serum half-life include "plasma half-life", "circulating half-life", "serum clearance", "plasma clearance" and "clearance half-life". The polypeptide is cleared by the action of one or more of the reticuloendothelial systems (RES), kidney, spleen or liver, by tissue factor, SEC receptor or other receptor mediated elimination, or by specific or unspecific proteolysis. Normally, clearance depends on size (relative to the cutoff for glomerular filtration), charge, attached carbohydrate chains, and the presence of cellular receptors for the protein. The functionality to be retained is normally selected from procoagulant, proteolytic or receptor binding activity. The functional in vivo half-life and the serum half-life may be determined by any suitable method known in the art.

[0078] The term "increased" as used about the functional in vivo half-life or serum half-life is used to indicate that the relevant half-life of the polypeptide variant is statistically significantly increased relative to that of rhFVIIa as determined under comparable conditions (typically determined in an experimental animal, such as rats, rabbits, pigs or monkeys).

[0079] The term "AUC_{iv}" or "Area Under the Curve when administered intravenously" is used in its normal meaning, i.e. as the area under the activity in serum-time curve, where the polypeptide variant has been administered intravenously, in particular when administered intravenously in rats. Typically, the activity measured is the "clotting activity" as defined hereinbefore. Once the experimental activity-time points have been determined, the AUC_{iv} may conveniently be calculated by a computer program, such as GraphPad Prism 3.01.

[0080] It will be understood that in order to make a direct comparison between the AUC_{iv}-values of different molecules (e.g. between the variants of the invention and a reference molecule such as rhFVIIa) the same amount of activity should be administered. Consequently, the AUC_{iv}-values are typically normalized (i.e. corrected for differences in the injected dose) and expressed as AUC_{iv}/dose administered.

[0081] The term "reduced sensitivity to proteolytic degradation" is primarily intended to mean that the polypeptide variant has reduced sensitivity to proteolytic degradation in

comparison to hFVIIa or rhFVIIa as determined under comparable conditions. Preferably, the proteolytic degradation is reduced by at least 10% (e.g. by 10-25% or by 10-50%), such as at least 25% (e.g. by 25-50%, by 25-75% or by 25-100%), more preferably by at least 35%, such as at least 50%, (e.g. by 50-75% or by 50-100%) even more preferably by at least 60%, such as by at least 75% (e.g. by 75-100%) or even at least 90%. Most preferably, the proteolytic degradation is reduced by at least 100%.

[0082] The term "renal clearance" is used in its normal meaning to indicate any clearance taking place by the kidneys, e.g. by glomerular filtration, tubular excretion or degradation in the tubular cells. Renal clearance depends on physical characteristics of the polypeptide, including size (diameter), hydrodynamic volume, symmetry, shape/rigidity, and charge. Normally, a molecular weight of about 67 kDa is considered to be a cut-off-value for renal clearance. Renal clearance may be established by any suitable assay, e.g. an established in vivo assay. Typically, renal clearance is determined by administering a labelled (e.g. radiolabelled or fluorescence labelled) polypeptide to a patient and measuring the label activity in urine collected from the patient. Reduced renal clearance is determined relative to a corresponding reference polypeptide, e.g. rhFVIIa, under comparable conditions. Preferably, the renal clearance rate of the polypeptide variant is reduced by at least 50%, preferably by at least 75%, and most preferably by at least 90% compared to rhFVIIa.

[0083] The terms "at least 25% of its side chain exposed to the surface of the molecule" and "at least 50% of its side chain exposed to the surface of the molecule" are defined with reference to Example 1, where the calculations, etc. are described in detail.

[0084] It should be noted that when the terms "at least 25% of its side chain exposed to the surface of the molecule" and "at least 50% of its side chain exposed to the surface of the molecule" are used in connection with introduction of an in vivo N-glycosylation site these terms refer to the surface accessibility of the amino acid side chain in the position where the sugar moiety is actually attached. In many cases it will be necessary to introduce a serine or a threonine residue in position +2 relative to the asparagine residue to which the sugar moiety is actually attached (unless, of course, this position is already occupied by a serine or a threonine residue) and these positions, where the serine or threonine residues are introduced, are allowed to be buried, i.e. to have less than 25% or 50% of their side chains exposed to the surface of the molecule.

[0085] The terms "tissue factor binding site", "active site region" and "ridge of the active site binding cleft" are defined with reference to Example 1 herein, wherein the above-mentioned sites/regions are determined.

[0086] The term "hydrophobic amino acid residue" includes the following amino acid residues: isoleucine (I), leucine (L), methionine (M), valine (V), phenylalanine (F), tyrosine (Y) and tryptophan (W).

[0087] The term "negatively charged amino acid residue" includes the following amino acid residues: Aspartic acid (D) and glutamic acid (E).

[0088] The term "positively charged amino acid residue" includes the following amino acid residues: Lysine (K), arginine (R) and histidine (H).

Variants of the Invention

[0089] In its broadest aspect the present invention relates to a FVII or FVIIa polypeptide variant having an amino acid sequence comprising 3-15 amino acid modifications relative to hFVII or hFVIIa having the amino acid sequence shown in SEQ ID NO:2, wherein said amino acid sequence of the variant comprises an amino acid substitution in position 10 and 32 and wherein a sugar moiety is covalently attached to an introduced in vivo N-glycosylation site located outside the Gla domain.

[0090] The modifications perfomed in the two above-indicated regions of the parent FVII polypeptide serve the following purposes:

[0091] The modifications performed in positions located outside the Gla domain (introduction of in vivo N-glycosylation site(s)) are preferably of such nature that the AUC_{iv}, the functional in vivo half-life and/or the serum half-life of the resulting variant is increased as compared to rhFVIIa.

[0092] The modifications perfomed in the Gla domain of the parent polypeptide are preferably of such nature that an increased phospholipid membrane binding affinity of the resulting molecule is achieved and/or of such nature the resulting molecule has an improved capability to activate FX to FXa and/or of such nature that a stronger clot is formed.

[0093] Without being limited by any particular theory, it is presently believed that the enhanced membrane affinity results in a higher local concentration of the activated polypeptide variants in close proximity to the other coagulation factors, particularly FX. Thus, the rate of activation of FX to FXa will be higher, simply due to a higher molar ratio of the activated FVII variant to FX. The increased activation rate of FX then results in a higher amount of active thrombin, and thus a higher rate of cross-linking of fibrin.

[0094] Thus, in preferred embodiments of the invention the parent FVII or FVIIa polypeptide has been modified so that the resulting activated polypeptide variant has (as compared to rhFVIIa):

[0095] i) an increased bioavailability (AUC_{iv}) and an increased phospholipid membrane binding affinity;

[0096] ii) an increased bioavailability (AUC_{iv}) and an increased capability to activate FX to FXa;

[0097] iii) an increased bioavailability (AUC_{iv}) and is capable of generating a stronger clot (increased AUC_{throm});

[0098] iv) an increased bioavailability (AUC_{iv}) and a reduced T_{max};

[0099] v) an increased functional in vivo half-life and an increased phospholipid membrane binding affinity;

[0100] vi) an increased functional in vivo half-life and an increased capability to activate FX to FXa;

[0101] vii) an increased functional in vivo half-life and is capable of generating a stronger clot (increased AUC_{throm});

[0102] viii) an increased functional in vivo half-life and a reduced T_{max};

[0103] ix) an increased serum half-life and an increased phospholipid membrane binding affinity;

[0104] x) an increased serum half-life and an increased capability to activate FX to FXa;

[0105] xi) an increased serum half-life and is capable of generating a stronger clot (increased AUC_{throm}); and/or

[0106] xii) an increased serum half-life and a reduced T_{max}.

[0107] Consequently, medical treatment with a polypeptide variant according to the invention offers a number of advantages over the currently available rhFVIIa compound (NovoSeven®), such as administration of lower dosage, longer duration between injections, increased clot strength and/or faster action.

[0108] Thus, preferred variants of the invention are such variants which, in their activated forms and when compared to rhFVIIa, generates in increased Area Under the Curve when administered intravenously (AUC_{iv}), in particular when administered intravenously in rats. More particularly, variants of the present invention, which are preferred, are such variants where the ratio between the AUC_{iv} of said variant, in its actvated form, and the AUC_{iv} of rhFVIIa is at least 1.25, such as at least 1.5, e.g. at least 1.75, more preferably at least 2, such as at least 3, even more preferably at least 4, such as at least 5, in particular when administered (intravenously) in rats.

[0109] This effect may in turn (but do not necessarily do so) correspond to an increased functional in vivo half-life and/or an increased serum half-life as compared to rhFVIIa. Accordingly, in another preferred embodiment of the invention, the ratio between the functional in vivo half-life or the serum half-life for the variant, in its activated form, and the functional in vivo half-life or the serum half-life for rhFVIIa is at least 1.25. More preferably, the ratio between the relevant half-life for the variant, in its activated form, and the relevant half-life for hFVIIa or rhFVIIa is at least 1.5, such as at least 1.75, e.g. at least 2, even more preferably at least 3, such as at least 4, e.g. at least 5.

[0110] As will be understood, the variants of the invention also possess, in addition to the above-mentioned functionality (i.e. increased AUC_{iv}, increased functional in vivo half-life and/or increased serum half-life), an increased phospholipid membrane binding affinity as compared to rhFVII, an an increased capability to activate FX to Fxa, a capability of generating a stronger clot (increased AUC_{throm}) and/or a reduced T_{max}.

[0111] Thus, in one preferred embodiment of the invention, the polypeptide variant has (in addition to an increased AUC_{iv}, increased functional in vivo half-life and/or increased serum half-life) an increased phospholipid membrane binding affinity relative to rhFVIIa. Membrane binding affinity may be measured by methods known in the art, such as by the Biacore® assay described in K. Nagata and H. Handa (Ads.), Real-Time Analysis of Biomolecular Interactions, Springer-Verlag, Tokyo, 2000, Chapter 6 entitled "Lipid-Protein Interactions". Alternatively, the membrane binding affinity may be measured as described in Example 1 in WO 99/20767.

[0112] In another preferred embodiment of the invention, the polypeptide variant (in addition to an increased AUC_{iv}, increased functional in vivo half-life and/or increased serum half-life), has an increased FX activation activity as compared to rhFVIIa, in particular when assayed in a TF-independent assay, such as the "TF-independent Factor X Activation Assay" disclosed herein. More particularly, it is preferred that the ratio between the FX activation activity of the polypeptide variant, in its activated form, and the FX activation activity of rhFVIIa is at least 1.25 when assayed in the "TF-independent Factor X Activation Assay" disclosed herein. More preferably, the ratio between the FX activation activity of the variant, in its activated form, and the FX activation activity of rhFVIIa is at least 1.5, such as at least 1.75, e.g. at least 2, even more preferably at least 3, such as at least 4, e.g. at least 5, still more preferably at least 6, such as at least 7, e.g. at least 8, most preferably at least 9, such as at least 10, when assayed in the "TF-independent Factor X Activation Assay" described herein.

[0113] In still another preferred embodiment of the invention, the polypeptide variant is (in addition to an increased AUC_{iv}, increased functional in vivo half-life and/or increased serum half-life), in its activated form, capable of generating a stronger clot as compared to rhFVIIa. This effect may be determined in the "Thrombogram Assay" described herein as an increase in the Area Under the Curve (AUC_{throm}). The AUC_{throm} is also sometimes denoted "total thrombin work" and constitutes a measure for the strength of the clot formed. More particularly, it is preferred that the ratio between the AUC_{throm}, generated by the variant in its activated form, and the AUC_{throm} generated by rhFVIIa is at least 1.15 when assayed in the "Thrombogram Assay" described herein. More preferably, the ratio is at least 1.2, such as at least 1.25, e.g. at least 1.3, even more preferably at least 1.4, such as at least 1.5, e.g. at least 1.6, most preferably at least 1.7, such as at least 1.8, e.g. at least 1.9 or at least 2.

[0114] In even another preferred embodiment of the invention the polypeptide variant has (in addition to an increased AUC_{iv}, increased functional in vivo half-life and/or increased serum half-life), in its activated form, a faster action. This effect may be determined in the "Thrombogram Assay" described herein as a reduction in the time needed to reach maximum thrombin level (T_{max}). Accordingly, preferred variants are such variants where the ratio between T_{max} for the variant, in its activated form, and T_{max} for rhFVIIa is at the most 0.95 when assayed in the "Thrombogram Assay" described herein. Preferably, the ratio is at the most 0.9, such as at the most 0.8, e.g. at the most 0.7, more preferably at the most 0.6, such as at the most 0.5.

Introduction of In Vivo N-Glycosylation Sites Located Outside the Gla Domain

[0115] A number of suitable modifications leading to an increase in AUC_{iv}, functional in vivo half-life and/or serum half-life is disclosed in WO 01/58935. The variants disclosed in WO 01/58935 are the result of a generally new strategy for developing improved FVII or FVIIa molecules, which may also be used for the parent FVII or FVIIa polypeptide of the present invention.

[0116] The position to be modified is preferably selected from a part of the FVII or FVIIa molecule that is located outside the tissue factor binding site, and/or outside the active site region, and/or outside the ridge of the active site binding cleft. These sites/regions are identified in Example 1 herein. It should be emphasized, however, that in certain situations, e.g. in case an inactivated polypeptide variant is

desired, it may be advantageous to perform modifications in or close to such regions. For example, it is contemplated that one or more in vivo N-glycosylation sites may advantageously be introduced in the active site region or at the ridge of the active site binding cleft of the FVII or FVIIa molecule. The active site region, the tissue factor binding site and the ridge of the active site binding cleft are defined in Example 1 herein and are constituted by the following residues:

[0117] I153, Q167, V168, L169, L170, L171, Q176, L177, C178, G179, G180, T181, V188, V189, S190, A191, A192, H193, C194, F195, D196, K197, 1198, W201, V228, I229, I230, P231, S232, T233, Y234, V235, P236, G237, T238, T239, N240, H241, D242, I243, A244, L245, L246, V281, S282, G283, W284, G285, Q286, T293, T324, E325, Y326, M327, F328, D338, S339, C340, K341, G342, D343, S344, G345, G346, P347, H348, L358, T359, G360, 1361, V362, S363, W364, G365, C368, V376, Y377, T378, R379, V380, Q382, Y383, W386, L387, L400 and F405 (active site region);

[0118] L13, K18, F31, E35, R36, L39, F40, I42, S43, S60, K62, D63, Q64, L65, I69, C70, F71, C72, L73, P74, F76, E77, G78, R79, E82, K85, Q88, I90, V92, N93, E94, R271, A274, F275, V276, R277, F278, R304, L305, M306, T307, Q308, D309, Q312, Q313, E325 and R379 (tissue factor binding site); and

[0119] N173, A175, K199, N200, N203, D289, R290, G291, A292, P321 and T370 (the ridge of the active site binding cleft).

[0120] The total number of amino acid residues to be modified outside the Gla domain in the parent FVII or FVIIa polypeptide (as compared to the amino acid sequence shown in SEQ ID NO:2) will typically not exceed 10. Preferably, the FVII or FVIIa variant comprises an amino acid sequence which differs in 1-10 amino acid residues from amino acid residues 46-406 shown in SEQ ID NO:2, typically in 1-8 or in 2-8 amino acid residues, e.g. in 1-5 or in 2-5 amino acid residues, such as in 1-4 or in 1-3 amino acid residues, e.g. in 1, 2 or 3 amino acid residues from amino acid residues 46-406 shown in SEQ ID NO:2.

[0121] Thus, the polypeptide variant of the invention may contain 1-10 (additional or introduced) in vivo N-glycosylation sites, typically 1-8 or 2-8 (additional or introduced) in vivo N-glycosylation sites, preferably 1-5 or 2-5 (additional or introduced) in vivo N-glycosylation sites, such as 1-4 or 1-3 (additional or introduced) in vivo N-glycosylation sites, e.g. 1, 2 or 3 (additional or introduced) in vivo N-glycosylation sites. Analogously, the polypeptide variant of the invention may contain 1-10 (additional or introduced) sugar moieties, typically 1-8 or 2-8 (additional or introduced) sugar moieties, preferably 1-5 or 2-5 (additional or introduced) sugar moieties, such as 1-4 or 1-3 (additional or introduced) sugar moieties, e.g. 1, 2 or 3 (additional or introduced) sugar moieites. It will be understood that the introduced sugar moiety/moieties will be covalently attached to the introduced in vivo N-glycosylation site(s)

[0122] When used in the present context, the term "naturally occurring glycosylation site" covers the glycosylation sites at postions N145, N322, S52 and S60. In a similar way, the term "naturally occurring in vivo O-glycosylation site" includes the positions S52 and S60, whereas the term "naturally occurring in vivo N-glycosylation site" includes positions N145 and N322.

[0123] It will be understood that in order to prepare a polypeptide variant, wherein the polypeptide variant comprises one or more sugar moieties covalently attached to one or more in vivo N-glycosylation sites, the polypeptide variant must be expressed in a host cell capable of attaching sugar (oligosaccharide) moieties at the glycosylation site(s) or alternatively subjected to in vitro glycosylation. Examples of glycosylating host cells are given in the section further below entitled "Coupling to a sugar moiety".

[0124] Examples of positions, wherein the in vivo N-glycosylation sites may be introduced include, but is not limited to, positions comprising an amino acid residue having an amino acid residue having at least 25% of its side chain exposed to the surface (as defined in Example 1 herein), such as in a position comprising an amino acid residue having at least 50% of its side chain exposed to the surface (as defined in Example 1 herein). In general, it is preferred that the in vivo N-glycosylation site is introduced by substitution, although insertion is also contemplated. The position is preferably selected from a part of the molecule that is located outside the tissue factor binding site and/or the active site region and/or outside the ridge of the active site cleft. These sites/regions are identified in Example 1 herein. It should be understood that when the term "at least 25% (or at least 50%) of its side chain exposed to the surface" is used in connection with introduction of an in vivo N-glycosylation site this term refers to the surface accessibility of the amino acid side chain in the position where the sugar moiety is actually attached. In many cases it will be necessary to introduce a serine or a threonine residue in position +2 relative to the asparagine residue to which the sugar moiety is actually attached (unless, of course, this position is already occupied by a serine or a threonine residue) and these positions, where the serine or threonine residues are introduced, are allowed to be buried, i.e. to have less than 25% of their side chains exposed to the surface.

[0125] Specific and preferred examples of such substitutions creating an in vivo N-glycosylation site include a substitution selected from the group consisting of A51N, G58N, T106N, K109N, G124N, K143N+N145T, A175T, I205S, I205T, V253N, T267N, T267N+S269T, S314N+K316S, S314N+K316T, R315N+V317S, R315N+V317T, K316N+G318S, K316N+G318T, G318N, D334N and combinations thereof. More preferably, the in vivo N-glycosylation site is introduced by a substitution selected from the group consisting of A51N, G58N, T106N, K109N, G124N, K143N+N145T, A175T, I205T, V253N, T267N+S269T, S314N+K316T, R315N+V317T, K316N+G318T, G318N, D334N and combinations thereof. Even more preferably, the in vivo N-glycosylation site is introduced by a substitution selected from the group consisting of T106N, A175T, I205T, V253N, T267N+S269T and combinations thereof, in particular I205T.

[0126] In one embodiment, only one in vivo N-glycosylation site has been introduced by substitution. In another embodiment, two or more (such as two) in vivo N-glycosylation sites have been introduced by substitution. Examples of preferred substitutions creating two in vivo N-glycosylation sites include substitutions selected from the group consisting of A51N+G58N, A51N+T106N, A51N+K109N, A51N+G124N, A51N+K143N+N145T, A51N+A175T, A51N+I205T, A51N+V253N, A51N+T267N+S269T, A51N+S314N+K316T, A51N+R315N+V317T,

A51N+K316N+G318T, A51N+G318N, A51N+D334N, G58N+T106N, G58N+K109N, G58N+G124N, G58N+K143N+N145T, G58N+A175T, G58N+I205T, G58N+V253N, G58N+T267N+S269T, G58N+S314N+K316T, G58N+R315N+V317T, G58N+K316N+G318T, G58N+G318N, G58N+D334N, T106N+K109N, T106N+G124N, T106N+K143N+N145T, T106N+A175T, T106N+I205T, T106N+V253N, T106N+T267N+S269T, T106N+S314N+K316T, T106N+R315N+V317T, T106N+K316N+G318T, T106N+G318N, T106N+D334N, K109N+G124N, K109N+K143N+N145T, K109N+A175T, K109N+I205T, K109N+V253N, K109N+T267N+S269T, K109N+S314N+K316T, K109N+R315N+V317T, K109N+K316N+G318T, K109N+G318N, K109N+D334N, G124N+K143N+N145T, G124N+A175T, G124N+I205T, G124N+V253N, G124N+T267N+S269T, G124N+S314N+K316T, G124N+R315N+V317T, G124N+K316N+G318T, G124N+G318N, G124N+D334N, K143N+N145T+A175T, K143N+N145T+I205T, K143N+N145T+V253N, K143N+N145T+T267N+S269T, K143N+N 145T+S314N+K316T, K143N+N145T+R315N+V317T, K143N+N145T+K316N+G318T, K143N+N145T+G318N, K143N+N145T+D334N, A175T+I205T, A175T+V253N, A175T+T267N+S269T, A175T+S314N+K316T, A175T+R315N+V317T, A175T+K316N+G318T, A175T+G318N, A175T+D334N, I205T+V253N, I205T+T267N+S269T, I205T+S314N+K316T, I205T+R315N+V317T, I205T+K316N+G318T, I205T+G318N, I205T+D334N, V253N+T267N+S269T, V253N+S314N+K316T, V253N+R315N+V317T, 253N+K316N+G318T, V253N+G318N, V253N+D334N, T267N+S269T+S314N+K316T, 267N+S269T+R315N+V317T, T267N+S269T+K316N+G318T, T267N+S269T+G318N, T267N+S269T+D334N, S314N+K316T+R315N+V317T, S314N+K316T+G318N, S314N+K316T+D334N, R315N+V317T+K316N+G318T, R315N+V317T+G318N, R315N+V317T+D334N and G318N+D334N. More preferably, the substitutions are selected from the group consisiting of T106N+A175T, T106N+I205T, T106N+V253N, T106N+T267N+S269T, A175T+I205T, A175T+V253N, A175T+T267N+S269T, I205T+V253N, I205T+T267N+S269T and V253N+T267N+S269T, even more preferably from the group consisiting of T106N+I205T, T106N+V253N and I205T+T267N+S269T.

[0127] In an even further embodiment, three or more (such as three) in vivo N-glycosylation sites have been introduced by substitution. Examples of preferred substitutions creating three in vivo N-glycosylation sites include substitutions selected from the group consisiting of I205T+V253N+T267N+S269T and T106N+I205T+V253N.

[0128] As discussed above, it is preferred that the in vivo N-glycosylation site is introduced in a position which does neither form part of the tissue factor binding site nor form part of the active site region and the ridge of the active site binding cleft as defined herein. It is envisaged that such glycosylation variants will primarily belong to the class of active polypeptide variants as defined hereinbefore.

[0129] It will be understood that as an alternative to introduction of in vivo N-glycosylation sites in the above-discussed positions one may introduce (either by substitution or by insertion) cysteine residues in the same positions, where the introduced cysteine residue is then subsequently covalently attached to a non-polypeptide moiety, such as PEG, in particular mPEG. Thus, examples of positions, wherein a cysteine residue may be introduced include, but is not limited to, positions comprising an amino acid residue having an amino acid residue having at least 25% of its side chain exposed to the surface (as defined in Example 1 herein), such as in a position comprising an amino acid residue having at least 50% of its side chain exposed to the surface (as defined in Example 1 herein). The position is preferably selected from a part of the molecule that is located outside the tissue factor binding site and/or the active site region and/or outside the ridge of the active site cleft. These sites/regions are identified in Example 1 herein. Thus, the above disclosure concerning introduction of in vivo N-glycosylation sites applies mutatis mutandis to introduction of cysteine residues.

[0130] It will be understood that the modifications in positions located outside the Gla domain discussed in the above section should be combined with one or more modifications in the Gla domain (see the section entitled "Modifications in the Gla domain" below).

Modifications in the Gla Domain

[0131] As will be understood the variants of the present invention comprises, in addition to at least one introduced in vivo N-glycosylation site located outside the Gla domain (cf. above), at least two substitutions in the Gla domain, namely a substitution in position 10 and position 32.

[0132] A number of suitable modifications leading to increased phospholipid membrane binding affinity is disclosed in WO 99/20767 and WO 00/66753.

[0133] In a preferred embodiment of the invention the substitution in position 10 is P10Q. In another preferred embodiment of the invention the substitution in position 32 is K32E. In a particularly preferred embodiment of the invention the variant comprises the following substitutions P10Q+K32E.

[0134] In an interesting embodiment of the invention the variant comprises, in addition to the substitutions in position 10 and 32, such as in addition to the substitutions P10Q+K32E, at least one further modification in the Gla domain.

[0135] In one preferred embodiment of the invention the further modification in the Gla domain comprises an amino acid substitution in position 33. Preferably, a hydrophobic amino acid residue is introduced by substitution in position 33, such as D33I, D33L, D33M, D33V, D33F, D33Y or D33W, in particular D33F. Accordingly, in one very interesting embodiment of the invention, the variant comprises the following substitutions P10Q+K32+D33F.

[0136] In another preferred embodiment of the invention the further modification in the Gla domain comprises an insertion of at least one (such as one) amino acid residue between position 3 and 4. It is preferred that the inserted amino acid residue is a hydrophobic amino acid residue. Most preferably the insertion is A3AY. Accordingly, in another very interesting embodiment of the invention, the variant comprises the following modifications A3AY+P10Q+K32E or A3AY+P10Q+K32E+D33F.

[0137] In still another preferred embodiment of the invention the further modification in the Gla domain comprises a substitution in position 34. It is preferred that a negatively charged amino acid residue is introduced by substitution in position 34. Most preferably the substitution is A34E. Accordingly, in still another very interesting embodiment of

the invention, the variant comprises the following modifications P10Q+K32E+A34E, P10Q+K32E+D33F+A34E, A3AY+P10Q+K32E+A34E or A3AY+P10Q+K32E+D33F+A34E.

[0138] The Gla domain may also contain modifications in other positions, in particular in positions 8, 11 and 28, such as R28F or R28E. On the other hand it should be understood that the Gla domain should not be modified to such an extent that the membrane binding properties are impaired. Accordingly, it is preferred that no modifications are made in the residues that become 7-carboxylated, i.e. it is preferred that no modifications are made in residues 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35. In a similar way, it is in general not preferred that non-polypeptide moieties, such as sugar moieties and/or PEG groups, are introduced in the Gla domain. Consequently, it is preferred that no modifications are made in the Gla domain that creates an in vivo N-glycosylation site.

[0139] Finally, it will be understood that the modifications in the Gla domain discussed in this section must be combined with one or more of the modifications disclosed in the section entitled "Introduction of in vivo N-glycosylation sites located outside the Gla domain" above.

[0140] Specific examples of such "combined" variants are given below.

[0141] In one embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+T106N.

[0142] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A175T.

[0143] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+I205T.

[0144] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+V253N.

[0145] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+T267+S269T.

[0146] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+T1106N+I205T.

[0147] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+T106N+V253N.

[0148] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+I205T+T267N+S269T.

[0149] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+T106N.

[0150] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A175T.

[0151] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+I205T.

[0152] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+V253N.

[0153] In a further preferred embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+T267+S269T.

[0154] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+T106N+I205T.

[0155] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+T106N+V253N.

[0156] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+I205T+T267N+S269T.

[0157] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+T106N.

[0158] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A175T.

[0159] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+I205T.

[0160] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+V253N.

[0161] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+T267+S269T.

[0162] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+T106N+I205T.

[0163] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+T106N+V253N.

[0164] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+I205T+T267N+S269T.

[0165] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+T 106N.

[0166] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A175T.

[0167] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+T205T.

[0168] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+V253N.

[0169] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+T267+S269T.

[0170] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+T106N+I205T.

[0171] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+T106N+V253N.

[0172] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+I205T+T267N+S269T.

[0173] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+T106N.

[0174] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+A175T.

[0175] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+I205T.

[0176] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+V253N.

[0177] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+T267+S269T.

[0178] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+T106N+I205T.

[0179] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+T106N+V253N.

[0180] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+I205T+T267N+S269T.

[0181] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E+T106N.

[0182] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E A175T.

[0183] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E I205T.

[0184] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E V253N.

[0185] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E T267+S269T.

[0186] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E T106N+I205T.

[0187] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E T106N+V253N.

[0188] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E I205T+T267N+S269T.

[0189] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E+T106N.

[0190] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E A175T.

[0191] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E I205T.

[0192] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E V253N.

[0193] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E T267+S269T.

[0194] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E T106N+I205T.

[0195] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E TI 06N+V253N.

[0196] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E I205T+T267N+S269T.

[0197] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E+T106N.

[0198] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E A175T.

[0199] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E I205T.

[0200] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E V253N.

[0201] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E T267+S269T.

[0202] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E T106N+I205T.

[0203] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E T106N+V253N.

[0204] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E I205T+T267N+S269T.

Other Modifications Outside the Gla Domain

[0205] In a further embodiment of the present invention, the FVII or FVIIa variant may, in addition to the modifications described in the sections above, also contain mutations,

which are already known to increase the intrinsic activity of the polypeptide, e.g. such as those described in WO 02/22776

[0206] Examples of preferred substitutions include substitutions selected from the group consisting of V158D, E296D, M298Q, L305V and K337A. More preferably, said substitutions are selected from the group consisting of V158D+E296D+M298Q+L305V+K337A, V158D+E296D+M298Q+K337A, V158D+E296D+M298Q+L305V, V158D+E296D+M298Q, M298Q, L305V+K337A, L305V and K337A.

[0207] In a further embodiment of the present invention, the FVII or FVIIa variant may, in addition to the modifications described in the sections above, also contain mutations, which are already known to cause a decreased inhibition by TFPI. One example includes the substitution K341Q disclosed by Neuenschwander et al, Biochemistry, 1995; 34:8701-8707.

[0208] Moreover, the variant may contain modifications which are belived to increase the TF binding affinity. Examples of such modifications include substitutions selected from the group consisting of L39E, L39Q, L39H, 142K, 142R, S43H, S43Q, K62E, K62R, L65Q, L65S, F71D, F71Y, F71E, F71Q, F71N, E82Q, E82N, E82K and F275H

[0209] As already indicated above, the variant may also contain conservative amino acid substitutions.

The Non-Polypeptide Moiety

[0210] Based on the present disclosure the skilled person will be aware that amino acid residues comprising other attachment groups may be introduced by substitution into the parent polypeptide, using the same approach as that illustrated above with in vivo N-glycosylation sites. For instance, one or more amino acid residues comprising an acid group (glutamic acid or aspartic acid), tyrosine or lysine may be introduced into the positions discussed above. In particular, one or more cysteine residues may be introduced in the positions discussed above.

[0211] As indicated further above the non-polypeptide moiety of the conjugated variant is preferably selected from the group consisting of a polymer molecule, a lipophilic compound, a sugar moiety (by way of in vivo glycosylation) and an organic derivatizing agent. All of these agents may confer desirable properties to the variant polypeptide, in particular increased AUC_{iv}, increased functional in vivo half-life and/or increased plasma half-life. The variant polypeptide is normally conjugated to only one type of non-polypeptide moiety, but may also be conjugated to two or more different types of non-polypeptide moieties, e.g. to a polymer molecule and a sugar moiety, to a lipophilic group and a sugar moiety, to an organic derivatizing agent and a sugar moiety, to a lipophilic group and a polymer molecule, etc. The conjugation to two or more different non-polypeptide moieties may be done simultaneous or sequentially.

Methods of Preparing a Conjugated Variant of the Invention

[0212] In the following sections "Conjugation to a lipophilic compound", "Conjugation to a polymer molecule", "Conjugation to a sugar moiety" and "Conjugation to an organic derivatizing agent" conjugation to specific types of non-polypeptide moieties is described. In general, a conjugated variant according to the invention may be produced by culturing an appropriate host cell under conditions conducive for the expression of the variant polypeptide, and recovering the variant polypeptide, wherein a) the variant polypeptide comprises at least one N- or O-glycosylation site and the host cell is an eukaryotic host cell capable of in vivo glycosylation, and/or b) the variant polypeptide is subjected to conjugation to a non-polypeptide moiety in vitro.

[0213] It will be understood that the conjugation should be designed so as to produce the optimal molecule with respect to the number of non-polypeptide moieties attached, the size and form of such molecules (e.g. whether they are linear or branched), and the attachment site(s) in the polypeptide. The molecular weight of the non-polypeptide moiety to be used may, e.g., be chosen on the basis of the desired effect to be achieved. For instance, if the primary purpose of the conjugation is to achieve a conjugated variant having a high molecular weight (e.g. to reduce renal clearance) it is usually desirable to conjugate as few high molecular weight non-polypeptide moieties as possible to obtain the desired molecular weight. When a high degree of shielding is desirable this may be obtained by use of a sufficiently high number of low molecular weight non-polypeptide moieties (e.g. with a molecular weight of from about 300 Da to about 5 kDa, such as a molecular weight of from 300 Da to 2 kDa).

Conjugation to a Polymer Molecule

[0214] The polymer molecule to be coupled to the variant polypeptide may be any suitable polymer molecule, such as a natural or synthetic homo-polymer or hetero-polymer, typically with a molecular weight in the range of about 300-100,000 Da, such as about 500-20,000 Da, more preferably in the range of about 500-15,000 Da, even more preferably in the range of about 2-12 kDa, such as in the range of about 3-10 kDa. When the term "about" is used herein in connection with a certain molecular weight, the word "about" indicates an approximate average molecular weight and reflects the fact that there will normally be a certain molecular weight distribution in a given polymer preparation.

[0215] Examples of homo-polymers include a polyol (i.e. poly-OH), a polyamine (i.e. poly-NH_2) and a polycarboxylic acid (i.e. poly-COOH). A hetero-polymer is a polymer comprising different coupling groups, such as a hydroxyl group and an amine group.

[0216] Examples of suitable polymer molecules include polymer molecules selected from the group consisting of polyalkylene oxide (PAO), including polyalkylene glycol (PAG), such as polyethylene glycol (PEG) and polypropylene glycol (PPG), branched PEGs, poly-vinyl alcohol (PVA), poly-carboxylate, poly-(vinylpyrolidone), polyethylene-co-maleic acid anhydride, polystyrene-co-maleic acid anhydride, dextran, including carboxymethyl-dextran, or any other biopolymer suitable for reducing immunogenicity and/or increasing functional in vivo half-life and/or serum half-life. Another example of a polymer molecule is human albumin or another abundant plasma protein. Generally, polyalkylene glycol-derived polymers are biocompatible, non-toxic, non-antigenic, non-immunogenic, have various water solubility properties, and are easily excreted from living organisms.

[0217] PEG is the preferred polymer molecule, since it has only few reactive groups capable of cross-linking compared

to, e.g., polysaccharides such as dextran. In particular, mono-functional PEG, e.g. methoxypolyethylene glycol (mPEG), is of interest since its coupling chemistry is relatively simple (only one reactive group is available for conjugating with attachment groups on the polypeptide). Consequently, as the risk of cross-linking is eliminated, the resulting conjugated variants are more homogeneous and the reaction of the polymer molecules with the variant polypeptide is easier to control.

[0218] To effect covalent attachment of the polymer molecule(s) to the variant polypeptide, the hydroxyl end groups of the polymer molecule must be provided in activated form, i.e. with reactive functional groups (examples of which include primary amino groups, hydrazide (HZ), thiol, succinate (SUC), succinimidyl succinate (SS), succinimidyl succinamide (SSA), succinimidyl propionate (SPA), succinimidyl butyrate (SBA), succinimidy carboxymethylate (SCM), benzotriazole carbonate (BTC), N-hydroxysuccinimide (NHS), aldehyde, nitrophenylcarbonate (NPC), and tresylate (TRES)). Suitable activated polymer molecules are commercially available, e.g. from Shearwater Polymers, Inc., Huntsville, Ala., USA, or from PolyMASC Pharmaceuticals plc, UK.

[0219] Alternatively, the polymer molecules can be activated by conventional methods known in the art, e.g. as disclosed in WO 90/13540. Specific examples of activated linear or branched polymer molecules for use in the present invention are described in the Shearwater Polymers, Inc. 1997 and 2000 Catalogs (Functionalized Biocompatible Polymers for Research and pharmaceuticals, Polyethylene Glycol and Derivatives, incorporated herein by reference).

[0220] Specific examples of activated PEG polymers include the following linear PEGs: NHS-PEG (e.g. SPA-PEG, SSPA-PEG, SBA-PEG, SS-PEG, SSA-PEG, SC-PEG, SG-PEG, and SCM-PEG), and NOR-PEG, BTC-PEG, EPOX-PEG, NCO-PEG, NPC-PEG, CDI-PEG, ALD-PEG, TRES-PEG, VS-PEG, IODO-PEG, and MAL-PEG, and branched PEGs such as PEG2-NHS and those disclosed in U.S. Pat. No. 5,932,462 and U.S. Pat. No. 5,643,575, both of which are incorporated herein by reference. Furthermore, the following publications, incorporated herein by reference, disclose useful polymer molecules and/or PEGylation chemistries: U.S. Pat. No. 5,824,778, U.S. Pat. No. 5,476,653, WO 97/32607, EP 229,108, EP 402,378, U.S. Pat. No. 4,902,502, U.S. Pat. No. 5,281,698, U.S. Pat. No. 5,122,614, U.S. Pat. No. 5,219,564, WO 92/16555, WO 94/04193, WO 94/14758, WO 94/17039, WO 94/18247, WO 94/28024, WO 95/00162, WO 95/11924, WO95/13090, WO 95/33490, WO 96/00080, WO 97/18832, WO 98/41562, WO 98/48837, WO 99/32134, WO 99/32139, WO 99/32140, WO 96/40791, WO 98/32466, WO 95/06058, EP 439 508, WO 97/03106, WO 96/21469, WO 95/13312, EP 921 131, U.S. Pat. No. 5,736,625, WO 98/05363, EP 809 996, U.S. Pat. No. 5,629,384, WO 96/41813, WO 96/07670, U.S. Pat. No. 5,473,034, U.S. Pat. No. 5,516,673, EP 605 963, U.S. Pat. No. 5,382,657, EP 510 356, EP 400 472, EP 183 503 and EP 154 316.

[0221] Specific examples of activated PEG polymers particularly preferred for coupling to cysteine residues, include the following linear PEGs: vinylsulfone-PEG (VS-PEG), preferably vinylsulfone-mPEG (VS-mPEG); maleimide-PEG (MAL-PEG), preferably maleimide-mPEG (MAL-mPEG) and orthopyridyl-disulfide-PEG (OPSS-PEG), preferably orthopyridyl-disulfide-mPEG (OPSS-mPEG). Typically, such PEG or mPEG polymers will have a size of about 5 kDa, about 10 kD, about 12 kDa or about 20 kDa.

[0222] The conjugation of the polypeptide variant and the activated polymer molecules is conducted by use of any conventional method, e.g. as described in the following references (which also describe suitable methods for activation of polymer molecules): Harris and Zalipsky, eds., Poly(ethylene glycol) Chemistry and Biological Applications, AZC, Washington; R. F. Taylor, (1991), "Protein immobilisation. Fundamental and applications", Marcel Dekker, N.Y.; S. S. Wong, (1992), "Chemistry of Protein Conjugation and Crosslinking", CRC Press, Boca Raton; G. T. Hermanson et al., (1993), "Immobilized Affinity Ligand Techniques", Academic Press, N.Y.).

[0223] The skilled person will be aware that the activation method and/or conjugation chemistry to be used depends on the attachment group(s) of the variant polypeptide (examples of which are given further above), as well as the functional groups of the polymer (e.g. being amine, hydroxyl, carboxyl, aldehyde, sulfydryl, succinimidyl, maleimide, vinysulfone or haloacetate). The PEGylation may be directed towards conjugation to all available attachment groups on the variant polypeptide (i.e. such attachment groups that are exposed at the surface of the polypeptide) or may be directed towards one or more specific attachment groups, e.g. the N-terminal amino group as described in U.S. Pat. No. 5,985,265 or to cysteine residues. Furthermore, the conjugation may be achieved in one step or in a stepwise manner (e.g. as described in WO is 99/55377).

[0224] For PEGylation to cysteine residues (see above) the FVII or FVIIa variant is usually treated with a reducing agent, such as dithiothreitol (DDT) prior to PEGylation. The reducing agent is subsequently removed by any conventional method, such as by desalting. Conjugation of PEG to a cysteine residue typically takes place in a suitable buffer at pH 6-9 at temperatures varying from 4° C. to 25° C. for periods up to 16 hours.

[0225] It will be understood that the PEGylation is designed so as to produce the optimal molecule with respect to the number of PEG molecules attached, the size and form of such molecules (e.g. whether they are linear or branched), and the attachment site(s) in the variant polypeptide. The molecular weight of the polymer to be used may e.g. be chosen on the basis of the desired effect to be achieved.

[0226] In connection with conjugation to only a single attachment group on the protein (e.g. the N-terminal amino group), it may be advantageous that the polymer molecule, which may be linear or branched, has a high molecular weight, preferably about 10-25 kDa, such as about 15-25 kDa, e.g. about 20 kDa.

[0227] Normally, the polymer conjugation is performed under conditions aimed at reacting as many of the available polymer attachment groups with polymer molecules. This is achieved by means of a suitable molar excess of the polymer relative to the polypeptide. Typically, the molar ratios of activated polymer molecules to polypeptide are up to about 1000-1, such as up to about 200-1, or up to about 100-1. In some cases the ration may be somewhat lower, however, such as up to about 50-1, 10-1, 5-1, 2-1 or 1-1 in order to obtain optimal reaction.

[0228] It is also contemplated according to the invention to couple the polymer molecules to the polypeptide through a linker. Suitable linkers are well known to the skilled person. A preferred example is cyanuric chloride (Abuchowski et al., (1977), J. Biol. Chem., 252, 3578-3581; U.S. Pat. No. 4,179,337; Shafer et al., (1986), J. Polym. Sci. Polym. Chem. Ed., 24, 375-378).

[0229] Subsequent to the conjugation, residual activated polymer molecules are blocked according to methods known in the art, e.g. by addition of primary amine to the reaction mixture, and the resulting inactivated polymer molecules are removed by a suitable method.

[0230] It will be understood that depending on the circumstances, e.g. the amino acid sequence of the variant polypeptide, the nature of the activated PEG compound being used and the specific PEGylation conditions, including the molar ratio of PEG to polypeptide, varying degrees of PEGylation may be obtained, with a higher degree of PEGylation generally being obtained with a higher ratio of PEG to variant polypeptide. The PEGylated variant polypeptides resulting from any given PEGylation process will, however, normally comprise a stochastic distribution of conjugated polypeptide variants having slightly different degrees of PEGylation.

Coupling to a Sugar Moiety

[0231] In order to achieve in vivo glycosylation of a FVII molecule comprising one or more glycosylation sites the nucleotide sequence encoding the variant polypeptide must be inserted in a glycosylating, eucaryotic expression host. The expression host cell may be selected from fungal (filamentous fungal or yeast), insect or animal cells or from transgenic plant cells. In one embodiment the host cell is a mammalian cell, such as a CHO cell, BHK or HEK, e.g. HEK 293, cell, or an insect cell, such as an SF9 cell, or a yeast cell, e.g. *S. cerevisiae* or *Pichia pastoris*, or any of the host cells mentioned hereinafter.

[0232] Covalent in vitro coupling of sugar moieties (such as dextran) to amino acid residues of the variant polypeptide may also be used, e.g. as described, for example in WO 87/05330 and in Aplin etl al., CRC Crit Rev. Biochem, pp. 259-306, 1981. The in vitro coupling of sugar moieties or PEG to protein- and peptide-bound Gln-residues can be carried out by transglutaminases (TGases). Transglutaminases catalyse the transfer of donor amine-groups to protein- and peptide-bound Gln-residues in a so-called cross-linking reaction. The donor-amine groups can be protein- or peptide-bound, such as the s-amino-group in Lys-residues or it can be part of a small or large organic molecule. An example of a small organic molecule functioning as amino-donor in TGase-catalysed cross-linking is putrescine (1,4-diaminobutane). An example of a larger organic molecule functioning as amino-donor in TGase-catalysed cross-linking is an amine-containing PEG (Sato et al., 1996, Biochemistry 35, 13072-13080).

[0233] TGases, in general, are highly specific enzymes, and not every Gln-residues exposed on the surface of a protein is accessible to TGase-catalysed cross-linking to amino-containing substances. On the contrary, only few Gln-residues are naturally functioning as TGase substrates but the exact parameters governing which Gln-residues are good TGase substrates remain unknown. Thus, in order to render a protein susceptible to TGase-catalysed cross-linking reactions it is often a prerequisite at convenient positions to add stretches of amino acid sequence known to function very well as TGase substrates. Several amino acid sequences are known to be or to contain excellent natural TGase substrates e.g. substance P, elafin, fibrinogen, fibronectin, α_2-plasmin inhibitor, α-caseins, and β-caseins.

Conjugation to an Organic Derivatizing Agent

[0234] Covalent modification of the variant polypeptide may be performed by reacting one or more attachment groups of the variant polypeptide with an organic derivatizing agent. Suitable derivatizing agents and methods are well known in the art. For example, cysteinyl residues most commonly are reacted with x-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, α-bromo-β-(4-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole. Histidyl residues are derivatized by reaction with diethylpyrocarbonateat pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful. The reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0. Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing α-amino-containing residues include imidoesters such as methyl picolinimidate, pyridoxal phosphate, pyridoxal, chloroborohydride, trinitrobenzenesulfonic acid, O-methylisourea, 2,4-pentanedione and transaminase-catalyzed reaction with glyoxylate. Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pKa of the guanidine functional group.

[0235] Furthermore, these reagents may react with the groups of lysine as well as the arginine guanidino group. Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R—N═C═N—R'), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl)carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Conjugation to a Lipophilic Compound

[0236] The variant polypeptide and the lipophilic compound may be conjugated to each other, either directly or by use of a linker. The lipophilic compound may be a natural compound such as a saturated or unsaturated fatty acid, a fatty acid diketone, a terpene, a prostaglandin, a vitamine, a carotenoide or steroide, or a synthetic compound such as a carbon acid, an alcohol, an amine and sulphonic acid with one or more alkyl-, aryl-, alkenyl- or other multiple unsaturated compounds. The conjugation between the variant polypeptide and the lipophilic compound, optionally

through a linker may be done according to methods known in the art, e.g. as described by Bodanszky in Peptide Synthesis, John Wiley, New York, 1976 and in WO 96/12505.

Methods of Preparing a Polypeptide Variant of the Invention

[0237] The polypeptide variant of the present invention may be produced by any suitable method known in the art. Such methods include constructing a nucleotide sequence encoding the polypeptide variant and expressing the sequence in a suitable transformed or transfected host. Preferably, the host cell is a gammacarboxylating host cell such as a mammalian cell. However, polypeptide variants of the invention may be produced, albeit less efficiently, by chemical synthesis or a combination of chemical synthesis or a combination of chemical synthesis and recombinant DNA technology.

[0238] A nucleotide sequence encoding a polypeptide of the invention may be constructed by isolating or synthesizing a nucleotide sequence encoding the parent FVII, such as hFVII with the amino acid sequence shown in SEQ ID NO:2 and then changing the nucleotide sequence so as to effect introduction (i.e. insertion or substitution) or removal (i.e. deletion or substitution) of the relevant amino acid residue(s).

[0239] The nucleotide sequence is conveniently modified by site-directed mutagenesis in accordance with conventional methods. Alternatively, the nucleotide sequence is prepared by chemical synthesis, e.g. by using an oligonucleotide synthesizer, wherein oligonucleotides are designed based on the amino acid sequence of the desired polypeptide, and preferably selecting those codons that are favored in the host cell in which the recombinant polypeptide will be produced. For example, several small oligonucleotides coding for portions of the desired polypeptide may be synthesized and assembled by PCR, ligation or ligation chain reaction (LCR) (Barany, PNAS 88:189-193, 1991). The individual oligonucleotides typically contain 5' or 3' overhangs for complementary assembly.

[0240] Alternative nucleotide sequence modification methods are available for producing polypeptide variants for high throughput screening, for instance methods which involve homologous cross-over such as disclosed in U.S. Pat. No. 5,093,257, and methods which involve gene shuffling, i.e. recombination between two or more homologous nucleotide sequences resulting in new nucleotide sequences having a number of nucleotide alterations when compared to the starting nucleotide sequences. Gene shuffling (also known as DNA shuffling) involves one or more cycles of random fragmentation and reassembly of the nucleotide sequences, followed by screening to select nucleotide sequences encoding polypeptides with desired properties. In order for homology-based nucleic acid shuffling to take place, the relevant parts of the nucleotide sequences are preferably at least 50% identical, such as at least 60% identical, more preferably at least 70% identical, such as at least 80% identical. The recombination can be performed in vitro or in vivo.

[0241] Examples of suitable in vitro gene shuffling methods are disclosed by Stemmer et al. (1994), Proc. Natl. Acad. Sci. USA; vol. 91, pp. 10747-10751; Stemmer (1994), Nature, vol. 370, pp. 389-391; Smith (1994), Nature vol. 370, pp. 324-325; Zhao et al., Nat. Biotechnol. 1998, March; 16(3): 258-61; Zhao H. and Arnold, F B, Nucleic Acids Research, 1997, Vol. 25. No. 6 pp. 1307-1308; Shao et al., Nucleic Acids Research 1998, Jan 15; 26(2): pp. 681-83; and WO 95/17413.

[0242] An example of a suitable in vivo shuffling method is disclosed in WO 97/07205. Other techniques for mutagenesis of nucleic acid sequences by in vitro or in vivo recombination are disclosed e.g. in WO 97/20078 and U.S. Pat. No. 5,837,458. Examples of specific shuffling techniques include "family shuffling", "synthetic shuffling" and "in silico shuffling".

[0243] Family shuffling involves subjecting a family of homologous genes from different species to one or more cycles of shuffling and subsequent screening or selection. Family shuffling techniques are disclosed e.g. by Crameri et al. (1998), Nature, vol. 391, pp. 288-291; Christians et al. (1999), Nature Biotechnology, vol. 17, pp. 259-264; Chang et al. (1999), Nature Biotechnology, vol. 17, pp. 793-797; and Ness et al. (1999), Nature Biotechnology, vol. 17, 893-896.

[0244] Synthetic shuffling involves providing libraries of overlapping synthetic oligonucleotides based e.g. on a sequence alignment of homologous genes of interest. The synthetically generated oligonucleotides are recombined, and the resulting recombinant nucleic acid sequences are screened and if desired used for further shuffling cycles. Synthetic shuffling techniques are disclosed in WO 00/42561.

[0245] In silico shuffling refers to a DNA shuffling procedure, which is performed or modelled using a computer system, thereby partly or entirely avoiding the need for physically manipulating nucleic acids. Techniques for in silico shuffling are disclosed in WO 00/42560.

[0246] Once assembled (by synthesis, site-directed mutagenesis or another method), the nucleotide sequence encoding the polypeptide is inserted into a recombinant vector and operably linked to control sequences necessary for expression of the FVII in the desired transformed host cell.

[0247] It should of course be understood that not all vectors and expression control sequences function equally well to express the nucleotide sequence encoding the polypeptide variants described herein. Neither will all hosts function equally well with the same expression system. However, one of skill in the art may make a selection among these vectors, expression control sequences and hosts without undue experimentation. For example, in selecting a vector, the host must be considered because the vector must replicate in it or be able to integrate into the chromosome. The vector's copy number, the ability to control that copy number, and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered. In selecting an expression control sequence, a variety of factors should also be considered. These include, for example, the relative strength of the sequence, its controllability, and its compatibility with the nucleotide sequence encoding the polypeptide, particularly as regards potential secondary structures. Hosts should be selected by consideration of their compatibility with the chosen vector, the toxicity of the product coded for by the nucleotide

sequence, their secretion characteristics, their ability to fold the polypeptide variant correctly, their fermentation or culture requirements, and the ease of purification of the products coded for by the nucleotide sequence.

[0248] The recombinant vector may be an autonomously replicating vector, i.e. a vector, which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector is one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

[0249] The vector is preferably an expression vector, in which the nucleotide sequence encoding the polypeptide variant of the invention is operably linked to additional segments required for transcription of the nucleotide sequence. The vector is typically derived from plasmid or viral DNA. A number of suitable expression vectors for expression in the host cells mentioned herein are commercially available or described in the literature. Useful expression vectors for eukaryotic hosts, include, for example, vectors comprising expression control sequences from SV40, bovine papilloma virus, adenovirus and cytomegalovirus. Specific vectors are, e.g., pcDNA3.1(+)\Hyg (Invitrogen, Carlsbad, Calif., USA) and pCI-neo (Stratagene, La Jola, Calif., USA). Useful expression vectors for yeast cells include the 2μ plasmid and derivatives thereof, the POT1 vector (U.S. Pat. No. 4,931,373), the pJSO37 vector described in Okkels, Ann. New York Acad. Sci. 782, 202-207, 1996, and pPICZ A, B or C (Invitrogen). Useful vectors for insect cells include pVL941, pBG311 (Cate et al., "Isolation of the Bovine and Human Genes for Mullerian Inhibiting Substance And Expression of the Human Gene In Animal Cells", Cell, 45, pp. 685-98 (1986), pBluebac 4.5 and pMelbac (both available from Invitrogen). Useful expression vectors for bacterial hosts include known bacterial plasmids, such as plasmids from *E. coli*, including pBR322, pET3a and pET12a (both from Novagen Inc., WI, USA), wider host range plasmids, such as RP4, phage DNAs, e.g., the numerous derivatives of phage lambda, e.g., NM989, and other DNA phages, such as M13 and filamentous single stranded DNA phages.

[0250] Other vectors for use in this invention include those that allow the nucleotide sequence encoding the polypeptide variant to be amplified in copy number. Such amplifiable vectors are well known in the art. They include, for example, vectors able to be amplified by DHFR amplification (see, e.g., Kaufman, U.S. Pat. No. 4,470,461, Kaufman and Sharp, "Construction Of A Modular Dihydrafolate Reductase cDNA Gene: Analysis Of Signals Utilized For Efficient Expression", Mol. Cell. Biol., 2, pp. 1304-19 (1982)) and glutamine synthetase ("GS") amplification (see, e.g., U.S. Pat. No. 5,122,464 and EP 338,841).

[0251] The recombinant vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. An example of such a sequence (when the host cell is a mammalian cell) is the SV40 origin of replication. When the host cell is a yeast cell, suitable sequences enabling the vector to replicate are the yeast plasmid 2μ replication genes REP 1-3 and origin of replication.

[0252] The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or the *Schizosaccharomyces pombe* TPI gene (described by P. R. Russell, Gene 40, 1985, pp. 125-130), or one which confers resistance to a drug, e.g. ampicillin, kanamycin, tetracyclin, chloramphenicol, neomycin, hygromycin or methotrexate. For *Saccharomyces cerevisiae*, selectable markers include ura3 and leu2. For filamentous fungi, selectable markers include amdS, pyrG, arcB, niaD and sC.

[0253] The term "control sequences" is defined herein to include all components, which are necessary or advantageous for the expression of the polypeptide variant of the invention. Each control sequence may be native or foreign to the nucleic acid sequence encoding the polypeptide variant. Such control sequences include, but are not limited to, a leader sequence, polyadenylation sequence, propeptide sequence, promoter, enhancer or upstream activating sequence, signal peptide sequence, and transcription terminator. At a minimum, the control sequences include a promoter.

[0254] A wide variety of expression control sequences may be used in the present invention. Such useful expression control sequences include the expression control sequences associated with structural genes of the foregoing expression vectors as well as any sequence known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof.

[0255] Examples of suitable control sequences for directing transcription in mammalian cells include the early and late promoters of SV40 and adenovirus, e.g. the adenovirus 2 major late promoter, the MT-1 (metallothionein gene) promoter, the human cytomegalovirus immediate-early gene promoter (CMV), the human elongation factor 1α (EF-1α) promoter, the *Drosophila* minimal heat shock protein 70 promoter, the Rous Sarcoma Virus (RSV) promoter, the human ubiquitin C (UbC) promoter, the human growth hormone terminator, SV40 or adenovirus E1b region polyadenylation signals and the Kozak consensus sequence (Kozak, M. *J Mol Biol* 1987 Aug. 20;196(4):947-50).

[0256] In order to improve expression in mammalian cells a synthetic intron may be inserted in the 5' untranslated region of the nucleotide sequence encoding the polypeptide. An example of a synthetic intron is the synthetic intron from the plasmid pCI-Neo (available from Promega Corporation, WI, USA).

[0257] Examples of suitable control sequences for directing transcription in insect cells include the polyhedrin promoter, the P10 promoter, the *Autographa californica* polyhedrosis virus basic protein promoter, the baculovirus immediate early gene 1 promoter and the baculovirus 39K delayed-early gene promoter, and the SV40 polyadenylation sequence. Examples of suitable control sequences for use in yeast host cells include the promoters of the yeast α-mating system, the yeast triose phosphate isomerase (TPI) promoter, promoters from yeast glycolytic genes or alcohol dehydrogenase genes, the ADH2-4c promoter, and the inducible GAL promoter. Examples of suitable control sequences for use in filamentous fungal host cells include the ADH3 promoter and terminator, a promoter derived from the genes encoding *Aspergillus oryzae* TAKA amylase triose phosphate isomerase or alkaline protease, an *A. niger* α-amylase, *A. niger* or *A. nidulans* glucoamylase, *A. nidu-*

lans acetamidase, *Rhizomucor miehei* aspartic proteinase or lipase, the TPI1 terminator and the ADH3 terminator. Examples of suitable control sequences for use in bacterial host cells include promoters of the lac system, the trp system, the TAC or TRC system, and the major promoter regions of phage lambda.

[0258] The presence or absence of a signal peptide will, e.g., depend on the expression host cell used for the production of the polypeptide variant to be expressed (whether it is an intracellular or extracellular polypeptide) and whether it is desirable to obtain secretion. For use in filamentous fungi, the signal peptide may conveniently be derived from a gene encoding an *Aspergillus* sp. amylase or glucoamylase, a gene encoding a *Rhizomucor miehei* lipase or protease or a *Humicola lanuginosa* lipase. The signal peptide is preferably derived from a gene encoding *A. oryzae* TAKA amylase, *A. niger* neutral α-amylase, *A. niger* acid-stable amylase, or *A. niger* glucoamylase. For use in insect cells, the signal peptide may conveniently be derived from an insect gene (cf. WO 90/05783), such as the *Lepidopteran manduca sexta* adipokinetic hormone precursor, (cf. U.S. Pat. No. 5,023,328), the honeybee melittin (Invitrogen), ecdysteroid UDPglucosyltransferase (egt) (Murphy et al., Protein Expression and Purification 4, 349-357 (1993) or human pancreatic lipase (hpl) (Methods in Enzymology 284, pp. 262-272, 1997). A preferred signal peptide for use in mammalian cells is that of hFVII or the murine Ig kappa light chain signal peptide (Coloma, M (1992) J. Imm. Methods 152:89-104). For use in yeast cells suitable signal peptides have been found to be the α-factor signal peptide from *S. cereviciae* (cf. U.S. Pat. No. 4,870,008), a modified carboxypeptidase signal peptide (cf. L. A. Valls et al., Cell 48, 1987, pp. 887-897), the yeast BAR1 signal peptide (cf. WO 87/02670), the yeast aspartic protease 3 (YAP3) signal peptide (cf. M. Egel-Mitani et al., Yeast 6, 1990, pp. 127-137), and the synthetic leader sequence TA57 (WO98/32867). For use in *E. coli* cells a suitable signal peptide have been found to be the signal peptide ompA (EP581821).

[0259] The nucleotide sequence of the invention encoding a polypeptide variant, whether prepared by site-directed mutagenesis, synthesis, PCR or other methods, may optionally include a nucleotide sequence that encode a signal peptide. The signal peptide is present when the polypeptide variant is to be secreted from the cells in which it is expressed. Such signal peptide, if present, should be one recognized by the cell chosen for expression of the polypeptide variant. The signal peptide may, e.g. be that normally associated with hFVII) or, alternatively, the signal peptide may be from another source than hFVII, such as any of those normally associated with other human wild-type vitamin K-dependent polypeptides. Furthermore, the signal peptide may be a signal peptide normally expressed from the host cell or one which is not normally expressed from the host cell. Accordingly, the signal peptide may be prokaryotic, e.g. derived from a bacterium such as *E. coli*, or eukaryotic, e.g. derived from a mammalian, or insect or yeast cell.

[0260] Any suitable host may be used to produce the polypeptide variant, including bacteria (although not particularly preferred), fungi (including yeasts), plant, insect, mammal, or other appropriate animal cells or cell lines, as well as transgenic animals or plants. Examples of bacterial host cells include grampositive bacteria such as strains of *Bacillus*, e.g. *B. brevis* or *B. subtilis, Pseudomonas* or *Streptomyces*, or gramnegative bacteria, such as strains of *E. coli*. The introduction of a vector into a bacterial host cell may, for instance, be effected by protoplast transformation (see, e.g., Chang and Cohen, 1979, *Molecular General Genetics* 168: 111-115), using competent cells (see, e.g., Young and Spizizin, 1961, *Journal of Bacteriology* 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, *Journal of Molecular Biology* 56: 209-221), electroporation (see, e.g., Shigekawa and Dower, 1988, *Biotechniques* 6: 742-751), or conjugation (see, e.g., Koehler and Thorne, 1987, *Journal of Bacteriology* 169: 5771-5278). Examples of suitable filamentous fungal host cells include strains of *Aspergillus*, e.g. *A. oryzae, A. niger*, or *A. nidulans, Fusarium* or *Trichoderma*. Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known per se. Suitable procedures for transformation of *Aspergillus* host cells are described in EP 238 023 and U.S. Pat. No. 5,679,543. Suitable methods for transforming *Fusarium* species are described by Malardier et al., 1989, Gene 78: 147-156 and WO 96/00787. Examples of suitable yeast host cells include strains of *Saccharomyces*, e.g. *S. cerevisiae, Schizosaccharomyces, Klyveromyces, Pichia*, such as *P. pastoris* or *P. methzanolica, Hansenula*, such as *H. Polymorpha* or *Yarrowia*. Yeast may be transformed using the procedures described by Becker and Guarente, In Abelson, J. N. and Simon, M. I., editors, *Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology*, Volume 194, pp 182-187, Academic Press, Inc., New York; Ito et al., 1983, *Journal of Bacteriology* 153: 163; Hinnen et al., 1978, *Proceedings of the National Academy of Sciences USA* 75: 1920: and as disclosed by Clontech Laboratories, Inc, Palo Alto, Calif., USA (in the product protocol for the Yeastmaker™ Yeast Transformation System Kit). Examples of suitable insect host cells include a *Lepidoptora* cell line, such as *Spodoptera frugiperda* (Sf9 or Sf21) or *Trichoplusioa ni* cells (High Five) (U.S. Pat. No. 5,077,214). Transformation of insect cells and production of heterologous polypeptides therein may be performed as described by Invitrogen. Examples of suitable mammalian host cells include Chinese hamster ovary (CHO) cell lines, (e.g. CHO-K1; ATCC CCL-61), Green Monkey cell lines (COS) (e.g. COS 1 (ATCC CRL-1650), COS 7 (ATCC CRL-1651)); mouse cells (e.g. NS/O), Baby Hamster Kidney (BHK) cell lines (e.g. ATCC CRL-1632 or ATCC CCL-10), and human cells (e.g. HEK 293 (ATCC CRL-1573)), as well as plant cells in tissue culture. Additional suitable cell lines are known in the art and available from public depositories such as the American Type Culture Collection, Rockville, Md. Also, the mammalian cell, such as a CHO cell, may be modified to express sialyltransferase, e.g. 1,6-sialyltransferase, e.g. as described in U.S. Pat. No. 5,047,335, in order to provide improved glycosylation of the polypeptide variant.

[0261] In order to increase secretion it may be of particular interest to produce the polypeptide variant of the invention together with an endoprotease, in particular a PACE (paired basic amino acid converting enzyme) (e.g. as described in U.S. Pat. No. 5,986,079), such as a Kex2 endoprotease (e.g. as described in WO 00/28065).

[0262] Methods for introducing exogeneous DNA into mammalian host cells include calcium phosphate-mediated transfection, electroporation, DEAE-dextran mediated transfection, liposome-mediated transfection, viral vectors

and the transfection method described by Life Technologies Ltd, Paisley, UK using Lipofectamin 2000. These methods are well known in the art and e.g. described by Ausbel et al. (eds.), 1996, Current Protocols in Molecular Biology, John Wiley & Sons, New York, USA. The cultivation of mammalian cells are conducted according to established methods, e.g. as disclosed in (Animal Cell Biotechnology, Methods and Protocols, Edited by Nigel Jenkins, 1999, Human Press Inc, Totowa, N.J., USA and Harrison M A and Rae I F, General Techniques of Cell Culture, Cambridge University Press 1997).

[0263] In the production methods of the present invention, the cells are cultivated in a nutrient medium suitable for production of the polypeptide variant using methods known in the art. For example, the cell may be cultivated by shake flask cultivation, small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermenters performed in a suitable medium and under conditions allowing the polypeptide to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or may be prepared according to published compositions (e.g., in catalogues of the American Type Culture Collection). If the polypeptide variant is secreted into the nutrient medium, the polypeptide can be recovered directly from the medium. If the polypeptide variant is not secreted, it can be recovered from cell lysates.

[0264] The resulting polypeptide variant may be recovered by methods known in the art. For example, the polypeptide variant may be recovered from the nutrient medium by conventional procedures including, but not limited to, centrifugation, filtration, extraction, spray drying, evaporation, or precipitation.

[0265] The polypeptides may be purified by a variety of procedures known in the art including, but not limited to, chromatography (e.g., ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (e.g., preparative isoelectric focusing), differential solubility (e.g., ammonium sulfate precipitation), HPLC, or extraction (see, e.g., *Protein Purification*, J.-C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989).

[0266] Single chain polypeptide variants of the invention can be purified and activated to two-chain polypeptide variants by a number of methods as described in the literature (Broze and Majerus, 1980, J. Biol. Chem. 255:1242-47 and Hedner and Kisiel, 1983, J. Clin. Invest. 71:1836-41). Another method whereby single chain polypeptide variant can be purified is by incorporation of Zn ions during purification as described in U.S. Pat. No. 5,700,914. In a preferred embodiment the polypeptide variant is purified as a single chain polypeptide variant. The single chain polypeptide variant is activated by either use of an immobilized enzyme (e.g. factors Ia, IXa, Xa and XIIa) or by autoactivation using a positively charged ion exchange matrix or the like.

[0267] It is advantageous to first purify the polypeptide variant in its single chain form, then PEGylate (if desired) and last activate by one of the methods described above or by autoactivation as described by Pedersen et al, 1989, Biochemistry 28: 9331-36. The advantage of carrying out PEGylation before activation is that PEGylation of the new aminoterminal formed by cleavage of R152-I153 is avoided. PEGylation of this new amino terminal would render the molecule inactive since the formation of a hydrogen bond between D242 and the amino terminal of I153 is necessary for activity.

Pharmaceutical Composition of the Invention and its Use

[0268] In a further aspect, the present invention relates to a composition, in particular to a pharmaceutical composition, comprising a polypeptide variant of the invention and a pharmaceutically acceptable carrier or excipient.

[0269] The polypeptide variant or the pharmaceutical composition according to the invention may be used as a medicament.

[0270] Due to the improved properties mentioned hereinbefore, the polypeptide variants of the invention, or the pharmaceutical composition of the invention, are particular useful for the treatment of uncontrollable bleeding events in trauma patients, thrombocytopenic patients, patients in anticoagulant treatment, and cirrhosis patients with variceal bleeds, or other upper gastrointestinal bleedings, and in patients undergoing orthotopic liver transplantation, or liver resection (allowing for transfusion free surgery).

[0271] Trauma is defined as an injury to living tissue caused by an extrinsic agent. It is the 4th leading cause of death in the US and places a large financial burden on the economy.

[0272] Trauma is classified as either blunt or penetrative. Blunt trauma results in internal compression, organ damage and internal haemorrhage whereas penetrative trauma (as the consequence of an agent penetrating the body and destroying tissue, vessels and organs) results in external haemorrhage.

[0273] Haemorrhage, as a result of trauma, can start a cascade of problems. For example physiological compensation mechanisms are initiated with initial peripheral and mesenteric vasoconstriction to shunt blood to the central circulation. If circulation is not restored, hypovolemia shock (multiple organ failure due to inadequate perfusion) ensues. Since tissues throughout the body become starved for oxygen, anaerobic metabolism begins. However, the concomitant lactic acid leads the blood pH to drop and metabolic acidosis develops. If acidosis is severe and uncorrected, the patient may develop multisystem failure and die.

[0274] Although the majority of trauma patients are hypothermic on arrival in the emergency room due to the environmental conditions at the scene, inadequate protection, intravenous fluid administration and ongoing blood loss worsen the hypothermic state. Deficiencies in coagulation factors can result from blood loss or transfusions. Meanwhile, acidosis and hypothermia interfere with blood clotting mechanisms. Thus coagulopathy develops, which in turn, may mask surgical bleeding sites and hamper the control of mechanical bleeding. Hypothermia, coagulopathy and acidosis are often characterised as the "trauma triad of death"

[0275] Trauma may be caused by several events. For example, road traffic accidents result in many different types of trauma. Whilst some road traffic accidents are likely to result in penetrative trauma, many road traffic accidents are

likely to inflict blunt trauma to both head and body. However, these various types of trauma can all result in coagulopathy in the patient. Road traffic accidents are the leading cause of accidental death in the US. There are over 42,000 deaths from them in the US each year. Many trauma patients die at the location of the accident either whilst being treated by the paramedics, before they arrive or in transit to the ER.

[0276] Another example includes gunshot wounds. Gunshot wounds are traumas that can result in massive bleeding. They are penetrative and destroy tissue as the bullet passes through the body, whether it be in the torso or a limb. In the US about 40,000 people a year die from gunshot wounds

[0277] A further example includes falls. Falls result in a similar profile of trauma type to road traffic accidents. By falling onto a solid object or the ground from height can cause both penetrative and decelerative blunt trauma. In the US, falls are a common cause of accidental death, numbering about 13,000.

[0278] A still further example includes machinery accidents. A smaller number of people die in the US from machinery accident related deaths, whether struck by, or entangled in machinery. The figures are small but significant—around 2,000.

[0279] A still further example includes stab wounds. Stab wounds are penetrative injuries that can also cause massive bleeding. The organs most likely to be damaged in a stab wound are the liver, small intestine and the colon.

[0280] Cirrhosis of the liver is the terminal sequel of prolonged repeated injury to the hepatic parenchyma. The end result is the formation of broad bands of fibrous tissue separating regenerative nodules that do not maintain the normal organization of liver lobules and thus cause deteriorated liver function. Patients have prolonged prothrombin times as a result of the depletion of vitamin K-dependent coagulation factors. Pathogenetically, liver cirrhosis should be regarded as the final common pathway of chronic liver injury, which can result from any form of intense repeated prolonged liver cell injury. Cirrhosis of the liver may be caused by direct liver injury, including chronic alcoholism, chronic viral hepatitis (types B, C, and D), and auto immune hepatitis as well as by indirect injury by way of bile duct damage, including primary biliary cirrhosis, primary sclerosing cholangitis and biliary atresia. Less common causes of cirrhosis include direct liver injury from inherited disease such as cystic fibrosis, alpha-1-antitrypsin deficiency, hemochromatosis, Wilson's disease, galactosemia, and glycogen storage disease.

[0281] Transplantation is primarily reserved for late stage cirrhotic patients, where it is the key intervention for treating the disease. To be eligible for transplantation, a patient must be classified as Child's B or C, as well as meet additional criteria for selection. Last year, in the US alone, 4,954 transplants were performed.

[0282] It has been estimated that there are 6,000 bleeding episodes associated with patients undergoing resection each year. This correlates with the reserved position of this procedure although seems slightly high in comparison with transplantation numbers.

[0283] Accurate data on the incidence of variceal bleeding is hard to obtain. The key facts known are that at the time of diagnosis, varices are present in about 60% of decompensated and 30% of compensated patients and that about 30% of these patients with varices will experience a bleed and that each episode of variceal bleeding is associated with a 30% risk of mortality.

[0284] Thus, in a further aspect the present invention relates to a polypeptide variant of the invention for the manufacture of a medicament for the treatment of diseases or disorder wherein clot formation is desirable. A still further aspect of the present invention relates to a method for treating a mammal having a disease or disorder wherein clot formation is desirable, comprising administering to a mammal in need thereof an effective amount of the polypeptide variant or the pharmaceutical composition of the invention.

[0285] Thrombocytopenia is caused by one of three mechanisms-decreased bone marrow production, increased splenic sequestration, or accelerated destruction of platelets. Thrombocytopenia is a risk factor for hemorrhage, and platelet transfusion reduces the incidence of bleeding. The threshold for prophylactic platelet transfusion is 10,000/μl. In patients without fever or infections, a threshold of 5000/μl may be sufficient to prevent spontaneous hemorrhage. For invasive procedures, 50,000/μl platelets is the usual target level. In patients who develop antibodies to platelets following repeated transfusions, bleeding can be extremely difficult to control.

[0286] Examples of diseases/disorders wherein increased clot formation is desirable include, but is not limited to, hemorrhages, including brain hemorrhages, as well as patient with severe uncontrolled bleedings, such as trauma. Further examples include patients undergoing living transplantations, patients undergoing resection and patients with variceal bleedings.

[0287] The polypeptide variants of the invention is administered to patients in a therapeutically effective dose, normally one approximately paralleling that employed in therapy with rFVII such as NovoSeven®, or at lower dosage. By "therapeutically effective dose" herein is meant a dose that is sufficient to produce the desired effects in relation to the condition for which it is administered. The exact dose will depend on the circumstances, and will be ascertainable by one skilled in the art using known techniques. Normally, the dose should be capable of preventing or lessening the severity or spread of the condition or indication being treated. It will be apparent to those of skill in the art that an effective amount of a polypeptide variant or composition of the invention depends, inter alia, upon the disease, the dose, the administration schedule, whether the polypeptide variant or composition is administered alone or in conjunction with other therapeutic agents, the plasma half-life of the compositions, and the general health of the patient. Preferably, the polypeptide variant or composition of the invention is administered in an effective dose, in particular a dose which is sufficient to normalize the coagulation disorder.

[0288] The polypeptide variant of the invention is preferably administered in a composition including a pharmaceutically acceptable carrier or excipient. "Pharmaceutically acceptable" means a carrier or excipient that does not cause any untoward effects in patients to whom it is administered. Such pharmaceutically acceptable carriers and excipients are well known in the art (see, for example, Remington's

Pharmaceutical Sciences, 18th edition, A. R. Gennaro, Ed., Mack Publishing Company [1990]; Pharmaceutical Formulation Development of Peptides and Proteins, S. Frokjaer and L. Hovgaard, Eds., Taylor & Francis [2000]; and Handbook of Pharmaceutical Excipients, 3rd edition, A. Kibbe, Ed., Pharmaceutical Press [2000]).

[0289] The polypeptide variant of the invention can be formulated into pharmaceutical compositions by well-known methods. Suitable formulations are described by Remington's Pharmaceutical Sciences by E. W. Martin (Mark Publ. Co., 16th Ed., 1980).

[0290] The polypeptide variant of the invention can be used "as is" and/or in a salt form thereof. Suitable salts include, but are not limited to, salts with alkali metals or alkaline earth metals, such as sodium, potassium, calcium and magnesium, as well as e.g. zinc salts. These salts or complexes may by present as a crystalline and/or amorphous structure.

[0291] The pharmaceutical composition of the invention may be administered alone or in conjunction with other therapeutic agents. These agents may be incorporated as part of the same pharmaceutical composition or may be administered separately from the polypeptide variant of the invention, either concurrently or in accordance with another treatment schedule. In addition, the polypeptide variant or pharmaceutical composition of the invention may be used as an adjuvant to other therapies.

[0292] A "patient" for the purposes of the present invention includes both humans and other mammals. Thus, the methods are applicable to both human therapy and veterinary applications. The pharmaceutical composition comprising the polypeptide variant of the invention may be formulated in a variety of forms, e.g. as a liquid, gel, lyophilized, or as a compressed solid. The preferred form will depend upon the particular indication being treated and will be apparent to one skilled in the art.

[0293] In particular, the pharmaceutical composition comprising the polypeptide variant of the invention may be formulated in lyophilised or stable soluble form. The polypeptide variant may be lyophilised by a variety of procedures known in the art. The polypeptide variant may be in a stable soluble form by the removal or shielding of proteolytic degradation sites as described herein. The advantage of obtaining a stable soluble preparation lies in easier handling for the patient and, in the case of emergencies, quicker action, which potentially can become life saving. The preferred form will depend upon the particular indication being treated and will be apparent to one of skill in the art.

[0294] The administration of the formulations of the present invention can be performed in a variety of ways, including, but not limited to, orally, subcutaneously, intravenously, intracerebrally, intranasally, transdermally, intraperitoneally, intramuscularly, intrapulmonary, vaginally, rectally, intraocularly, or in any other acceptable manner. The formulations can be administered continuously by infusion, although bolus injection is acceptable, using techniques well known in the art, such as pumps or implantation. In some instances the formulations may be directly applied as a solution or spray.

Parentals

[0295] A preferred example of a pharmaceutical composition is a solution designed for parenteral administration. Although in many cases pharmaceutical solution formulations are provided in liquid form, appropriate for immediate use, such parenteral formulations may also be provided in frozen or in lyophilized form. In the former case, the composition must be thawed prior to use. The latter form is often used to enhance the stability of the active compound contained in the composition under a wider variety of storage conditions, as it is recognized by those skilled in the art that lyophilized preparations are generally more stable than their liquid counterparts. Such lyophilized preparations are reconstituted prior to use by the addition of one or more suitable pharmaceutically acceptable diluents such as sterile water for injection or sterile physiological saline solution.

[0296] In case of parenterals, they are prepared for storage as lyophilized formulations or aqueous solutions by mixing, as appropriate, the polypeptide variant having the desired degree of purity with one or more pharmaceutically acceptable carriers, excipients or stabilizers typically employed in the art (all of which are termed "excipients"), for example buffering agents, stabilizing agents, preservatives, isotonifiers, non-ionic surfactants or detergents, antioxidants and/or other miscellaneous additives.

[0297] Buffering agents help to maintain the pH in the range which approximates physiological conditions. They are typically present at a concentration ranging from about 2 mM to about 50 mM. Suitable buffering agents for use in the present invention include both organic and inorganic acids and salts thereof such as citrate buffers (e.g., monosodium citrate-disodium citrate mixture, citric acid-trisodium citrate mixture, citric acid-monosodium citrate mixture, etc.), succinate buffers (e.g., succinic acid-monosodium succinate mixture, succinic acid-sodium hydroxide mixture, succinic acid-disodium succinate mixture, etc.), tartrate buffers (e.g., tartaric acid-sodium tartrate mixture, tartaric acid-potassium tartrate mixture, tartaric acid-sodium hydroxide mixture, etc.), fumarate buffers (e.g., fumaric acid-monosodium fumarate mixture, fumaric acid-disodium fumarate mixture, monosodium fumarate-disodium fumarate mixture, etc.), gluconate buffers (e.g., gluconic acid-sodium glyconate mixture, gluconic acid-sodium hydroxide mixture, gluconic acid-potassium glyuconate mixture, etc.), oxalate buffer (e.g., oxalic acid-sodium oxalate mixture, oxalic acid-sodium hydroxide mixture, oxalic acid-potassium oxalate mixture, etc.), lactate buffers (e.g., lactic acid-sodium lactate mixture, lactic acid-sodium hydroxide mixture, lactic acid-potassium lactate mixture, etc.) and acetate buffers (e.g., acetic acid-sodium acetate mixture, acetic acid-sodium hydroxide mixture, etc.). Additional possibilities are phosphate buffers, histidine buffers and trimethylamine salts such as Tris.

[0298] Stabilizers refer to a broad category of excipients, which can range in function from a bulking agent to an additive which solubilizes the therapeutic agent or helps to prevent denaturation or adherence to the container wall. Typical stabilizers can be polyhydric sugar alcohols (enumerated above); amino acids such as arginine, lysine, glycine, glutamine, asparagine, histidine, alanine, ornithine, L-leucine, 2-phenylalanine, glutamic acid, threonine, etc., organic sugars or sugar alcohols, such as lactose, trehalose,

stachyose, mannitol, sorbitol, xylitol, ribitol, myoinisitol, galactitol, glycerol and the like, including cyclitols such as inositol; polyethylene glycol; amino acid polymers; sulfur-containing reducing agents, such as urea, glutathione, thioctic acid, sodium thioglycolate, thioglycerol, α-monothioglycerol and sodium thiosulfate; low molecular weight polypeptides (i.e. <10 residues); proteins such as human serum albumin, bovine serum albumin, gelatin or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; monosaccharides such as xylose, mannose, fructose and glucose; disaccharides such as lactose, maltose and sucrose; trisaccharides such as raffinose, and polysaccharides such as dextran. Stabilizers are typically present in the range of from 0.1 to 10,000 parts by weight based on the active protein weight.

[0299] Preservatives are added to retard microbial growth, and are typically added in amounts of about 0.2%-1% (w/v). Suitable preservatives for use with the present invention include phenol, benzyl alcohol, meta-cresol, methyl paraben, propyl paraben, octadecyldimethylbenzyl ammonium chloride, benzalkonium halides (e.g. benzalkonium chloride, bromide or iodide), hexamethonium chloride, alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol and 3-pentanol.

[0300] Isotonicifiers are added to ensure isotonicity of liquid compositions and include polyhydric sugar alcohols, preferably trihydric or higher sugar alcohols, such as glycerin, erythritol, arabitol, xylitol, sorbitol and mannitol. Polyhydric alcohols can be present in an amount between 0.1% and 25% by weight, typically 1% to 5%, taking into account the relative amounts of the other ingredients.

[0301] Non-ionic surfactants or detergents (also known as "wetting agents") may be present to help solubilizing the therapeutic agent as well as to protect the therapeutic polypeptide against agitation-induced aggregation, which also permits the formulation to be exposed to shear surface stress without causing denaturation of the polypeptide. Suitable non-ionic surfactants include polysorbates (20, 80, etc.), polyoxamers (184, 188 etc.), Pluronic® polyols, polyoxyethylene sorbitan monoethers (Tween®-20, Tween®-80, etc.).

[0302] Additional miscellaneous excipients include bulking agents or fillers (e.g. starch), chelating agents (e.g. EDTA), antioxidants (e.g., ascorbic acid, methionine, vitamin E) and cosolvents.

[0303] The active ingredient may also be entrapped in microcapsules prepared, for example, by coascervation techniques or by interfacial polymerization, for example hydroxymethylcellulose, gelatin or poly-(methylmethacylate) microcapsules, in colloidal drug delivery systems (for example liposomes, albumin micro spheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, supra.

[0304] Parenteral formulations to be used for in vivo administration must be sterile. This is readily accomplished, for example, by filtration through sterile filtration membranes.

Sustained Release Preparations

[0305] Examples of sustained-release preparations include semi-permeable matrices of solid hydrophobic polymers containing the polypeptide variant, the matrices having a suitable form such as a film or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate) or poly(vinylalcohol)), polylactides, copolymers of L-glutamic acid and ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the ProLease® technology or Lupron Depot® (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(−)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for long periods such as up to or over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated polypeptides remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37° C., resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization is depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S—S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

[0306] The invention is further described in the following non-limiting examples.

Materials and Methods

Accessible Surface Area (ASA)

[0307] The computer program Access (B. Lee and F. M. Richards, J. Mol. Biol. 55: 379-400 (1971)) version 2 (© 1983 Yale University) is used to compute the accessible surface area (ASA) of the individual atoms in the structure. This method typically uses a probe-size of 1.4 Å and defines the Accessible Surface Area (ASA) as the area formed by the center of the probe. Prior to this calculation all water molecules and all hydrogen atoms should be removed from the coordinate set, as should other atoms not directly related to the protein.

Fractional ASA of Side Chain

[0308] The fractional ASA of the side chain atoms is computed by division of the sum of the ASA of the atoms in the side chain with a value representing the ASA of the side chain atoms of that residue type in an extended Ala-x-Ala tripeptide (See Hubbard, Campbell & Thornton (1991) J. Mol. Biol. 220,507-530). For this example the CA atom is regarded as a part of the side chain of Glycine residues but not for the remaining residues. The following table is used as standard 100% ASA for the side chain:

Ala	69.23Å^2
Arg	200.35Å^2
Asn	106.25Å^2
Asp	102.06Å^2
Cys	96.69Å^2
Gln	140.58Å^2
Glu	134.61Å^2
Gly	32.28Å^2
His	147.00Å^2

-continued

Ile	137.91Å^2
Leu	140.76Å^2
Lys	162.50Å^2
Met	156.08Å^2
Phe	163.90Å^2
Pro	119.65Å^2
Ser	78.16Å^2
Thr	101.67Å^2
Trp	210.89Å^2
Tyr	176.61Å^2
Val	114.14Å^2

[0309] Residues not detected in the structure are defined as having 100% exposure as they are thought to reside in flexible regions. The gamma-carboxy glutamic acids at positions 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35 are all defined as being 100% exposed.

Determining Distances Between Atoms

[0310] The distance between atoms is most easily determined using molecular graphics software e.g. InsightII® v. 98.0, MSI INC.

Active Site Region

[0311] The active site region is defined as any residues having at least one atom within 10 Å of any atom in the catalytic triad (residues H193, D242, S344).

Determination of Tissue Factor Binding Site

[0312] The TF binding site is defined as comprising of all residues having their accessible surface area changed upon TF binding. This is determined by at least two ASA calculations; one on the isolated ligand(s) in the ligand(s)/receptor(s) complex and one on the complete ligand(s)/receptor(s) complex.

Measurement of Reduced Sensitivity to Proteolytic Degradation

[0313] Proteolytic degradation can be measured using the assay described in U.S. Pat. No. 5,580,560, Example 5, where proteolysis is autoproteolysis.

[0314] Furthermore, reduced proteolysis can be tested in an in vivo model using radiolabelled samples and comparing proteolysis of rhFVIIa and the polypeptide variant of the invention by withdrawing blood samples and subjecting these to SDS-PAGE and autoradiography.

[0315] Irrespectively of the assay used for determining proteolytic degradation, "reduced proteolytic degradation" is intended to mean a measurable reduction in cleavage compared to is that obtained by rhFVIIa as measured by gel scanning of Coomassie stained SDS-PAGE gels, HPLC or as measured by conserved catalytic activity in comparison to wild type using the tissue factor independent activity assay decribed below.

Determination of the Molecular Weight of Polypeptide Variants

[0316] The molecular weight of polypeptide variants is determined by either SDS-PAGE, get filtration, Western Blots, matrix assisted laser desorption mass spectrometry or equilibrium centrifugation, e.g. SDS-PAGE according to Laemmli, U.K., Nature Vol 227 (1970), pp. 680-85.

TF-Independent Factor X Activation Assay

[0317] This assay has been described in detail on page 39826 in Nelsestuen et al., J Biol Chem, 2001; 276:39825-39831.

[0318] Briefly, the molecule to be assayed (either hFVIIa, rhFVIIa or the polypeptide variant of the invention in its activated form) is mixed with a source of phospholipid (phosphatidylcholine and phosphatidylserine in a ratio of 8:2 or phosphatidylcholine, phosphatidylserine and phosphatidylethanol in a ratio of 4:2:4) and Factor X in Tris buffer containing BSA. After a specified incubation time the reaction is stopped by addition of excess EDTA. The concentration of factor Xa is then measured from absorbance change at 405 nm after addition of a chromogenic substrate (S-2222, Chromogenix). After correction from background the tissue factor independent activity of rhFVIIa (a_{wt}) is determined as the absorbance change after 10 minutes and the tissue factor independent activity of the polypeptide variant of the invention ($a_{variant}$) is also determined as the absorbance change after 10 minutes. The ratio between the activity of the polypeptide variant, in its activated form, and the activity of rhFVIIa is defined as $a_{variant}/a_{wt}$.

Clotting Assay

[0319] Clotting activity is measured in one-stage assays and clotting times are recorded on a Thrombotrack IV coagulometer (MEDINOR). FVII depleted human plasma (American Diagnostica) is reconstituted and equilibrated at room temperature for 15-20 minutes. 50 µl of plasma is then transferred to the coagulometer cups.

[0320] hFVIIa, rhFVIIa or variants are diluted in Glyoxaline Buffer (5.7 mM barbiturate, 4.3 mM sodium citrate, 117 mM NaCl, 1 mg/mL BSA, pH 7.35). The samples are added to the cup in 50 µl and incubated at 37° C. for 2 minutes.

[0321] Thromboplastin (MEDINOR) is reconstituted with water and $CaCl_2$ is added. The reaction is initiated by adding 0.1 ml thromboplastin containing 4.5 mM $CaCl_2$.

[0322] Data are analysed using PRISM software.

TF-Independent Clotting Assay

[0323] This assay is performed as described above under "Clotting Assay" but without addition of thromboplastin.

Amidolytic Assay

[0324] The ability of the variants to cleave small peptide substrates can be measured using the chromogenic substrate S-2288 (D-Ile-Pro-Arg-p-nitroanilide). The amidolytic activity may be measured both in the presence and absence of antithrombin III (ATIII).

[0325] HFVIIa, rhFVIIa or variant is diluted to 90 nM in assay buffer (50 mM Na-Hepes pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 0.1% BSA, IU/ml Heparin). Furthermore, soluble TF (sTF) is diluted to 450 nM in assay buffer. ATIII is diluted to 900 nM in assay buffer. 120 µl of assay buffer is mixed with 20 µl of the FVIIa sample, 20 µl sTF and 20 µl ATIII or assay buffer. The final concentrations of FVIIa, sTF and ATIII are 10, 50 and 100 nM, respectively. After 5 min incubation at room temperature with gentle shaking, followed by 10 min incubation at 37° C., the reaction is started by addition of the S-2288 substrate to 1 mM and the absorption at 405 nm is determined at several time points.

Thrombogram Assay

[0326] The effect of hFVIIa, rhFVIIa or variant on thrombin generation in human plasma is tested in a modified version of the assay described on page 589 in Hemker et al. in Thromb Haemost 2000; 83: 589-91. Briefly, the molecule to be assayed (either hFVIIa, rhFVIIa or variant) is mixed with normal platelet rich plasma (PRP), normal platelet poor plasma (PPP) or FVII depleted PPP with or without the addition of recombinant human tissue factor (rTF), relipidated rTF or another source of TF (such as thromboplastin). A source of phospholipid (phosphatidylcholine and phosphatidylserine in a ratio of 8:2 or phosphatidylcholine, phosphatidylserine and phosphatidylethanol in a ratio of 4:2:4) can be added.

[0327] The reaction is started by addition of a fluoregenic thrombin substrate and calcium chloride. The fluorescence is measured continuously and the thrombin amidolytic activity is calculated by calculating the slope of the fluorescence curve (the increase in fluorescence over time). In this way the time until maximum thrombin amidolytic activity is obtained (T_{max}) and total thrombin work (area under the curve (AUC)) can be calculated.

[0328] The following procedure is used: PRP is obtained by centrifuging freshly drawn blood at 250 g, 15° C. for 10 min. Blood coagulation is inhibited either by using citrate (13 mM tri-sodium citrate), corn trypsin inhibitor (50-100 μg/ml blood) or a combination of citrate and corn trypsin inhibitor. The platelet count is adjusted to 3×10^8/mL using buffer or autologous platelet poor plasma (PPP). PPP is obtained by double centrifugation of PRP at 1000 g, 15° C. for 10 min. FVII depletion is done by incubating PPP with a FVII specific monoclonal antibody coupled to a solid phase.

[0329] Per well of a 96-well microtiter plate 80 μl PRP is added and 20 μL buffer containing rhFVII or variant to be tested in final concentrations between 0.1 and 100 nM. rTF is added in 5 μL assay buffer to a final concentration of 1 pM. The assay buffer consists of 20 mM Hepes, 150 mM NaCl and 60 mg/ml BSA in distilled water. The reaction is started by adding 20 μL of the substrate solution containing 0.1 M calcium chloride. The assay plate and reagents are prewarmed to 37° C. and the reaction takes place at this temperature. The fluorimeter used is a BMG Fluormeter with a excitation filter at 390 nm and an emission filter at 460 nm. The fluorescence is measured in each well of 96-well clear bottom plates in 20-40 second interval over 30-180 minutes. Data are analyzed using PRISM Software.

ELISA Assay

[0330] FVII/FVIIa (or variant) concentrations are determined by ELISA. Wells of a microtiter plate are coated with an antibody directed against the protease domain using a solution of 2 μg/ml in PBS (100 μl per well). After 2 hours coating at R.T., the wells are washed 4 times with THT buffer (100 mM NaCl, 50 mM Tris-HCl pH 7.2 0.05% Tween-20). Subsequently, 200 μl of 1% Casein (diluted from 2.5% stock using 100 mM NaCl, 50 mM Tris-HCl pH 7.2) is added per well for blocking. After 1 hr incubation at R.T., the wells are emptied, and 100 μl of sample (optionally diluted in dilution buffer (THT+0.1% Casein)) is added. After another incubation of 1 hr at room temperature, the wells are washed 4 times with THT buffer, and 100 μl of a biotin-labelled antibody directed against the EGF-like domain (1 μg/ml) is added. After another 1 hr incubation at R.T., followed by 4 more washes with THT buffer, 100 μl of streptavidin-horse radish peroxidase (DAKO A/S, Glostrup, Denmark, 1/10000 diluted) is added. After another 1 hr incubation at R.T., followed by 4 more washes with THT buffer, 100 μl of TMB (3,3',5,5'-tetramethylbenzidine, Kemen-Tech A/S, Denmark) is added. After 30 min incubation at R.T. in the dark, 100 μl of 1 M H_2SO_4 is added and OD_{450nm} is determined. A standard curve is prepared using rhFVIIa (NovoSeven®).

[0331] Alternatively, FVII/FVIIa or variants may be quantified through the Gla domain rather than through the protease domain. In this ELISA set-up, wells are coated overnight with an antibody directed against the EGF-like domain and for detection, a calcium-dependent biotin-labelled monoclonal anti-Gla domain antibody is used (2 μg/ml, 100 μl per well). In this set-up, 5 mM $CaCl_2$ is added to the THT and dilution buffers.

Whole Blood Assay

[0332] The whole blood clotting assay is performed as described by Elg et al. Thrombosis Res. 2001, 101(3):159-170.

Reconstituted Coagulation Assay

[0333] The reconstituted coagulation assay is performed as described by Van't Veer et al. Blood 2000, 95(4), 1330-1335.

EXAMPLES

Example 1

[0334] The X-ray structure of hFVIIa in complex with soluble tissue factor by Banner et. al., J Mol Biol, 1996; 285:2089 is used for this example. It is noted that the numbering of residues in the reference does not follow the sequence. Here we have used the sequential numbering according to SEQ ID NO:2. The gamma-carboxy glutamic acids at positions 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35 are all here named GLU (three letter abbreviation) or E (one letter abbreviation). Residues 143-152 are not present in the structure.

Surface Exposure

[0335] Performing fractional ASA calculations on FVII fragments alone combined with the definition of accessibilities of non standard and/or missing residues described in the methods resulted in the following residues having more than 25% of their side chain exposed to the surface: A1, N2, A3, F4, L5, E6, E7, L8, R9, P10, S12, L13, E14, E16, K18, E19, E20, Q21, S23, F24, E25, E26, R8, E29, F31, K32, D33, A34, E35, R36, K38, L39, W41, I42, S43, S45, G47, D48, Q49, A51, S52, S53, Q56, G58, S60, K62, D63, Q64, L65, Q66, S67, I69, F71, L73, P74, A75, E77, G78, R79, E82, T83, H84, K85, D86, D87, Q88, L89, I90, V92, N93, E94, G97, E99, S103, D104, H105, T106, G107, T108, K109, S111, R113, E116, G117, S119, L120, L121, A122, D123, G124, V125, S126, T128, P129, T130, V131, E132, I140, L141, E142, K143, R144, N145, A146, S147, K148, P149, Q150, G151, R152, G155, K157, V158, P160, K161, E163, L171, N173, G174, A175, N184, T185, I186, H193, K197, K199, N200, R202, N203, I205, S214, E215, H216, D217,

G218, D219, S222, R224, S232, T233, V235, P236, G237, T238, T239, N240, H249, Q250, P251, V253, T255, D256, E265, R266, T267, E270, R271, F275, V276, R277, F278, L280, L287, L288, D289, R290, G291, A292, T293, L295, E296, N301, M306, T307, Q308, D309, L311, Q312, Q313, R315, K316, V317, G318, D319, S320, P321, N322, T324, E325, Y326, Y332, S333, D334, S336, K337, K341, G342, H351, R353, G354, Q366, G367, T370, V371, G372, R379, E385, Q388, K389, R392, S393, E394, P395, R396, P397, G398, V399, L400, L401, R402, P404 and P406 (A1-S45 are located in the Gla domain, the remaining positions are located outside the Gla domain).

[0336] The following residues had more than 50% of their side chain exposed to the surface: A1, A3, F4, L5, E6, E7, L8, R9, P10, E14, E16, K18, E19, E20, Q21, S23, E25, E26, E29, K32, A34, E35, R36, K38, L39, I42, S43, G47, D48, A51, S52, S53, Q56, G58, S60, K62, L65, Q66, S67, I69, F71, L73, P74, A75, E77, G78, R79, E82, H84, K85, D86, D87, Q88, L89, I90, V92, N93, E94, G97, T106, G107, T108, K109, S111, E116, S119, L121, A122, D123, G124, V131, E132, L141, E142, K143, R144, N145, A146, S147, K148, P149, Q150, G151, R152, G155, K157, P160, N173, G174, A175, K197, K199, N200, R202, S214, E215, H216, G218, R224, V235, P236, G237, T238, H249, Q250, V253, D256, T267, F275, R277, F278, L288, D289, R290, G291, A292, T293, L295, N301, M306, Q308, D309, L311, Q312, Q313, R315, K316, G318, D319, N322, E325, D334, K341, G354, G367, V371, E385, K389, R392, E394, R396, P397, G398, R402, P404 and P406 (A1-S43 are located in the Gla domain, the remaining positions are located outside the Gla domain).

Tissue Factor Binding Site

[0337] Performing ASA calculations the following residues in human FVII change their ASA in the complex. These residues were defined as constituting the receptor binding site: L13, K18, F31, E35, R36, L39, F40, I42, S43, S60, K62, D63, Q64, L65, I69, C70, F71, C72, L73, P74, F76, E77, G78, R79, E82, K85, Q88, I90, V92, N93, E94, R271, A274, F275, V276, R277, F278, R304, L305, M306, T307, Q308, D309, Q312, Q313, E325 and R379.

Active Site Region

[0338] The active site region is defined as any residue having at least one atom within a distance of 10 Å from any atom in the catalytic triad (residues H193, D242, S344): 1153, Q167, V168, L169, L170, L171, Q176, L177, C178, G179, G180, T181, V188, V189, S190, A191, A192, H193, C194, F195, D196, K197, I198, W201, V228, I229, I230, P231, S232, T233, Y234, V235, P236, G237, T238, T239, N240, H241, D242, I243, A244, L245, L246, V281, S282, G283, W284, G285, Q286, T293, T324, E325, Y326, M327, F328, D338, S339, C340, K341, G342, D343, S344, G345, G346, P347, H348, L358, T359, G360, 1361, V362, S363, W364, G365, C368, V376, Y377, T378, R379, V380, Q382, Y383, W386, L387, L400 and F405.

The Ridge of the Active Site Binding Cleft

[0339] The ridge of the active site binding cleft region was defined by visual inspection of the FVIIa structure 1FAK-.pdb as: N173, A175, K199, N200, N203, D289, R290, G291, A292, P321 and T370.

Example 2

Design of an Expression Cassette for Expression of rhFVII in Mammalian Cells

[0340] The DNA sequence shown in SEQ ID NO:1, encompassing the short form of the full length cDNA encoding hFVII with its native short signal peptide (Hagen et al., 1986. PNAS 83:2412), was synthesized in order to facilitate high expression in mammalian cells. First the ATG start codon context was modified according to the Kozak consensus sequence (Kozak, M. *J Mol Biol* 1987 Aug. 20;196(4):947-50), so that there is a perfect match to the consensus sequence upstream of the ATG start codon. Secondly the open reading frame of the native cDNA was modified by making a bias in the codon usage towards the codons frequently used in highly expressed human genes. Further, two translational stop codons were inserted at the end of the open reading frame in order to facilitate efficient translational stop. The fully synthetic and expression optimized hFVII gene was assembled from 70-mer DNA oligonucleotides and finally amplified using end primers inserting BamHI and HindIII sites at the 5' and 3, ends respectively using standard PCR techniques, which resulted in the following sequence:

```
ggatcccgccaccatggtcagccaggccctccgcctcctgtgcctgctcc
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catggcgtcctgcatcgccggcgccgggccaatgcctttctggaagagct
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ggcgtctacacgcgcgtcagccagtacattgagtggctgcagaagctcat
gcggagcgaaccccggcccggggtgctcctgcgggcccctttccctgat
aaaagctt
```

[0341] A vector for the cloning of the generated PCR product encompassing the expression cassette for hFVII was prepared by cloning the intron from pCINeo (Promega). The synthetic intron from pCI-Neo was amplified using standard PCR conditions and the primers:

```
CBProFpr174: 5'-AGCTGGCTAGCCACTGGGCAGGTAAGTATCA-3'
and

CBProFpr175: 5'-TGGCGGGATCCTTAAGAGCTGTAATTGAACT-3'
```

resulting in a 332 bp PCR fragment. The fragment was cut with NheI and BamHI before cloning into pcDNA3.1/HygR (obtained from Invitrogen) resulting in PF#34.

[0342] The expression cassette for hFVII was cloned between the BamHI and HindIII sites of PF#34, resulting in plasmid PF#226.

Example 3

Expression of Polypeptide Variants in CHO K1 Cells

[0343] The cell line CHO K1 (ATCC # CCL-61) is seeded at 50% confluence in T-25 flasks using MEMO, 10% FCS (Gibco/BRL Cat # 10091), P/S and 5 μg/ml phylloquinone and allowed to grow until confluent. The confluent mono cell layer is transfected with 5 μg of the relevant plasmid described above using the Lipofectamine 2000 transfection agent (Life technologies) according to the manufacturer's instructions. Twenty four hours post transfection a sample is drawn and quantified using e.g. an ELISA recognizing the EGF1 domain of hFVII. At this time point relevant selection (e.g. Hygromycin B) may be applied to the cells with the purpose of generating a pool of stable transfectants. When using CHO K1 cells and the Hygromycin B resistance gene as selectable marker on the plasmid, this is usually achieved within one week.

Example 4

Generation of CHO K1 Cells Stably Expressing Polypeptide Variants.

[0344] A vial of CHO-K1 transfectant pool is thawed and the cells seeded in a 175 cm^2 tissue flask containing 25 ml of MEMα, 10% FCS, phylloquinone (5 μg/ml), 100 U/l penicillin, 100 μg/l streptomycin and grown for 24 hours. The cells are harvested, diluted and plated in 96 well microtiter plates at a cell density of ½-1 cell/well. After a week of growth, colonies of 20-100 cells are present in the wells and those wells containing only one colony are labelled. After a further two weeks, the media in all wells containing only one colony is substituted with 200 μl fresh medium. After 24 hours, a medium sample is withdrawn and analysed by e.g. ELISA. High producing clones are selected and used to produce FVII or variant on large scale.

Example 5

Purification of Polypeptide Variants and Subsequent Activation

[0345] FVII and FVII variants are purified as follows: The procedure is performed at 4° C. The harvested culture media from large-scale production is ultrafiltered using a Millipore TFF system with 30 KDa cut-off Pellicon membranes. After concentration of the medium, citrate is added to 5 mM and the pH is adjusted to 8.6. If necessary, the conductivity is lowered to below 10 mS/cm. Subsequently, the sample is applied to a Q-sepharose FF column, equilibrated with 50 mM NaCl, 10 mM Tris pH 8.6. After washing the column with 100 mM NaCl, 10 mM Tris pH 8.6, followed by 150 mM NaCl, 10 mM Tris pH 8.6, FVII is eluted using 10 mM Tris, 25 mM NaCl, 35 mM $CaCl_2$, pH 8.6.

[0346] For the second chromatographic step, an affinity column is prepared by coupling of a monoclonal Calcium-dependent antiGla-domain antibody to CNBr-activated Sepharose FF. About 5.5 mg antibody is coupled per ml resin. The column is equilibrated with 10 mM Tris, 100 mM NaCl, 35 mM $CaCl_2$, pH 7.5. NaCl is added to the sample to a concentration of 100 mM NaCl and the pH is adjusted to 7.4-7.6. After O/N application of the sample, the column is washed with 100 mM NaCl, 35 mM $CaCl_2$, 10 mM Tris pH 7.5, and the FVII protein is eluted with 100 mM NaCl, 50 mM citrate, 75 mM Tris pH 7.5.

[0347] For the third chromatographic, the conductivity of the sample is lowered to below 10 mS/cm, if necessary, and the pH is adjusted to 8.6. The sample is then applied to a Q-sepharose column (equilibrated with 50 mM NaCl, 10 mM Tris pH 8.6) at a density around 3-5 mg protein per ml gel to obtain efficient activation. After application, the column is washed with 50 mM NaCl, 10 mM Tris pH 8.6 for about 4 hours with a flow of 3-4 column volumes (cv) per hour. The FVII protein is eluted using a gradient of 0-100% of 500 mM NaCl, 10 mM Tris pH 8.6 over 40 cv. FVII containing fractions are pooled.

[0348] For the final chromatographic step, the conductivity is lowered to below 10 mS/cm. Subsequently, the sample is applied to a Q-sepharose column (equilibrated with 140 mM NaCl, 10 mM glycylglycine pH 8.6) at a concentration of 3-5 mg protein per ml gel. The column is then washed with 140 mM NaCl, 10 mM glycylglycine pH 8.6 and FVII is eluted with 140 mM NaCl, 15 mM $CaCl_2$, 10 mM glycylglycine pH 8.6. The eluate is diluted to 10 mM $CaCl_2$ and the pH is adjusted 6.8-7.2. Finally, Tween-80 is added to 0.01% and the pH is adjusted to 5.5 for storage at −80° C.

SEQUENCE LISTING

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<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
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<222> LOCATION: (115)..(1335)

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                                                          Ala
                                                           1

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Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu Cys
             5                  10                  15

aaa gag gaa cag tgc agc ttt gag gaa gcc cgg gag att ttc aaa gac      213
Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys Asp
         20                  25                  30

gct gag cgg acc aaa ctg ttt tgg att agc tat agc gat ggc gat cag      261
Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp Gln
     35                  40                  45

tgc gcc tcc agc cct tgc cag aac ggg ggc tcc tgc aaa gac cag ctg      309
Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln Leu
 50                  55                  60                  65

cag agc tat atc tgc ttc tgc ctg cct gcc ttt gag ggg cgc aat tgc      357
Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn Cys
                 70                  75                  80

gaa acc cat aag gat gac cag ctg att tgc gtc aac gaa aac ggg ggc      405
Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly Gly
             85                  90                  95

tgc gag cag tac tgc agc gat cac acg ggc acg aag cgg agc tgc cgc      453
Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys Arg
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tgc cac gaa ggc tat agc ctc ctg gct gac ggg gtg tcc tgc acg ccc      501
Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr Pro
    115                 120                 125

acg gtg gaa tac cct tgc ggg aag att ccc att cta gaa aag cgg aac      549
Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg Asn
130                 135                 140                 145

gct agc aaa ccc cag ggc cgg atc gtc ggc ggg aag gtc tgc cct aag      597
Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro Lys
                150                 155                 160

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Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln Leu
            165                 170                 175

tgc ggc ggg acc ctc atc aat acc att tgg gtc gtg tcc gcc gct cac      693
Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala His
        180                 185                 190

tgc ttc gat aag att aag aat tgg cgg aac ctc atc gct gtg ctc ggc      741
Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu Gly
    195                 200                 205

gaa cac gat ctg tcc gag cat gac ggg gac gaa cag tcc cgc cgg gtg      789
Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg Val
210                 215                 220                 225

gct cag gtc atc att ccc tcc acc tat gtg cct ggc acg acc aat cac      837
Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn His
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gat atc gct ctg ctc cgc ctc cac cag ccc gtc gtg ctc acc gat cac      885
Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp His
            245                 250                 255

gtc gtg cct ctg tgc ctg cct gag cgg acc ttt agc gaa cgc acg ctg      933
Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr Leu
        260                 265                 270

gct ttc gtc cgc ttt agc ctc gtg tcc ggc tgg ggc cag ctg ctc gac      981
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    275                 280                 285
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290                 295                 300                 305

atg acc cag gac tgc ctg cag cag tcc cgc aaa gtg ggg gac tcc ccc     1077
Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser Pro
                310                 315                 320

aat atc acg gag tat atg ttt tgc gct ggc tat agc gat ggc tcc aag     1125
Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser Lys
            325                 330                 335

gat agc tgc aag ggg gac tcc ggc ggg ccc cat gcc acg cac tat cgc     1173
Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr Arg
        340                 345                 350

ggg acc tgg tac ctc acc ggg atc gtc agc tgg ggc cag ggc tgc gcc     1221
Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys Ala
    355                 360                 365

acg gtg ggg cac ttt ggc gtc tac acg cgc gtc agc cag tac att gag     1269
Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile Glu
370                 375                 380                 385

tgg ctg cag aag ctc atg cgg agc gaa ccc cgg ccc ggg gtg ctc ctg     1317
Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu Leu
                390                 395                 400

cgg gcc cct ttc cct tga taa                                         1338
Arg Ala Pro Phe Pro
            405


<210> SEQ ID NO 2
<211> LENGTH: 406
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 2

Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu
  1               5                  10                  15

Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys
             20                  25                  30

Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp
         35                  40                  45

Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln
     50                  55                  60

Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn
 65                  70                  75                  80

Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly
                 85                  90                  95

Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys
            100                 105                 110

Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr
        115                 120                 125

Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg
    130                 135                 140

Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro
145                 150                 155                 160

Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln
                165                 170                 175

Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala
            180                 185                 190
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-continued

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His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu
        195                 200                 205

Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg
    210                 215                 220

Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn
225                 230                 235                 240

His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp
                245                 250                 255

His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr
            260                 265                 270

Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu
        275                 280                 285

Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg
    290                 295                 300

Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser
305                 310                 315                 320

Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser
                325                 330                 335

Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr
            340                 345                 350

Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys
        355                 360                 365

Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile
    370                 375                 380

Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu
385                 390                 395                 400

Leu Arg Ala Pro Phe Pro
                405


<210> SEQ ID NO 3
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Primer
      CBProFpr174

<400> SEQUENCE: 3

agctggctag ccactgggca ggtaagtatc a                                  31


<210> SEQ ID NO 4
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Primer
      CBProFpr175

<400> SEQUENCE: 4

tggcgggatc cttaagagct gtaattgaac t                                   31
```

1. A Factor VII (FVII) or Factor VIIa (FVIIa) polypeptide variant having an amino acid sequence comprising 3-15 amino acid modifications relative to human Factor VII (hFVII) or human Factor VIIa (hFVIIa) having the amino acid sequence shown in SEQ ID NO:2, wherein said amino acid sequence of the variant comprises an amino acid substitution in position 10 and 32 and wherein a sugar moiety is covalently attached to an introduced in vivo N-glycosylation site located outside the Gla domain.

2. The variant according to claim 1, wherein said substitution in position 10 is P10Q.

3. The variant according to claim 1, wherein said substitution in position 32 is K32E.

4. The variant according to claim 1, wherein said substitution in position 10 is P10Q and said substitution in position 32 is K32E.

5. The variant according to claim 1, wherein said variant comprises at least one further amino acid modification in the Gla domain.

6-15. (canceled)

16. The variant according to claim 5, wherein said further modification in the Gla domain comprises an amino acid substitution in position 34.

17. The variant according to claim 16, wherein a negatively charged amino acid residue is introduced by substitution in position 34.

18. The variant according to claim 17, wherein said substitution is A34E.

19. The variant according to claim 18, wherein said variant comprises the substitutions P10Q+K32E+A34E.

20-25. (canceled)

26. The variant according to claim 1, wherein said in vivo N-glycosylation site is introduced by substitution.

27-28. (canceled)

29. The variant according to claim 26, wherein said in vivo N-glycosylation site is introduced by a substitution selected from the group consisting of A51N, G58N, T106N, K109N, G124N, K143N+N145T, A175T, I205S, I205T, V253N, T267N, T267N+S269T, S314N+K316S, S314N+K316T, R315N+V317S, R315N+V317T, K316N+G318S, K316N+G318T, G318N, D334N and combinations thereof.

30. The variant according to claim 29, wherein said in vivo N-glycosylation site is introduced by a substitution selected from the group consisting of A51N, G58N, T106N, K109N, G124N, K143N+N145T, A175T, I205T, V253N, T267N+S269T, S314N+K316T, R315N+V317T, K316N+G318T, G318N, D334N and combinations thereof.

31. The variant according to claim 30, wherein said in vivo N-glycosylation site is introduced by a substitution selected from the group consisting of T106N, A175T, I205T, V253N, T267N+S269T and combinations thereof.

32. The variant according to claim 16, wherein one in vivo N-glycosylation site has been introduced by substitution.

33. The variant according to claim 16, wherein two or more in vivo N-glycosylation sites have been introduced by substitution.

34-40. (canceled)

41. The variant according to claim 1, wherein said variant is in its activated form.

42. The variant according to claim 1, wherein said variant, in its activated form, has at least 10% of the amidolytic activity of rhFVIIa when assayed in the "Amidolytic Assay" described herein.

43. The variant according to claim 1, wherein said variant, in its activated form, has at least 10% of the clotting activity of rhFVIIa when assayed in the "Clotting Assay" described herein.

44. A nucleotide sequence encoding the variant of claim 1.

45. An expression vector comprising the nucleotide sequence of claim 44.

46. A host cell comprising the nucleotide sequence of claim 44.

47. The host cell according to claim 46, wherein said host cell is a gammacarboxylating cell capable of it vivo glycosylation.

48. A pharmaceutical composition comprising the variant of claim 1, and a pharmaceutical acceptable carrier or excipient.

49-54. (canceled)

55. A method for treating a mammal having a disease or a disorder wherein clot formation is desirable, comprising administering to a mammal in need thereof an effective amount of the pharmaceutical composition of claim 48.

56. The method according to claim 55, wherein said disease or disorder is selected from the group consisting of hemorrhages, including brain hemorrhages, severe uncontrolled bleedings, such as trauma, bleedings in patients undergoing living transplantations, bleeding in patients undergoing resection and variceal bleedings.

57-59. (canceled)

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(54) FACTOR VII OR VIIA POLYPEPTIDE VARIANTS

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(57) **ABSTRACT**

The present invention relates to novel polypeptide variants of factor VII (FVII) or factor VIIa (FVIIa) polypeptides, where said variants comprise an amino acid substitution in position 10 and 32 and where said variants further comprise a sugar moiety covalently attached to an introduced in vivo N-glycosylation site located outside of the Gla domain. Such polypeptide variants are useful in therapy, in particular for the treatment of a variety of coagulation-related disorders, such as trauma.

FACTOR VII OR VIIA POLYPEPTIDE VARIANTS

FIELD OF THE INVENTION

[0001] The present invention relates to novel polypeptide variants of factor VII (FVII) or factor VIIa (FVIIa) polypeptides, where said variants comprise an amino acid substitution in position 10 and 32 and where said variants further comprise a sugar moiety covalently attached to an introduced in vivo N-glycosylation site.

[0002] The present invention also relates to use of such polypeptide variants in therapy, in particular for the treatment of a variety of coagulation-related disorders.

BACKGROUND OF THE INVENTION

[0003] Blood coagulation is a process consisting of a complex interaction of various blood components (or factors) that eventually results in a fibrin clot. Generally, the blood components participating in what has been referred to as the "coagulation cascade" are proenzymes or zymogens, i.e. enzymatically inactive proteins that are converted into an active form by the action of an activator. One of these coagulation factors is FVII.

[0004] FVII is a vitamin K-dependent plasma protein synthesized in the liver and secreted into the blood as a single-chain glycoprotein with a molecular weight of 53 kDa (Broze & Majerus, J. Biol. Chem 1980; 255:1242-1247). The FVII zymogen is converted into an activated form (FVIIa) by proteolytic cleavage at a single site, R152-I153, resulting in two chains linked by a single disulfide bridge. FVIIa in complex with tissue factor (FVIIa complex) is able to convert both factor IX and factor X into their activated forms, followed by reactions leading to rapid thrombin production and fibrin formation (Østerud & Rapaport, Proc Natl Acad Sci USA 1977; 74:5260-5264).

[0005] FVII undergoes post-translational modifications, including vitamin K-dependent carboxylation resulting in ten γ-carboxyglutamic acid residues in the N-terminal region of the molecule. Thus, residue number 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35 shown in SEQ ID NO:2 are γ-carboxyglutamic acids residues in the Gla domain important for FVII activity. Other post-translational modifications include sugar moiety attachment at two naturally occurring N-glycosylation sites at position 145 and 322, respectively, and at two naturally occurring O-glycosylation sites at position 52 and 60, respectively.

[0006] The gene coding for human FVII (hFVII) has been mapped to chromosome 13 at q34-qter 9 (de Grouchy et al., Hum Genet 1984; 66:230-233). It contains nine exons and spans 12.8 Kb (O'Hara et al., Proc Natl Acad Sci USA 1987; 84:5158-5162). The gene organisation and protein structure of FVII are similar to those of other vitamin K-dependent procoagulant proteins, with exons 1a and 1b encoding for signal sequence; exon 2 the propeptide and Gla domain; exon 3 a short hydrophobic region; exons 4 and 5 the epidermal growth factor-like domains; and exon 6 through 8 the serine protease catalytic domain (Yoshitake et al., Biochemistry 1985; 24: 3736-3750).

[0007] Reports exist on experimental three-dimensional structures of hFVIIa (Pike et al., PNAS. U.S.A., 1999; 96:8925-30 and Kemball-Cook et al., J. Struct. Biol, 1999; 127:213-223); of hFVIIa in complex with soluble tissue factor using X-ray crystallographic methods (Banner et al., Nature, 1996; 380:41 and Zhang et al., J. Mol. Biol, 1999; 285: 2089); and of smaller fragments of hFVII (Muranyi et al., Biochemistry, 1998; 37:10605 and Kao et al., Biochemistry, 1999; 38:7097).

[0008] Some protein-engineered variants of FVII have been reported. See, e.g., Dickinson & Ruf, J Bio Chem, 1997; 272:19875-19879, Kemball-Cook et al., J Biol Chem, 1998; 273:8516-8521, Bharadwaj et al., J Biol Chem, 1996; 271:30685-30691, Ruf et al., Biochemistry, 1999; 38:1957-1966; WO 99/20767; WO 00/11416; WO 02/22776; WO 02/38162; WO 01/83725; WO 01/58935; U.S. Pat. No. 5,580,560.

[0009] Reports exist on expression of FVII in BHK or other mammalian cells (WO 92/15686, WO 91/11514 and WO 88/10295) and co-expression of FVII and kex2 endoprotease in eukaryotic cells (WO 00/28065).

[0010] Commercial preparations of human recombinant FVIIa are sold as NovoSeven®. NovoSeven® is indicated for the treatment of bleeding episodes in hemophilia A or B patients. NovoSeven® is the only rhFVIIa for effective and reliable treatment of bleeding episodes available on the market.

[0011] An inactive form of FVII in which arginine 152 and/or isoleucine 153 is/are modified has been reported in WO91/1154. These amino acids are located at the activation site. WO 96/12800 describes inactivation of FVIIa by a serine proteinase inhibitor; inactivation by carbamylation of FVIIa at the α-amino acid group I153 has been described by Petersen et al., Eur J Biochem, 1999; 261:124-129. The inactivated form is capable of competing with wild-type FVII or FVIIa for binding to tissue factor and inhibiting clotting activity. The inactivated form of FVIIa is suggested to be used for treatment of patients being in hypercoagulable states, such as patients with sepsis, in risk of myocardial infarction or of thrombotic stroke.

[0012] A circulating rhFVIIa half-life of 2.3 hours was reported in "Summary Basis for Approval for NovoSeven®", FDA reference number 96-0597. Relatively high doses and frequent administration are necessary to reach and sustain the desired therapeutic or prophylactic effect. As a consequence adequate dose regulation is difficult to obtain and the need of frequent intravenous administrations imposes restrictions on the patient's way of living.

[0013] In connection with treatment of uncontrolled bleedings, such as trauma, it is believed that factor VIIa is capable of activating factor X to factor Xa without binding to tissue factor, and this activation reaction is believed to occur primarily on activated blood platelets (Hedner et al. Blood Coagulation & Fibrinolysis, 2000; 11;107-111). However, hFVIIa or rhFVIIa has a low activity towards factor X in the absence of tissue factor and, consequently, treatment of uncontrolled bleeding, for example in trauma patients, requires relatively high and multiple doses of hFVIIa or rhFVIIa. Therefore, in order to treat uncontrolled bleedings more efficiently (to minimize blood loss) there is need for improved FVIIa molecules, which possess a high activity toward factor X in the absence of tissue factor. Such improved FVIIa molecules will exhibit a lowered clotting time (or faster action) as compared to rhFVIIa when administered in connection with uncontrolled bleedings.

[0014] A molecule with a longer circulation half-life would decrease the number of necessary administrations. Given the association of current the rhFVIIa product with frequent injections, and the potential for obtaining more optimal therapeutic FVIIa levels with concomitant enhanced therapeutic effect, there is a clear need for improved FVII- or FVIIa-like molecules.

[0015] One way to increase the circulation half-life of a protein is to ensure that renal clearance of the protein is reduced. This may be achieved by conjugating the protein to a chemical moiety, which is capable of conferring reduced renal clearance to the protein.

[0016] Furthermore, attachment of a chemical moiety to the protein or substitution of amino acids exposed to proteolysis may effectively block a proteolytic enzyme from contact leading to proteolytic degradation of the protein. Polyethylene glycol (PEG) is one such chemical moiety that has been used in the preparation of therapeutic protein products. WO 98/32466 suggests that FVII, among many other proteins, may be PEGylated but does not contain any further information in this respect. WO 01/58935 discloses a new strategy for developing FVII or FVIIa molecules having inter alia an increased half-life.

[0017] As indicated above, another problem in current rhFVIIa treatment is the relative instability of the molecule with respect to proteolytic degradation. Proteolytic degradation is a major obstacle for obtaining a preparation in solution as opposed to a lyophilized product. The advantage of obtaining a stable soluble preparation lies in easier handling for the patient, and, in the case of emergencies, quicker action, which potentially can become life saving. Attempts to prevent proteolytic degradation by site directed mutagenesis at major proteolytic sites have been disclosed in WO 88/10295.

[0018] One object of the present invention is to provide improved FVII or FVIIa molecules (FVII or FVIIa variants) with a longer circulation half-life (thereby decreasing the number of necessary administrations) and which are capable of activating factor X to factor Xa (without binding to tissue factor) more efficiently than hFVIIa or rhFVIIa (thereby being able to treat uncontrolled bleadings, such as a trauma, more efficiently).

[0019] Another object of the present invention is to provide improved FVII or FVII molecules (FVII or FVIIa variants) with an increased bioavailability (such as an increased Area Under the Curve as compared to rhFVIIa when administered intravenously) and which are capable of activating factor X to factor Xa (without binding to tissue factor) more efficiently than hFVIIa or rhFVIIa (thereby being able to treat uncontrolled bleadings, such as a trauma, more efficiently).

[0020] These objects are met by the FVII or FVIIa variants provided herein.

BRIEF DISCLOSURE OF THE INVENTION

[0021] In its broadest aspect the present invention relates to a FVII or FVIIa polypeptide variant having an amino acid sequence comprising 3-15 amino acid modifications relative to hFVII or hFVIIa having the amino acid sequence shown in SEQ ID NO:2, wherein said amino acid sequence of the variant comprises an amino acid substitution in position 10 and 32 and wherein a sugar moiety is covalently attached to an introduced in vivo N-glycosylation site located outside the Gla domain.

[0022] Another aspect of the invention relates to a nucleotide sequence encoding the polypeptide variant of the invention.

[0023] In a further aspect the invention relates to an expression vector comprising the nucleotide sequence of the invention.

[0024] In a still further aspect the invention relates to a host cell comprising the nucleotide sequence of the invention or the expression vector of the invention.

[0025] In an even further aspect the invention relates to a pharmaceutical composition comprising the polypeptide variant of the invention, and a pharmaceutical acceptable carrier or excipient.

[0026] Still another aspect of the invention relates to the polypeptide variant of the invention, or the pharmaceutical composition of the invention, for use as a medicament.

[0027] Further aspects of the present invention will be apparent from the below description as well as from the appended claims.

DETAILED DISCLOSURE OF THE INVENTION

Definitions

[0028] In the context of the present application and invention the following definitions apply:

[0029] The term "conjugate" (or interchangeably "conjugated polypeptide") is intended to indicate a heterogeneous (in the sense of composite or chimeric) molecule formed by the covalent attachment of one or more polypeptide(s) to one or more non-polypeptide moieties such as polymer molecules, lipophilic compounds, sugar moieties or organic derivatizing agents. Preferably, the conjugate is soluble at relevant concentrations and conditions, i.e. soluble in physiological fluids such as blood. Examples of conjugated polypeptides of the invention include glycosylated and/or PEGylated polypeptides.

[0030] The term "covalent attachment" or "covalently attached" means that the polypeptide variant and the non-polypeptide moiety are either directly covalently joined to one another, or else are indirectly covalently joined to one another through an intervening moiety or moieties, such as a bridge, spacer, or linkage moiety or moieties.

[0031] The term "non-polypeptide moiety" is intended to mean a molecule, different from a peptide polymer composed of amino acid monomers and linked together by peptide bonds, which molecule is capable of conjugating to an attachment group of the polypeptide variant of the invention. Preferred examples of such molecules include polymer molecules, sugar moieties, lipophilic compounds or organic derivatizing agents. When used in the context of a conjugated variant of the invention it will be understood that the non-polypeptide moiety is linked to the polypeptide part of the conjugated variant through an attachment group of the polypeptide. As explained above, the non-polypeptide moiety can be directly covalently joined to the attachment group or it can be indirectly covalently joined to the attachment

group through an intervening moiety or moieties, such as a bridge spacer or linker moiety or moieties.

[0032] A "polymer molecule" is a molecule formed by covalent linkage of two or more monomers, wherein none of the monomers is an amino acid residue, except where the polymer is human albumin or another abundant plasma protein. The term "polymer" may be used interchangeably with the term "polymer molecule". The term is also intended to cover carbohydrate molecules attached by in vitro glycosylation, i.e. a synthetic glycosylation performed in vitro normally involving covalently linking a carbohydrate molecule to an attachment group of the polypeptide variant, optionally using a cross-linking agent. In vitro glycosylation is discussed in detail further below.

[0033] The term "sugar moiety" is intended to indicate a carbohydrate-containing molecule comprising one or more monosaccharide residues, capable of being attached to the polypeptide variant (to produce a polypeptide variant conjugate in the form of a glycosylated polypeptide variant) by way of in vivo glycosylation. The term "in vivo glycosylation" is intended to mean any attachment of a sugar moiety occurring in vivo, i.e. during posttranslational processing in a glycosylating cell used for expression of the polypeptide variant, e.g. by way of N-linked and O-linked glycosylation. The exact oligosaccharide structure depends, to a large extent, on the glycosylating organism in question.

[0034] An "N-glycosylation site" has the sequence N-X-S/T/C, wherein X is any amino acid residue except proline, N is asparagine and S/T/C is either serine, threonine or cysteine, preferably serine or threonine, and most preferably threonine. Preferably, the amino acid residue in position +3 relative to the asparagines residue is not a proline residue.

[0035] An "O-glycosylation site" is the OH-group of a serine or threonine residue.

[0036] The term "attachment group" is intended to indicate a functional group of the polypeptide variant, in particular of an amino acid residue thereof or a carbohydrate moiety, capable of attaching a non-polypeptide moiety such as a polymer molecule, a lipophilic molecule, a sugar moiety or an organic derivatizing agent. Useful attachment groups and their matching non-polypeptide moieties are apparent from the table below.

Attachment group	Amino acid	Examples of non-polypeptide moiety	Conjugation method/-Activated PEG	Reference
—NH_2	N-terminal, Lys	Polymer, e.g. PEG, with amide or imine group	mPEG-SPA Tresylated mPEG	Shearwater Inc. Delgado et al, critical reviews in Therapeutic Drug Carrier Systems 9(3, 4): 249–304 (1992)
—COOH	C-terminal, Asp, Glu	Polymer, e.g. PEG, with ester or amide group	mPEG-Hz	Shearwater Inc.
		Carbohydrate moiety	In vitro coupling	
—SH	Cys	Polymer, e.g. PEG, with disulfide, maleimide or vinyl sulfone group	PEG-vinylsulphone PEG-maleimide	Shearwater Inc. Delgado et al, critical reviews in Therapeutic
		Carbohydrate moiety	In vitro coupling	Drug Carrier Systems 9(3, 4): 249–304 (1992)
—OH	Ser, Thr, Lys, OH—	Sugar moiety PEG with ester, ether, carbamate, carbonate	In vivo O-linked glycosylation	
—$CONH_2$	Asn as part of a N-glycosylation site	Sugar moiety Polymer, e.g. PEG	In vivo N-glycosylation	
Aromatic residue	Phe, Tyr, Trp	Carbohydrate moiety	In vitro coupling	
—$CONH_2$	Gln	Carbohydrate moiety	In vitro coupling	Yan and Wold, Biochemistry, 1984, Jul 31; 23(16): 3759–65
Aldehyde Ketone	Oxidized oligosaccharide	Polymer, e.g. PEG, PEG-hydrazide	PEGylation	Andresz et al., 1978, Makromol. Chem. 179: 301, WO 92/16555, WO 00/23114
Guanidino	Arg	Carbohydrate moiety	In vitro coupling	Lundblad and Noyes, Chimical Reagents for Protein Modification, CRC Press Inc., Florida, USA
Imidazole ring	His	Carbohydrate moiety	In vitro coupling	As for guanidine

[0037] For in vivo N-glycosylation, the term "attachment group" is used in an unconventional way to indicate the amino acid residues constituting a N-glycosylation site (with the sequence N-X-S/T/C, wherein X is any amino acid residue except proline, N is asparagine and S/T/C is either serine, threonine or cysteine, preferably serine or threonine, and most preferably threonine). Although the asparagine residue of the N-glycosylation site is the one to which the sugar moiety is attached during glycosylation, such attachment cannot be achieved unless the other amino acid residues of the N-glycosylation site is present.

[0038] Accordingly, when the non-polypeptide moiety is a sugar moiety and the conjugation is to be achieved by in vivo N-glycosylation, the term "amino acid residue comprising an attachment group for a non-polypeptide moiety"

as used in connection with alterations of the amino acid sequence of the polypeptide is to be understood as meaning that one or more amino acid residues constituting an in vivo N-glycosylation site are to be altered in such a manner that a functional in vivo N-glycosylation site is introduced into the amino acid sequence.

[0039] In the present application, amino acid names and atom names (e.g. CA, CB, CD, CG, SG, NZ, N, O, C, etc) are used as defined by the Protein DataBank (PDB) (www-.pdb.org) based on the IUPAC nomenclature (IUPAC Nomenclature and Symbolism for Amino Acids and Peptides (residue names, atom names, etc.), *Eur. J. Biochem.,* 138, 9-37 (1984) together with their corrections in *Eur. J. Biochem.,* 152, 1 (1985)).

[0040] The term "amino acid residue" is intended to indicate an amino acid residue contained in the group consisting of alanine (Ala or A), cysteine (Cys or C), aspartic acid (Asp or D), glutamic acid (Glu or E), phenylalanine (Phe or F), glycine (Gly or G), histidine (His or H), isoleucine (Ile or I), lysine (Lys or K), leucine (Leu or L), methionine (Met or M), asparagine (Asn or N), proline (Pro or P), glutamine (Gln or Q), arginine (Arg or R), serine (Ser or S), threonine (Thr or T), valine (Val or V), tryptophan (Trp or W), and tyrosine (Tyr or Y) residues.

[0041] The terminology used for identifying amino acid positions is illustrated as follows: I205 indicates that position 205 is occupied by an isoleucine residue in the amino acid sequence shown in SEQ ID NO:2. I205T indicates that the isoleucine residue of position 205 has been substituted with a threonine residue. Alternative substitutions are indicated with a "/", e.g. I205SIT means an amino acid sequence in which isoleucine in position 205 is substituted with either serine or threonine. Multiple substitutions are indicated with a "+", e.g. K143N+N145T means a substitution of the lysine residue in position 143 with an asparagine residue and a substitution of the asparagine residue in position 145 with a threonine residue. Insertion of an additional amino acid residue is indicated in the following way: Insertion of a tyrosine residue after A3 (i.e. in position 4) is indicated by A3AY (leading to insertion of a tyrosine residue in position 4). A deletion of an amino acid residue is indicated by an asterix. For example, deletion of a valine residue in position 172 is indicated by V172*. Simultaneous insertion and substitution are indicated in the following way: Substitution of an alanine residue in position 175 with a threonine residue followed by insertion of a leucine residue after position 175 is indicated A175TL.

[0042] Unless otherwise indicated, the numbering of amino acid residues made herein is made relative to the amino acid sequence of the hFVII/hFVIIa polypeptide (SEQ ID NO:2).

[0043] The term "differs from" as used in connection with specific mutations is intended to allow for additional differences being present apart from the specified amino acid difference. For instance, in addition to the introduction of in vivo N-glycosylation sites (located outside the Gla domain), the polypeptide may comprise other modifications that are not related to introduction of such amino acid residues. In a similar way, in addition to the modifications performed in the Gla domain aiming at increasing the phospholipid membrane binding affinity, the polypeptide may contain other modifications that are not necessarily related to this effect.

[0044] Thus, in addition to the amino acid modifications disclosed herein, it will be understood that the amino acid sequence of the polypeptide variant of the invention may, if desired, contain other alterations, i.e. other substitutions, insertions or deletions. These may, for example, include truncation of the N- and/or C-terminus by one or more amino acid residues (e.g. by 1-10 amino acid residues), or addition of one or more extra residues at the N- and/or C-terminus, e.g. addition of a methionine residue at the N-terminus or introduction of a cysteine residue near or at the C-terminus, as well as "conservative amino acid substitutions", i.e. substitutions performed within groups of amino acids with similar characteristics, e.g. small amino acids, acidic amino acids, polar amino acids, basic amino acids, hydrophobic amino acids and aromatic amino acids.

[0045] Examples of such conservative substitutions are shown in the below table.

1	Alanine (A)	Glycine (G)	Serine (S)	Threonine (T)
2	Aspartic acid (D)	Glutamic acid (E)		
3	Asparagine (N)	Glutamine (Q)		
4	Arginine (R)	Histidine (H)	Lysine (K)	
5	Isoleucine (I)	Leucine (L)	Methionine (M)	Valine (V)
6	Phenylalanine (F)	Tyrosine (Y)	Tryptophan (W)	

[0046] Still other examples of additional modifications are disclosed in the section entitled "Other modifications outside the Gla domain" below.

[0047] The term "nucleotide sequence" is intended to indicate a consecutive stretch of two or more nucleotide molecules. The nucleotide sequence may be of genomic, cDNA, RNA, semisynthetic, synthetic origin, or any combinations thereof.

[0048] The term "polymerase chain reaction" or "PCR" generally refers to a method for amplification of a desired nucleotide sequence in vitro, as described, for example, in U.S. Pat. No. 4,683,195. In general, the PCR method involves repeated cycles of primer extension synthesis, using oligonucleotide primers capable of hybridising preferentially to a template nucleic acid.

[0049] The term "vector" refers to a plasmid or other nucleotide sequences that are capable of replicating within a host cell or being integrated into the host cell genome, and as such, are useful for performing different functions in conjunction with compatible host cells (a vector-host system): to facilitate the cloning of the nucleotide sequence, i.e. to produce usable quantities of the sequence, to direct the expression of the gene product encoded by the sequence and to integrate the nucleotide sequence into the genome of the host cell. The vector will contain different components depending upon the function it is to perform.

[0050] "Cell", "host cell", "cell line" and "cell culture" are used interchangeably herein and all such terms should be understood to include progeny resulting from growth or culturing of a cell.

[0051] "Transformation" and "transfection" are used interchangeably to refer to the process of introducing DNA into a cell.

[0052] "Operably linked" refers to the covalent joining of two or more nucleotide sequences, by means of enzymatic ligation or otherwise, in a configuration relative to one another such that the normal function of the sequences can be performed. For example, the nucleotide sequence encoding a presequence or secretory leader is operably linked to a nucleotide sequence coding for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide: a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the nucleotide sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, then synthetic oligonucleotide adaptors or linkers are used, in conjunction with standard recombinant DNA methods.

[0053] In the context of the present invention the terms "modification" or "amino acid modification" is intended to cover replacement of an amino acid side chain, substitution of an amino acid residue, deletion of an amino acid residue and/or insertion of an amino acid residue.

[0054] The terms "mutation" and "substitution" are used interchangeably herein.

[0055] The term "introduce" refers to introduction of an amino acid residue by substitution of an existing amino acid residue or, alternatively, by insertion of an additional amino acid residue.

[0056] The term "remove" refers to removal of an amino acid residue by substitution of the amino acid residue to be removed by another amino acid residue or, alternatively, by deletion (without substitution) of the amino acid residue to be removed

[0057] The term "FVII" or "FVII polypeptide" refers to a FVII molecule provided in single chain form. One example of a FVII polypeptide is wild-type human FVII (hFVII) shown in SEQ ID NO:2. It should be understood, however, that the term "FVII polypeptide" also covers hFVII-like molecules, such as fragments or variants of SEQ ID NO:2, in particular variants where the sequence comprises at least one, such as 1-15, e.g., 1-10, amino acid modifications as compared to SEQ ID NO:2.

[0058] The term "FVIIa" or "FVIIa polypeptide" refers to a FVIIa molecule provided in its activated two-chain form. When the amino acid sequence of SEQ ID NO:2 is used to describe the amino acid sequence of FVIIa it will be understood that the peptide bond between R152 and I153 of the single-chain form has been cleaved, and that one of the chains comprises amino acid residues 1-152, the other chain amino acid residues 153-406.

[0059] The terms "rFVII" and "rFVIIa" refer to FVII and FVIIa polypeptides produced by recombinant techniques.

[0060] The terms "hFVII" and "hFVIIa" refer to human wild-type FVII and FVIIa, respectively, having the amino acid sequence shown in SEQ ID NO:2

[0061] The terms "rhFVII" and "rhFVIIa" refer to human wild-type FVII and FVIIa, having the amino acid sequence shown in SEQ ID NO:2, produced by recombinant means. An example of rhFVIIa is NovoSeven®.

[0062] When used herein, the term "Gla domain" is intended to cover amino acid residues no. 1 to 45 of SEQ ID NO:2.

[0063] Accordingly, the term "located outside the Gla domain" covers amino acid residue no. 46-406 of SEQ ID NO:2.

[0064] The abbreviations "TF" and "TFPI" mean Tissue Factor and Tissue Factor Pathway Inhibitor, respectively.

[0065] The term "protease domain" is used about residues 153-406 counted from the N-terminus.

[0066] The term "catalytic site" is used to mean the catalytic triad consisting of S344, D242 and H193 of the polypeptide variant.

[0067] The term "parent" is intended to indicate the molecule to be modified/improved in accordance with the present invention. Although the parent polypeptide to be modified by the present invention may be any FVII or FVIIa polypeptide, and thus be derived from any origin, e.g. a non-human mammalian origin, it is preferred that the parent polypeptide is hFVII or hFVIIa

[0068] A "variant" is a polypeptide, which differs in one or more amino acid residues from its parent polypeptide, normally in 3-15 amino acid residues (e.g. in 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acid residues), such as in 3-10 amino acid residues, e.g. in 3-8 or 3-5 amino acid residues. In other words, a "variant" typically contains 3-15 amino acid modifications (for example 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acid modifications), such as 3-10 amino acid modifications, e.g. 3-8 or 3-5 amino acid modifications relative to the parent polypeptide. In the present context, the term "modification" encompasses insertions, deletions, substitutions and combinations thereof. It will be understood that a polypeptide variant according to the present invention will be modified in at least three positions, namely in at least position 10 and 32 (located in the Gla domain) and in at least one position located outside the Gla domain, where said at least one modification creates an in vivo N-glycosylation site.

[0069] The term "clotting activity" is used to mean the activity measured in the "Clotting Assay" described herein. In order to exhibit "clotting activity" a variant of the invention, in its activated form, should have at least 10% of the clotting acitivty of rhFVIIa when assayed in the "Clotting Assay" described herein. In a preferred embodiment of the invention the variant, in its activated form, has at least 20% of the clotting activity of rhFVIIa, such as at least 30%, e.g. at least 40%, more preferably at least 50%, such as at least 60%, e.g. at least 70%, even more preferably at least 80%, such as at least 90% of the clotting activity of rhFVIIa when assayed in the "Clotting Assay" described herein. In an interesting embodiment the variant, in its activated form, has substantially the same clotting activity as rhFVIIs, such as a clotting activity of 75-125% of the clotting acitivity of rhFVIIa.

[0070] The term "amidolytic activity" is used to mean the activity measured in the "Amidolytic Assay" described herein. In order to exhibit "amidolytic activity" a variant of the invention, in its activated form, should have at least 10%

of the amidolytic acitivty of rhFVIIa when assayed in the "Amidolytic Assay" described herein. In a preferred embodiment of the invention the variant, in its activated form, has at least 20% of the amidolytic activity of rhFVIIa, such as at least 30%, e.g. at least 40%, more preferably at least 50%, such as at least 60%, e.g. at least 70%, even more preferably at least 80%, such as at least 90% of the amidolytic activity of rhFVIIa when assayed in the "Amidolytic Assay" described herein. In an interesting embodiment the variant, in its activated form, has substantially the same amidolytic activity as rhFVIIs, such as an amidolytic activity of 75-125% of the amidolytic acitivity of rhFVIIa.

[0071] In the present context, the term "activity" is also used in connection with the variants capability of activating FX to FXa. This activity is also denoted "FX activation activity" or "FXa generation activity".

[0072] The term "increased FX activiation activity" or "increased FXa generation activity" is used to indicate that a variant of the invention, in its activated form, has a statistically signifycantly increased capability to activate FX to FXa as compared to rhFVIIa or. To what extent a variant of the invention (in its activated form) has an increased FX activation activity may conveniently be determined in the "TF-independent Factor X Activation Assay" described herein.

[0073] The term "stronger clot" or "increased clot strength" is used to indicate that the strength of the clot generated by the polypeptide variant is statistically significantly increased relative to that generated by rhFVIIa as determined under comparable conditions. This effect may be determined as the Area Under the Curve (AUC_{throm}) generated by the variant of the invention, in its activated form, when assayed in the "Thrombogram Assay" disclosed herein. In a similar way, the term "increased AUC_{throm}" is used to indicate that the Area Under the Curve generated by the variant (in its activated form) is statistically significantly increased relative to that generated by rhFVIIa as determined under comparable conditions and when measured in the "Thrombogram Assay" described herein

[0074] The term "T_{max}" is used about the time it takes to obtain the maximum thrombin activity level in the "Thrombogram Assay".

[0075] The term "immunogenicity" as used in connection with a given substance is intended to indicate the ability of the substance to induce a response from the immune system. The immune response may be a cell or antibody mediated response (see, e.g., Roitt: Essential Immunology (8th Edition, Blackwell) for further definition of immunogenicity). Normally, reduced antibody reactivity will be an indication of reduced immunogenicity. The reduced immunogenicity may be determined by use of any suitable method known in the art, e.g. in vivo or in vitro.

[0076] The term "functional in vivo half-life" is used in its normal meaning, i.e. the time at which 50% of the biological activity of the polypeptide is still present in the body/target organ, or the time at which the activity of the polypeptide is 50% of the initial value.

[0077] As an alternative to determining functional in vivo half-life, "serum half-life" may be determined, i.e. the time at which 50% of the polypeptide circulates in the plasma or bloodstream prior to being cleared. Determination of serum half-life is often more simple than determining the functional in vivo half-life and the magnitude of serum half-life is usually a good indication of the magnitude of functional in vivo half-life. Alternatively terms to serum half-life include "plasma half-life", "circulating half-life", "serum clearance", "plasma clearance" and "clearance half-life". The polypeptide is cleared by the action of one or more of the reticuloendothelial systems (RES), kidney, spleen or liver, by tissue factor, SEC receptor or other receptor mediated elimination, or by specific or unspecific proteolysis. Normally, clearance depends on size (relative to the cutoff for glomerular filtration), charge, attached carbohydrate chains, and the presence of cellular receptors for the protein. The functionality to be retained is normally selected from procoagulant, proteolytic or receptor binding activity. The functional in vivo half-life and the serum half-life may be determined by any suitable method known in the art.

[0078] The term "increased" as used about the functional in vivo half-life or serum half-life is used to indicate that the relevant half-life of the polypeptide variant is statistically significantly increased relative to that of rhFVIIa as determined under comparable conditions (typically determined in an experimental animal, such as rats, rabbits, pigs or monkeys).

[0079] The term "AUC_{iv}" or "Area Under the Curve when administered intravenously" is used in its normal meaning, i.e. as the area under the activity in serum-time curve, where the polypeptide variant has been administered intravenously, in particular when administered intravenously in rats. Typically, the activity measured is the "clotting activity" as defined hereinbefore. Once the experimental activity-time points have been determined, the AUC_{iv} may conveniently be calculated by a computer program, such as GraphPad Prism 3.01.

[0080] It will be understood that in order to make a direct comparison between the AUC_{iv}-values of different molecules (e.g. between the variants of the invention and a reference molecule such as rhFVIIa) the same amount of activity should be administered. Consequently, the AUC_{iv}-values are typically normalized (i.e. corrected for differences in the injected dose) and expressed as AUC_{iv}/dose administered.

[0081] The term "reduced sensitivity to proteolytic degradation" is primarily intended to mean that the polypeptide variant has reduced sensitivity to proteolytic degradation in comparison to hFVIIa or rhFVIIa as determined under comparable conditions. Preferably, the proteolytic degradation is reduced by at least 10% (e.g. by 10-25% or by 10-50%), such as at least 25% (e.g. by 25-50%, by 25-75% or by 25-100%), more preferably by at least 35%, such as at least 50%, (e.g. by 50-75% or by 50-100%) even more preferably by at least 60%, such as by at least 75% (e.g. by 75-100%) or even at least 90%. Most preferably, the proteolytic degradation is reduced by at least 100%.

[0082] The term "renal clearance" is used in its normal meaning to indicate any clearance taking place by the kidneys, e.g. by glomerular filtration, tubular excretion or degradation in the tubular cells. Renal clearance depends on physical characteristics of the polypeptide, including size (diameter), hydrodynamic volume, symmetry, shape/rigidity, and charge. Normally, a molecular weight of about 67 kDa is considered to be a cut-off-value for renal clearance.

Renal clearance may be established by any suitable assay, e.g. an established in vivo assay. Typically, renal clearance is determined by administering a labelled (e.g. radiolabelled or fluorescence labelled) polypeptide to a patient and measuring the label activity in urine collected from the patient. Reduced renal clearance is determined relative to a corresponding reference polypeptide, e.g. rhFVIIa, under comparable conditions. Preferably, the renal clearance rate of the polypeptide variant is reduced by at least 50%, preferably by at least 75%, and most preferably by at least 90% compared to rhFVIIa.

[0083] The terms "at least 25% of its side chain exposed to the surface of the molecule" and "at least 50% of its side chain exposed to the surface of the molecule" are defined with reference to Example 1, where the calculations, etc. are described in detail.

[0084] It should be noted that when the terms "at least 25% of its side chain exposed to the surface of the molecule" and "at least 50% of its side chain exposed to the surface of the molecule" are used in connection with introduction of an in vivo N-glycosylation site these terms refer to the surface accessibility of the amino acid side chain in the position where the sugar moiety is actually attached. In many cases it will be necessary to introduce a serine or a threonine residue in position +2 relative to the asparagine residue to which the sugar moiety is actually attached (unless, of course, this position is already occupied by a serine or a threonine residue) and these positions, where the serine or threonine residues are introduced, are allowed to be buried, i.e. to have less than 25% or 50% of their side chains exposed to the surface of the molecule.

[0085] The terms "tissue factor binding site", "active site region" and "ridge of the active site binding cleft" are defined with reference to Example 1 herein, wherein the above-mentioned sites/regions are determined.

[0086] The term "hydrophobic amino acid residue" includes the following amino acid residues: isoleucine (I), leucine (L), methionine (M), valine (V), phenylalanine (F), tyrosine (Y) and tryptophan (W).

[0087] The term "negatively charged amino acid residue" includes the following amino acid residues: Aspartic acid (D) and glutamic acid (E).

[0088] The term "positively charged amino acid residue" includes the following amino acid residues: Lysine (K), arginine (R) and histidine (H).

Variants of the invention

[0089] In its broadest aspect the present invention relates to a FVII or FVIIa polypeptide variant having an amino acid sequence comprising 3-15 amino acid modifications relative to hFVII or hFVIIa having the amino acid sequence shown in SEQ ID NO:2, wherein said amino acid sequence of the variant comprises an amino acid substitution in position 10 and 32 and wherein a sugar moiety is covalently attached to an introduced in vivo N-glycosylation site located outside the Gla domain.

[0090] The modifications performed in the two above-indicated regions of the parent FVII polypeptide serve the following purposes:

[0091] The modifications performed in positions located outside the Gla domain (introduction of in vivo N-glycosylation site(s)) are preferably of such nature that the AUC_{iv}, the functional in vivo half-life and/or the serum half-life of the resulting variant is increased as compared to rhFVIIa.

[0092] The modifications performed in the Gla domain of the parent polypeptide are preferably of such nature that an increased phospholipid membrane binding affinity of the resulting molecule is achieved and/or of such nature the resulting molecule has an improved capability to activate FX to FXa and/or of such nature that a stronger clot is formed.

[0093] Without being limited by any particular theory, it is presently believed that the enhanced membrane affinity results in a higher local concentration of the activated polypeptide variants in close proximity to the other coagulation factors, particularly FX. Thus, the rate of activation of FX to FXa will be higher, simply due to a higher molar ratio of the activated FVII variant to FX. The increased activation rate of FX then results in a higher amount of active thrombin, and thus a higher rate of cross-linking of fibrin.

[0094] Thus, in preferred embodiments of the invention the parent FVII or FVIIa polypeptide has been modified so that the resulting activated polypeptide variant has (as compared to rhFVIIa):

[0095] i) an increased bioavailability (AUC_{iv}) and an increased phospholipid membrane binding affinity;

[0096] ii) an increased bioavailability (AUC_{iv}) and an increased capability to activate FX to FXa;

[0097] iii) an increased bioavailability (AUC_{iv}) and is capable of generating a stronger clot (increased AUC_{throm});

[0098] iv) an increased bioavailability (AUC_{iv}) and a reduced T_{max};

[0099] v) an increased functional in vivo half-life and an increased phospholipid membrane binding affinity;

[0100] vi) an increased functional in vivo half-life and an increased capability to activate FX to FXa;

[0101] vii) an increased functional in vivo half-life and is capable of generating a stronger clot (increased AUC_{throm});

[0102] viii) an increased functional in vivo half-life and a reduced T_{max};

[0103] ix) an increased serum half-life and an increased phospholipid membrane binding affinity;

[0104] x) an increased serum half-life and an increased capability to activate FX to FXa;

[0105] xi) an increased serum half-life and is capable of generating a stronger clot (increased AUC_{throm}); and/or

[0106] xii) an increased serum half-life and a reduced T_{max}.

[0107] Consequently, medical treatment with a polypeptide variant according to the invention offers a number of advantages over the currently available rhFVIIa compound (NovoSeven®), such as administration of lower dosage, longer duration between injections, increased clot strength and/or faster action.

[0108] Thus, preferred variants of the invention are such variants which, in their activated forms and when compared

to rhFVIIa, generates in increased Area Under the Curve when administered intravenously (AUC_{iv}), in particular when administered intravenously in rats. More particularly, variants of the present invention, which are preferred, are such variants where the ratio between the AUC_{iv} of said variant, in its actvated form, and the AUC_{iv} of rhFVIIa is at least 1.25, such as at least 1.5, e.g. at least 1.75, more preferably at least 2, such as at least 3, even more preferably at least 4, such as at least 5, in particular when administered (intravenously) in rats.

[0109] This effect may in turn (but do not necessarily do so) correspond to an increased functional in vivo half-life and/or an increased serum half-life as compared to rhFVIIa. Accordingly, in another preferred embodiment of the invention, the ratio between the functional in vivo half-life or the serum half-life for the variant, in its activated form, and the functional in vivo half-life or the serum half-life for rhFVIIa is at least 1.25. More preferably, the ratio between the relevant half-life for the variant, in its activated form, and the relevant half-life for hFVIIa or rhFVIIa is at least 1.5, such as at least 1.75, e.g. at least 2, even more preferably at least 3, such as at least 4, e.g. at least 5.

[0110] As will be understood, the variants of the invention also possess, in addition to the above-mentioned functionality (i.e. increased AUC_{iv}, increased functional in vivo half-life and/or increased serum half-life), an increased phospholipid membrane binding affinity as compared to rhFVII, an an increased capability to activate FX to Fxa, a capability of generating a stronger clot (increased AUC_{throm}) and/or a reduced T_{max}.

[0111] Thus, in one preferred embodiment of the invention, the polypeptide variant has (in addition to an increased AUC_{iv}, increased functional in vivo half-life and/or increased serum half-life) an increased phospholipid membrane binding affinity relative to rhFVIIa. Membrane binding affinity may be measured by methods known in the art, such as by the Biacore® assay described in K. Nagata and H. Handa (Ads.), Real-Time Analysis of Biomolecular Interactions, Springer-Verlag, Tokyo, 2000, Chapter 6 entitled "Lipid-Protein Interactions". Alternatively, the membrane binding affinity may be measured as described in Example 1 in WO 99/20767.

[0112] In another preferred embodiment of the invention, the polypeptide variant (in addition to an increased AUC_{iv}, increased functional in vivo half-life and/or increased serum half-life), has an increased FX activation activity as compared to rhFVIIa, in particular when assayed in a TF-independent assay, such as the "TF-independent Factor X Activation Assay" disclosed herein. More particularly, it is preferred that the ratio between the FX activation activity of the polypeptide variant, in its activated form, and the FX activation activity of rhFVIIa is at least 1.25 when assayed in the "TF-independent Factor X Activation Assay" disclosed herein. More preferably, the ratio between the FX activation activity of the variant, in its activated form, and the PX activation activity of rhFVIIa is at least 1.5, such as at least 1.75, e.g. at least 2, even more preferably at least 3, such as at least 4, e.g. at least 5, still more preferably at least 6, such as at least 7, e.g. at least 8, most preferably at least 9, such as at least 10, when assayed in the "TF-independent Factor X Activation Assay" described herein.

[0113] In still another preferred embodiment of the invention, the polypeptide variant is (in addition to an increased AUC_{iv}, increased functional in vivo half-life and/or increased serum half-life), in its activated form, capable of generating a stronger clot as compared to rhFVIIa. This effect may be determined in the "Thrombogram Assay" described herein as an increase in the Area Under the Curve (AUC_{throm}). The AUC_{throm} is also sometimes denoted "total thrombin work" and constitutes a measure for the strength of the clot formed. More particularly, it is preferred that the ratio between the AUC_{throm}, generated by the variant in its activated form, and the AUC_{throm} generated by rhFVIIa is at least 1.15 when assayed in the "Thrombogram Assay" described herein. More preferably, the ratio is at least 1.2, such as at least 1.25, e.g. at least 1.3, even more preferably at least 1.4, such as at least 1.5, e.g. at least 1.6, most preferably at least 1.7, such as at least 1.8, e.g. at least 1.9 or at least 2.

[0114] In even another preferred embodiment of the invention the polypeptide variant has (in addition to an increased AUC_{iv}, increased functional in vivo half-life and/or increased serum half-life), in its activated form, a faster action. This effect may be determined in the "Thrombogram Assay" described herein as a reduction in the time needed to reach maximum thrombin level (T_{max}). Accordingly, preferred variants are such variants where the ratio between T_{max} for the variant, in its activated form, and T_{max} for rhFVIIa is at the most 0.95 when assayed in the "Thrombogram Assay" described herein. Preferably, the ratio is at the most 0.9, such as at the most 0.8, e.g. at the most 0.7, more preferably at the most 0.6, such as at the most 0.5.

Introduction of In Vivo N-Glycosylation Sites Located Outside the Gla Domain

[0115] A number of suitable modifications leading to an increase in AUC_{iv}, functional in vivo half-life and/or serum half-life is disclosed in WO 01/58935. The variants disclosed in WO 01/58935 are the result of a generally new strategy for developing improved FVII or FVIIa molecules, which may also be used for the parent FVII or FVIIa polypeptide of the present invention.

[0116] The position to be modified is preferably selected from a part of the FVII or FVIIa molecule that is located outside the tissue factor binding site, and/or outside the active site region, and/or outside the ridge of the active site binding cleft. These sites/regions are identified in Example 1 herein. It should be emphasized, however, that in certain situations, e.g. in case an inactivated polypeptide variant is desired, it may be advantageous to perform modifications in or close to such regions. For example, it is contemplated that one or more in vivo N-glycosylation sites may advantageously be introduced in the active site region or at the ridge of the active site binding cleft of the FVII or FVIIa molecule. The active site region, the tissue factor binding site and the ridge of the active site binding cleft are defined in Example 1 herein and are constituted by the following residues:

[0117] I153, Q167, V168, L169, L170, L171, Q176, L177, C178, G179, G180, T181, V188, V189, S190, A191, A192, H193, C194, F195, D196, K197, I198, W201, V228, I229, I230, P231, S232, T233, Y234, V235, P236, G237, T238, T239, N240, H241, D242, I243, A244, L245, L246, V281, S282, G283, W284, G285, Q286, T293, T324, E325, Y326, M327, F328, D338, S339, C340, K341, G342, D343, S344, G345, G346, P347, H348, L358, T359, G360, I361, V362,

S363, W364, G365, C368, V376, Y377, T378, R379, V380, Q382, Y383, W386, L387, L400 and F405 (active site region);

[0118] L13, K18, F31, E35, R36, L39, F40, I42, S43, S60, K62, D63, Q64, L65, I69, C70, F71, C72, L73, P74, F76, E77, G78, R79, E82, K85, Q88, I90, V92, N93, E94, R271, A274, F275, V276, R277, F278, R304, L305, M306, T307, Q308, D309, Q312, Q313, E325 and R379 (tissue factor binding site); and

[0119] N173, A175, K199, N200, N203, D289, R290, G291, A292, P321 and T370 (the ridge of the active site binding cleft).

[0120] The total number of amino acid residues to be modified outside the Gla domain in the parent FVII or FVIIa polypeptide (as compared to the amino acid sequence shown in SEQ ID NO:2) will typically not exceed 10. Preferably, the FVII or FVIIa variant comprises an amino acid sequence which differs in 1-10 amino acid residues from amino acid residues 46-406 shown in SEQ ID NO:2, typically in 1-8 or in 2-8 amino acid residues, e.g. in 1-5 or in 2-5 amino acid residues, such as in 1-4 or in 1-3 amino acid residues, e.g. in 1, 2 or 3 amino acid residues from amino acid residues 46-406 shown in SEQ ID NO:2.

[0121] Thus, the polypeptide variant of the invention may contain 1-10 (additional or introduced) in vivo N-glycosylation sites, typically 1-8 or 2-8 (additional or introduced) in vivo N-glycosylation sites, preferably 1-5 or 2-5 (additional or introduced) in vivo N-glycosylation sites, such as 1-4 or 1-3 (additional or introduced) in vivo N-glycosylation sites, e.g. 1, 2 or 3 (additional or introduced) in vivo N-glycosylation sites. Analogously, the polypeptide variant of the invention may contain 1-10 (additional or introduced) sugar moieties, typically 1-8 or 2-8 (additional or introduced) sugar moieties, preferably 1-5 or 2-5 (additional or introduced) sugar moieties, such as 1-4 or 1-3 (additional or introduced) sugar moieties, e.g. 1, 2 or 3 (additional or introduced) sugar moieites. It will be understood that the introduced sugar moiety/moieties will be covalently attached to the introduced in vivo N-glycosylation site(s)

[0122] When used in the present context, the term "naturally occurring glycosylation site" covers the glycosylation sites at postions N145, N322, S52 and S60. In a similar way, the term "naturally occurring in vivo O-glycosylation site" includes the positions S52 and S60, whereas the term "naturally occurring in vivo N-glycosylation site" includes positions N145 and N322.

[0123] It will be understood that in order to prepare a polypeptide variant, wherein the polypeptide variant comprises one or more sugar moieties covalently attached to one or more in vivo N-glycosylation sites, the polypeptide variant must be expressed in a host cell capable of attaching sugar (oligosaccharide) moieties at the glycosylation site(s) or alternatively subjected to in vitro glycosylation. Examples of glycosylating host cells are given in the section further below entitled "Coupling to a sugar moiety".

[0124] Examples of positions, wherein the in vivo N-glycosylation sites may be introduced include, but is not limited to, positions comprising an amino acid residue having an amino acid residue having at least 25% of its side chain exposed to the surface (as defined in Example 1 herein), such as in a position comprising an amino acid residue having at least 50% of its side chain exposed to the surface (as defined in Example 1 herein). In general, it is preferred that the in vivo N-glycosylation site is introduced by substitution, although insertion is also contemplated. The position is preferably selected from a part of the molecule that is located outside the tissue factor binding site and/or the active site region and/or outside the ridge of the active site cleft. These sites/regions are identified in Example 1 herein. It should be understood that when the term "at least 25% (or at least 50%) of its side chain exposed to the surface" is used in connection with introduction of an in vivo N-glycosylation site this term refers to the surface accessibility of the amino acid side chain in the position where the sugar moiety is actually attached. In many cases it will be necessary to introduce a serine or a threonine residue in position +2 relative to the asparagine residue to which the sugar moiety is actually attached (unless, of course, this position is already occupied by a serine or a threonine residue) and these positions, where the serine or threonine residues are introduced, are allowed to be buried, i.e. to have less than 25% of their side chains exposed to the surface.

[0125] Specific and preferred examples of such substitutions creating an in vivo N-glycosylation site include a substitution selected from the group consisting of A51N, G58N, T106N, K109N, G124N, K143N+N145T, A175T, I205S, I205T, V253N, T267N, T267N+S269T, S314N+K316S, S314N+K316T, R315N+V317S, R315N+V317T, K316N+G318S, K316N+G318T, G318N, D334N and combinations thereof. More preferably, the in vivo N-glycosylation site is introduced by a substitution selected from the group consisting of A51N, G58N, T106N, K109N, G124N, K143N+N145T, A175T, I205T, V253N, T267N+S269T, S314N+K316T, R315N+V317T, K316N+G318T, G318N, D334N and combinations thereof. Even more preferably, the in vivo N-glycosylation site is introduced by a substitution selected from the group consisting of T106N, A175T, I205T, V253N, T267N+S269T and combinations thereof, in particular I205T.

[0126] In one embodiment, only one in vivo N-glycosylation site has been introduced by substitution. In another embodiment, two or more (such as two) in vivo N-glycosylation sites have been introduced by substitution. Examples of preferred substitutions creating two in vivo N-glycosylation sites include substitutions selected from the group consisting of A51N+G58N, A51N+T106N, A51N+K109N, A51N+G124N, A51N+K143N+N145T, A51N+A175T, A51N+I205T, A51N+V253N, A51N+T267N+S269T, A51N+S314N+K316T, A51N+R315N+V317T, A51N+K316N+G318T, A51N+G318N, A51N+D334N, G58N+T106N, G58N+K109N, G58N+G124N, G58N+K143N+N145T, G58N+A175T, G58N+I205T, G58N+V253N, G58N+T267N+S269T, G58N+S314N+K316T, G58N+R315N+V317T, G58N+K316N+G318T, G58N+G318N, G58N+D334N, T106N+K109N, T106N+G124N, T106N+K143N+N145T, T106N+A175T, T106N+I205T, T106N+V253N, T106N+T267N+S269T, T106N+S314N+K316T, T106N+R315N+V317T, T106N+K316N+G318T, T106N+G318N, T106N+D334N, K109N+G124N, K109N+K143N+N145T, K109N+A175T, K109N+I205T, K109N+V253N, K109N+T267N+S269T, K109N+S314N+K316T, K109N+R315N+V317T, K109N+K316N+G318T, K109N+G318N, K109N+D334N, G124N+K143N+N145T, G124N+A175T, G124N+I205T, G124N+V253N, G124N+T267N+S269T, G124N+S314N+K316T, G124N+R315N+V317T,

G124N+K316N+G318T, G124N+G318N, G124N+D334N, K143N+N145T+A175T, K143N+N145T+I205T, K143N+N145T+V253N, K143N+N145T+T267N+S269T, K143N+N 145T+S314N+K316T, K143N+N145T+R315N+V317T, K143N+N145T+K316N+G318T, K143N+N145T+G318N, K143N+N145T+D334N, A175T+I205T, A175T+V253N, A175T+T267N+S269T, A175T+S314N+K316T, A175T+R315N+V317T, A175T+K316N+G318T, A175T+G318N, A175T+D334N, I205T+V253N, I205T+T267N+S269T, I205T+S314N+K316T, I205T+R315N+V317T, I205T+K316N+G318T, I205T+G318N, I205T+D334N, 253N+T267N+S269T, V253N+S314N+K316T, V253N+R315N+V317T, 253N+K316N+G318T, V253N+G318N, V253N+D334N, T267N+S269T+S314N+K316T, 267N+S269T+R315N+V317T, T267N+S269T+K316N+G318T, T267N+S269T+G318N, T267N+S269T+D334N, S314N+K316T+R315N+V317T, S314N+K316T+G318N, S314N+K316T+D334N, R315N+V317T+K316N+G318T, R315N+V317T+G318N, R315N+V317T+D334N and G318N+D334N. More preferably, the substitutions are selected from the group consisiting of T106N+A175T, T106N+I205T, T106N+V253N, T106N+T267N+S269T, A175T+I205T, A175T+V253N, A175T+T267N+S269T, I205T+V253N, I205T+T267N+S269T and V253N+T267N+S269T, even more preferably from the group consisiting of T106N+I205T, T106N+V253N and I205T+T267N+S269T.

[0127] In an even further embodiment, three or more (such as three) in vivo N-glycosylation sites have been introduced by substitution. Examples of preferred substitutions creating three in vivo N-glycosylation sites include substitutions selected from the group consisiting of I205T+V253N+T267N+S269T and T106N+I205T+V253N.

[0128] As discussed above, it is preferred that the in vivo N-glycosylation site is introduced in a position which does neither form part of the tissue factor binding site nor form part of the active site region and the ridge of the active site binding cleft as defined herein. It is envisaged that such glycosylation variants will primarily belong to the class of active polypeptide variants as defined hereinbefore.

[0129] It will be understood that as an alternative to introduction of in vivo N-glycosylation sites in the above-discussed positions one may introduce (either by substitution or by insertion) cysteine residues in the same positions, where the introduced cysteine residue is then subsequently covalently attached to a non-polypeptide moiety, such as PEG, in particular mPEG. Thus, examples of positions, wherein a cysteine residue may be introduced include, but is not limited to, positions comprising an amino acid residue having an amino acid residue having at least 25% of its side chain exposed to the surface (as defined in Example 1 herein), such as in a position comprising an amino acid residue having at least 50% of its side chain exposed to the surface (as defined in Example 1 herein). The position is preferably selected from a part of the molecule that is located outside the tissue factor binding site and/or the active site region and/or outside the ridge of the active site cleft. These sites/regions are identified in Example 1 herein. Thus, the above disclosure concerning introduction of in vivo N-glycosylation sites applies mutatis mutandis to introduction of cysteine residues.

[0130] It will be understood that the modifications in positions located outside the Gla domain discussed in the above section should be combined with one or more modifications in the Gla domain (see the section entitled "Modifications in the Gla domain" below).

Modifications in the Gla Domain

[0131] As will be understood the variants of the present invention comprises, in addition to at least one introduced in vivo N-glycosylation site located outside the Gla domain (cf. above), at least two substitutions in the Gla domain, namely a substitution in position 10 and position 32.

[0132] A number of suitable modifications leading to increased phospholipid membrane binding affinity is disclosed in WO 99/20767 and WO 00/66753.

[0133] In a preferred embodiment of the invention the substitution in position 10 is P10Q. In another preferred embodiment of the invention the substitution in position 32 is K32E. In a particularly preferred embodiment of the invention the variant comprises the following substitutions P10Q+K32E.

[0134] In an interesting embodiment of the invention the variant comprises, in addition to the substitutions in position 10 and 32, such as in addition to the substitutions P10Q+K32E, at least one further modification in the Gla domain.

[0135] In one preferred embodiment of the invention the further modification in the Gla domain comprises an amino acid substitution in position 33. Preferably, a hydrophobic amino acid residue is introduced by substitution in position 33, such as D33I, D33L, D33M, D33V, D33F, D33Y or D33W, in particular D33F. Accordingly, in one very interesting embodiment of the invention, the variant comprises the following substitutions P10Q+K32+D33F.

[0136] In another preferred embodiment of the invention the further modification in the Gla domain comprises an insertion of at least one (such as one) amino acid residue between position 3 and 4. It is preferred that the inserted amino acid residue is a hydrophobic amino acid residue. Most preferably the insertion is A3AY. Accordingly, in another very interesting embodiment of the invention, the variant comprises the following modifications A3AY+P10Q+K32E or A3AY+P10Q+K32E+D33F.

[0137] In still another preferred embodiment of the invention the further modification in the Gla domain comprises a substitution in position 34. It is preferred that a negatively charged amino acid residue is introduced by substitution in position 34. Most preferably the substitution is A34E. Accordingly, in still another very interesting embodiment of the invention, the variant comprises the following modifications P10Q+K32E+A34E, P10Q+K32E+D33F+A34E, A3AY+P10Q+K32E+A34E or A3AY+P10Q+K32E+D33F+A34E.

[0138] The Gla domain may also contain modifications in other positions, in particular in positions 8, 11 and 28, such as R28F or R28E. On the other hand it should be understood that the Gla domain should not be modified to such an extent that the membrane binding properties are impaired. Accordingly, it is preferred that no modifications are made in the residues that become γ-carboxylated, i.e. it is preferred that no modifications are made in residues 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35. In a similar way, it is in general not preferred that non-polypeptide moieties, such as sugar moieties and/or PEG groups, are introduced in the Gla domain.

Consequently, it is preferred that no modifications are made in the Gla domain that creates an in vivo N-glycosylation site.

[0139] Finally, it will be understood that the modifications in the Gla domain discussed in this section must be combined with one or more of the modifications disclosed in the section entitled "Introduction of in vivo N-glycosylation sites located outside the Gla domain" above.

[0140] Specific examples of such "combined" variants are given below.

[0141] In one embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+T106N.

[0142] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A175T.

[0143] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+I205T.

[0144] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+V253N.

[0145] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+T267+S269T.

[0146] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+T1106N+I205T.

[0147] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+T106N+V253N.

[0148] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+I205T+T267N+S269T.

[0149] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+T106N.

[0150] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A175T.

[0151] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+I205T.

[0152] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+V253N.

[0153] In a further preferred embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+T267+S269T.

[0154] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+T106N+I205T.

[0155] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+T106N+V253N.

[0156] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+I205T+T267N+S269T.

[0157] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+T106N.

[0158] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A175T.

[0159] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+I205T.

[0160] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+V253N.

[0161] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+T267+S269T.

[0162] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+T106N+I205T.

[0163] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+T106N+V253N.

[0164] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+I205T+T267N+S269T.

[0165] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+T106N.

[0166] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A175T.

[0167] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+T205T.

[0168] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+V253N.

[0169] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+T267+S269T.

[0170] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+T106N+I205T.

[0171] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+T106N+V253N.

[0172] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+I205T+T267N+S269T.

[0173] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+T106N.

[0174] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+A175T.

[0175] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+I205T.

[0176] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+V253N.

[0177] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+T267+S269T.

[0178] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+T106N+I205T.

[0179] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+T106N+V253N.

[0180] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+I205T+T267N+S269T.

[0181] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E+T106N.

[0182] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E A175T.

[0183] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E I205T.

[0184] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E V253N.

[0185] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E T267+S269T.

[0186] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E T106N+I205T.

[0187] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E T106N+V253N.

[0188] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E I205T+T267N+S269T.

[0189] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E+T106N.

[0190] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E A175T.

[0191] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E I205T.

[0192] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E V253N.

[0193] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34ET267+S269T.

[0194] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E T106N+I205T.

[0195] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E T106N+V253N.

[0196] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E I205T+T267N+S269T.

[0197] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E+T106N.

[0198] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E A175T.

[0199] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E I205T.

[0200] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E V253N.

[0201] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E T267+S269T.

[0202] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E T106N+I205T.

[0203] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E T106N+V253N.

[0204] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E I205T+T267N+S269T.

Other Modifications Outside the Gla Domain

[0205] In a further embodiment of the present invention, the FVII or FVIIa variant may, in addition to the modifications described in the sections above, also contain mutations, which are already known to increase the intrinsic activity of the polypeptide, e.g. such as those described in WO 02/22776

[0206] Examples of preferred substitutions include substitutions selected from the group consisting of V158D, E296D, M298Q, L305V and K337A. More preferably, said substitutions are selected from the group consisting of V158D+E296D+M298Q+L305V+K337A, V158D+E296D+M298Q+K337A, V158D+E296D+M298Q+L305V, V158D+E296D+M298Q, M298Q, L305V+K337A, L305V and K337A.

[0207] In a further embodiment of the present invention, the FVII or FVIIa variant may, in addition to the modifica-

tions described in the sections above, also contain mutations, which are already known to cause a decreased inhibition by TFPI. One example includes the substitution K341Q disclosed by Neuenschwander et al, Biochemistry, 1995; 34:8701-8707.

[0208] Moreover, the variant may contain modifications which are belived to increase the TF binding affinity. Examples of such modifications include substitutions selected from the group consisting of L39E, L39Q, L39H, 142K, 142R, S43H, S43Q, K62E, K62R, L65Q, L65S, F71D, F71Y, F71E, F71Q, F71N, E82Q, E82N, E82K and F275H

[0209] As already indicated above, the variant may also contain conservative amino acid substitutions.

The Non-Polypeptide Moiety

[0210] Based on the present disclosure the skilled person will be aware that amino acid residues comprising other attachment groups may be introduced by substitution into the parent polypeptide, using the same approach as that illustrated above with in vivo N-glycosylation sites. For instance, one or more amino acid residues comprising an acid group (glutamic acid or aspartic acid), tyrosine or lysine may be introduced into the positions discussed above. In particular, one or more cysteine residues may be introduced in the positions discussed above.

[0211] As indicated further above the non-polypeptide moiety of the conjugated variant is preferably selected from the group consisting of a polymer molecule, a lipophilic compound, a sugar moiety (by way of in vivo glycosylation) and an organic derivatizing agent. All of these agents may confer desirable properties to the variant polypeptide, in particular increased AUC_{iv}, increased functional in vivo half-life and/or increased plasma half-life. The variant polypeptide is normally conjugated to only one type of non-polypeptide moiety, but may also be conjugated to two or more different types of non-polypeptide moieties, e.g. to a polymer molecule and a sugar moiety, to a lipophilic group and a sugar moiety, to an organic derivatizing agent and a sugar moiety, to a lipophilic group and a polymer molecule, etc. The conjugation to two or more different non-polypeptide moieties may be done simultaneous or sequentially.

Methods of Preparing a Conjugated Variant of the Invention

[0212] In the following sections "Conjugation to a lipophilic compound", "Conjugation to a polymer molecule", "Conjugation to a sugar moiety" and "Conjugation to an organic derivatizing agent" conjugation to specific types of non-polypeptide moieties is described. In general, a conjugated variant according to the invention may be produced by culturing an appropriate host cell under conditions conducive for the expression of the variant polypeptide, and recovering the variant polypeptide, wherein a) the variant polypeptide comprises at least one N- or O-glycosylation site and the host cell is an eukaryotic host cell capable of in vivo glycosylation, and/or b) the variant polypeptide is subjected to conjugation to a non-polypeptide moiety in vitro.

[0213] It will be understood that the conjugation should be designed so as to produce the optimal molecule with respect to the number of non-polypeptide moieties attached, the size and form of such molecules (e.g. whether they are linear or branched), and the attachment site(s) in the polypeptide. The molecular weight of the non-polypeptide moiety to be used may, e.g., be chosen on the basis of the desired effect to be achieved. For instance, if the primary purpose of the conjugation is to achieve a conjugated variant having a high molecular weight (e.g. to reduce renal clearance) it is usually desirable to conjugate as few high molecular weight non-polypeptide moieties as possible to obtain the desired molecular weight. When a high degree of shielding is desirable this may be obtained by use of a sufficiently high number of low molecular weight non-polypeptide moieties (e.g. with a molecular weight of from about 300 Da to about 5 kDa, such as a molecular weight of from 300 Da to 2 kDa).

Conjugation to a Polymer Molecule

[0214] The polymer molecule to be coupled to the variant polypeptide may be any suitable polymer molecule, such as a natural or synthetic homo-polymer or hetero-polymer, typically with a molecular weight in the range of about 300-100,000 Da, such as about 500-20,000 Da, more preferably in the range of about 500-15,000 Da, even more preferably in the range of about 2-12 kDa, such as in the range of about 3-10 kDa. When the term "about" is used herein in connection with a certain molecular weight, the word "about" indicates an approximate average molecular weight and reflects the fact that there will normally be a certain molecular weight distribution in a given polymer preparation.

[0215] Examples of homo-polymers include a polyol (i.e. poly-OH), a polyamine (i.e. poly-NH_2) and a polycarboxylic acid (i.e. poly-COOH). A hetero-polymer is a polymer comprising different coupling groups, such as a hydroxyl group and an amine group.

[0216] Examples of suitable polymer molecules include polymer molecules selected from the group consisting of polyalkylene oxide (PAO), including polyalkylene glycol (PAG), such as polyethylene glycol (PEG) and polypropylene glycol (PPG), branched PEGs, poly-vinyl alcohol (PVA), poly-carboxylate, poly-(vinylpyrolidone), polyethylene-co-maleic acid anhydride, polystyrene-co-maleic acid anhydride, dextran, including carboxymethyl-dextran, or any other biopolymer suitable for reducing immunogenicity and/or increasing functional in vivo half-life and/or serum half-life. Another example of a polymer molecule is human albumin or another abundant plasma protein. Generally, polyalkylene glycol-derived polymers are biocompatible, non-toxic, non-antigenic, non-immunogenic, have various water solubility properties, and are easily excreted from living organisms.

[0217] PEG is the preferred polymer molecule, since it has only few reactive groups capable of cross-linking compared to, e.g., polysaccharides such as dextran. In particular, mono-functional PEG, e.g. methoxypolyethylene glycol (mPEG), is of interest since its coupling chemistry is relatively simple (only one reactive group is available for conjugating with attachment groups on the polypeptide). Consequently, as the risk of cross-linking is eliminated, the resulting conjugated variants are more homogeneous and the reaction of the polymer molecules with the variant polypeptide is easier to control.

[0218] To effect covalent attachment of the polymer molecule(s) to the variant polypeptide, the hydroxyl end groups

of the polymer molecule must be provided in activated form, i.e. with reactive functional groups (examples of which include primary amino groups, hydrazide (HZ), thiol, succinate (SUC), succinimidyl succinate (SS), succinimidyl succinamide (SSA), succinimidyl propionate (SPA), succinimidyl butyrate (SBA), succinimidy carboxymethylate (SCM), benzotriazole carbonate (BTC), N-hydroxysuccinimide (NHS), aldehyde, nitrophenylcarbonate (NPC), and tresylate (TRES)). Suitable activated polymer molecules are commercially available, e.g. from Shearwater Polymers, Inc., Huntsville, Ala., USA, or from PolyMASC Pharmaceuticals plc, UK.

[0219] Alternatively, the polymer molecules can be activated by conventional methods known in the art, e.g. as disclosed in WO 90/13540. Specific examples of activated linear or branched polymer molecules for use in the present invention are described in the Shearwater Polymers, Inc. 1997 and 2000 Catalogs (Functionalized Biocompatible Polymers for Research and pharmaceuticals, Polyethylene Glycol and Derivatives, incorporated herein by reference).

[0220] Specific examples of activated PEG polymers include the following linear PEGs: NHS-PEG (e.g. SPA-PEG, SSPA-PEG, SBA-PEG, SS-PEG, SSA-PEG, SC-PEG, SG-PEG, and SCM-PEG), and NOR-PEG, BTC-PEG, EPOX-PEG, NCO-PEG, NPC-PEG, CDI-PEG, ALD-PEG, TRES-PEG, VS-PEG, IODO-PEG, and MAL-PEG, and branched PEGs such as PEG2-NHS and those disclosed in U.S. Pat. No. 5,932,462 and U.S. Pat. No. 5,643,575, both of which are incorporated herein by reference. Furthermore, the following publications, incorporated herein by reference, disclose useful polymer molecules and/or PEGylation chemistries: U.S. Pat. No. 5,824,778, U.S. Pat. No. 5,476,653, WO 97/32607, EP 229,108, EP 402,378, U.S. Pat. No. 4,902,502, U.S. Pat. No. 5,281,698, U.S. Pat. No. 5,122,614, U.S. Pat. No. 5,219,564, WO 92/16555, WO 94/04193, WO 94/14758, WO 94/17039, WO 94/18247, WO 94/28024, WO 95/00162, WO 95/11924, WO95/13090, WO 95/33490, WO 96/00080, WO 97/18832, WO 98/41562, WO 98/48837, WO 99/32134, WO 99/32139, WO 99/32140, WO 96/40791, WO 98/32466, WO 95/06058, EP 439 508, WO 97/03106, WO 96/21469, WO 95/13312, EP 921 131, U.S. Pat. No. 5,736,625, WO 98/05363, EP 809 996, U.S. Pat. No. 5,629,384, WO 96/41813, WO 96/07670, U.S. Pat. No. 5,473,034, U.S. Pat. No. 5,516,673, EP 605 963, U.S. Pat. No. 5,382,657, EP 510 356, EP 400 472, EP 183 503 and EP 154 316.

[0221] Specific examples of activated PEG polymers particularly preferred for coupling to cysteine residues, include the following linear PEGs: vinylsulfone-PEG (VS-PEG), preferably vinylsulfone-mPEG (VS-mPEG); maleimide-PEG (MAL-PEG), preferably maleimide-mPEG (MAL-mPEG) and orthopyridyl-disulfide-PEG (OPSS-PEG), preferably orthopyridyl-disulfide-mPEG (OPSS-mPEG). Typically, such PEG or mPEG polymers will have a size of about 5 kDa, about 10 kD, about 12 kDa or about 20 kDa.

[0222] The conjugation of the polypeptide variant and the activated polymer molecules is conducted by use of any conventional method, e.g. as described in the following references (which also describe suitable methods for activation of polymer molecules): Harris and Zalipsky, eds., Poly(ethylene glycol) Chemistry and Biological Applications, AZC, Washington; R. F. Taylor, (1991), "Protein immobilisation. Fundamental and applications", Marcel Dekker, N.Y.; S. S. Wong, (1992), "Chemistry of Protein Conjugation and Crosslinking", CRC Press, Boca Raton; G. T. Hermanson et al., (1993), "Immobilized Affinity Ligand Techniques", Academic Press, N.Y.).

[0223] The skilled person will be aware that the activation method and/or conjugation chemistry to be used depends on the attachment group(s) of the variant polypeptide (examples of which are given further above), as well as the functional groups of the polymer (e.g. being amine, hydroxyl, carboxyl, aldehyde, sulfydryl, succinimidyl, maleimide, vinysulfone or haloacetate). The PEGylation may be directed towards conjugation to all available attachment groups on the variant polypeptide (i.e. such attachment groups that are exposed at the surface of the polypeptide) or may be directed towards one or more specific attachment groups, e.g. the N-terminal amino group as described in U.S. Pat. No. 5,985,265 or to cysteine residues. Furthermore, the conjugation may be achieved in one step or in a stepwise manner (e.g. as described in WO 99/55377).

[0224] For PEGylation to cysteine residues (see above) the FVII or FVIIa variant is usually treated with a reducing agent, such as dithiothreitol (DDT) prior to PEGylation. The reducing agent is subsequently removed by any conventional method, such as by desalting. Conjugation of PEG to a cysteine residue typically takes place in a suitable buffer at pH 6-9 at temperatures varying from 4° C. to 25° C. for periods up to 16 hours.

[0225] It will be understood that the PEGylation is designed so as to produce the optimal molecule with respect to the number of PEG molecules attached, the size and form of such molecules (e.g. whether they are linear or branched), and the attachment site(s) in the variant polypeptide. The molecular weight of the polymer to be used may e.g. be chosen on the basis of the desired effect to be achieved.

[0226] In connection with conjugation to only a single attachment group on the protein (e.g. the N-terminal amino group), it may be advantageous that the polymer molecule, which may be linear or branched, has a high molecular weight, preferably about 10-25 kDa, such as about 15-25 kDa, e.g. about 20 kDa.

[0227] Normally, the polymer conjugation is performed under conditions aimed at reacting as many of the available polymer attachment groups with polymer molecules. This is achieved by means of a suitable molar excess of the polymer relative to the polypeptide. Typically, the molar ratios of activated polymer molecules to polypeptide are up to about 1000-1, such as up to about 200-1, or up to about 100-1. In some cases the ration may be somewhat lower, however, such as up to about 50-1, 10-1, 5-1, 2-1 or 1-1 in order to obtain optimal reaction.

[0228] It is also contemplated according to the invention to couple the polymer molecules to the polypeptide through a linker. Suitable linkers are well known to the skilled person. A preferred example is cyanuric chloride (Abuchowski et al., (1977), J. Biol. Chem., 252, 3578-3581; U.S. Pat. No. 4,179,337; Shafer et al., (1986), J. Polym. Sci. Polym. Chem. Ed., 24, 375-378).

[0229] Subsequent to the conjugation, residual activated polymer molecules are blocked according to methods known in the art, e.g. by addition of primary amine to the reaction

mixture, and the resulting inactivated polymer molecules are removed by a suitable method.

[0230] It will be understood that depending on the circumstances, e.g. the amino acid sequence of the variant polypeptide, the nature of the activated PEG compound being used and the specific PEGylation conditions, including the molar ratio of PEG to polypeptide, varying degrees of PEGylation may be obtained, with a higher degree of PEGylation generally being obtained with a higher ratio of PEG to variant polypeptide. The PEGylated variant polypeptides resulting from any given PEGylation process will, however, normally comprise a stochastic distribution of conjugated polypeptide variants having slightly different degrees of PEGylation.

Coupling to a Sugar Moiety

[0231] In order to achieve in vivo glycosylation of a FVII molecule comprising one or more glycosylation sites the nucleotide sequence encoding the variant polypeptide must be inserted in a glycosylating, eucaryotic expression host. The expression host cell may be selected from fungal (filamentous fungal or yeast), insect or animal cells or from transgenic plant cells. In one embodiment the host cell is a mammalian cell, such as a CHO cell, BHK or HEK, e.g. HEK 293, cell, or an insect cell, such as an SF9 cell, or a yeast cell, e.g. *S. cerevisiae* or *Pichia pastoris*, or any of the host cells mentioned hereinafter.

[0232] Covalent in vitro coupling of sugar moieties (such as dextran) to amino acid residues of the variant polypeptide may also be used, e.g. as described, for example in WO 87/05330 and in Aplin etl al., CRC Crit Rev. Biochem, pp. 259-306,1981. The in vitro coupling of sugar moieties or PEG to protein- and peptide-bound Gln-residues can be carried out by transglutaminases (TGases). Transglutaminases catalyse the transfer of donor amine-groups to protein- and peptide-bound Gln-residues in a so-called cross-linking reaction. The donor-amine groups can be protein- or peptide-bound, such as the ϵ-amino-group in Lys-residues or it can be part of a small or large organic molecule. An example of a small organic molecule functioning as amino-donor in TGase-catalysed cross-linking is putrescine (1,4-diaminobutane). An example of a larger organic molecule functioning as amino-donor in TGase-catalysed cross-linking is an amine-containing PEG (Sato et al., 1996, Biochemistry 35, 13072-13080).

[0233] TGases, in general, are highly specific enzymes, and not every Gln-residues exposed on the surface of a protein is accessible to TGase-catalysed cross-linking to amino-containing substances. On the contrary, only few Gln-residues are naturally functioning as TGase substrates but the exact parameters governing which Gln-residues are good TGase substrates remain unknown. Thus, in order to render a protein susceptible to TGase-catalysed cross-linking reactions it is often a prerequisite at convenient positions to add stretches of amino acid sequence known to function very well as TGase substrates. Several amino acid sequences are known to be or to contain excellent natural TGase substrates e.g. substance P, elafin, fibrinogen, fibronectin, α_2-plasmin inhibitor, α-caseins, and β-caseins.

Conjugation to an Organic Derivatizing Agent

[0234] Covalent modification of the variant polypeptide may be performed by reacting one or more attachment groups of the variant polypeptide with an organic derivatizing agent. Suitable derivatizing agents and methods are well known in the art. For example, cysteinyl residues most commonly are reacted with x-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, α-bromo-β-(4-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole. Histidyl residues are derivatized by reaction with diethylpyrocarbonateat pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful. The reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0. Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing α-amino-containing residues include iinidoesters such as methyl picolinimidate, pyridoxal phosphate, pyridoxal, chloroborohydride, trinitrobenzenesulfonic acid, O-methylisourea, 2,4-pentanedione and transaminase-catalyzed reaction with glyoxylate. Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pKa of the guanidine functional group.

[0235] Furthermore, these reagents may react with the groups of lysine as well as the arginine guanidino group. Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R—N═C═N—R'), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl)carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Conjugation to a Lipophilic Compound

[0236] The variant polypeptide and the lipophilic compound may be conjugated to each other, either directly or by use of a linker. The lipophilic compound may be a natural compound such as a saturated or unsaturated fatty acid, a fatty acid diketone, a terpene, a prostaglandin, a vitamine, a carotenoide or steroide, or a synthetic compound such as a carbon acid, an alcohol, an amine and sulphonic acid with one or more alkyl-, aryl-, alkenyl- or other multiple unsaturated compounds. The conjugation between the variant polypeptide and the lipophilic compound, optionally through a linker may be done according to methods known in the art, e.g. as described by Bodanszky in Peptide Synthesis, John Wiley, New York, 1976 and in WO 96/12505.

Methods of Preparing a Polypeptide Variant of the Invention

[0237] The polypeptide variant of the present invention may be produced by any suitable method known in the art. Such methods include constructing a nucleotide sequence encoding the polypeptide variant and expressing the sequence in a suitable transformed or transfected host.

Preferably, the host cell is a gammacarboxylating host cell such as a mammalian cell. However, polypeptide variants of the invention may be produced, albeit less efficiently, by chemical synthesis or a combination of chemical synthesis or a combination of chemical synthesis and recombinant DNA technology.

[0238] A nucleotide sequence encoding a polypeptide of the invention may be constructed by isolating or synthesizing a nucleotide sequence encoding the parent FVII, such as hFVII with the amino acid sequence shown in SEQ ID NO:2 and then changing the nucleotide sequence so as to effect introduction (i.e. insertion or substitution) or removal (i.e. deletion or substitution) of the relevant amino acid residue(s).

[0239] The nucleotide sequence is conveniently modified by site-directed mutagenesis in accordance with conventional methods. Alternatively, the nucleotide sequence is prepared by chemical synthesis, e.g. by using an oligonucleotide synthesizer, wherein oligonucleotides are designed based on the amino acid sequence of the desired polypeptide, and preferably selecting those codons that are favored in the host cell in which the recombinant polypeptide will be produced. For example, several small oligonucleotides coding for portions of the desired polypeptide may be synthesized and assembled by PCR, ligation or ligation chain reaction (LCR) (Barany, PNAS 88:189-193, 1991). The individual oligonucleotides typically contain 5' or 3' overhangs for complementary assembly.

[0240] Alternative nucleotide sequence modification methods are available for producing polypeptide variants for high throughput screening, for instance methods which involve homologous cross-over such as disclosed in U.S. Pat. No. 5,093,257, and methods which involve gene shuffling, i.e. recombination between two or more homologous nucleotide sequences resulting in new nucleotide sequences having a number of nucleotide alterations when compared to the starting nucleotide sequences. Gene shuffling (also known as DNA shuffling) involves one or more cycles of random fragmentation and reassembly of the nucleotide sequences, followed by screening to select nucleotide sequences encoding polypeptides with desired properties. In order for homology-based nucleic acid shuffling to take place, the relevant parts of the nucleotide sequences are preferably at least 50% identical, such as at least 60% identical, more preferably at least 70% identical, such as at least 80% identical. The recombination can be performed in vitro or in vivo.

[0241] Examples of suitable in vitro gene shuffling methods are disclosed by Stemmer et al. (1994), Proc. Natl. Acad. Sci. USA; vol. 91, pp. 10747-10751; Stemmer (1994), Nature, vol. 370, pp. 389-391; Smith (1994), Nature vol. 370, pp. 324-325; Zhao et al., Nat. Biotechnol. 1998, March; 16(3): 258-61; Zhao H. and Arnold, F B, Nucleic Acids Research, 1997, Vol. 25. No. 6 pp. 1307-1308; Shao et al., Nucleic Acids Research 1998, Jan. 15; 26(2): pp. 681-83; and WO 95/17413.

[0242] An example of a suitable in vivo shuffling method is disclosed in WO 97/07205. Other techniques for mutagenesis of nucleic acid sequences by in vitro or in vivo recombination are disclosed e.g. in WO 97/20078 and U.S. Pat. No. 5,837,458. Examples of specific shuffling techniques include "family shuffling", "synthetic shuffling" and "in silico shuffling".

[0243] Family shuffling involves subjecting a family of homologous genes from different species to one or more cycles of shuffling and subsequent screening or selection. Family shuffling techniques are disclosed e.g. by Crameri et al. (1998), Nature, vol. 391, pp. 288-291; Christians et al. (1999), Nature Biotechnology, vol. 17, pp. 259-264; Chang et al. (1999), Nature Biotechnology, vol. 17, pp. 793-797; and Ness et al. (1999), Nature Biotechnology, vol. 17, 893-896.

[0244] Synthetic shuffling involves providing libraries of overlapping synthetic oligonucleotides based e.g. on a sequence alignment of homologous genes of interest. The synthetically generated oligonucleotides are recombined, and the resulting recombinant nucleic acid sequences are screened and if desired used for further shuffling cycles. Synthetic shuffling techniques are disclosed in WO 00/42561.

[0245] In silico shuffling refers to a DNA shuffling procedure, which is performed or modelled using a computer system, thereby partly or entirely avoiding the need for physically manipulating nucleic acids. Techniques for in silico shuffling are disclosed in WO 00/42560.

[0246] Once assembled (by synthesis, site-directed mutagenesis or another method), the nucleotide sequence encoding the polypeptide is inserted into a recombinant vector and operably linked to control sequences necessary for expression of the FVII in the desired transformed host cell.

[0247] It should of course be understood that not all vectors and expression control sequences function equally well to express the nucleotide sequence encoding the polypeptide variants described herein. Neither will all hosts function equally well with the same expression system. However, one of skill in the art may make a selection among these vectors, expression control sequences and hosts without undue experimentation. For example, in selecting a vector, the host must be considered because the vector must replicate in it or be able to integrate into the chromosome. The vector's copy number, the ability to control that copy number, and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered. In selecting an expression control sequence, a variety of factors should also be considered. These include, for example, the relative strength of the sequence, its controllability, and its compatibility with the nucleotide sequence encoding the polypeptide, particularly as regards potential secondary structures. Hosts should be selected by consideration of their compatibility with the chosen vector, the toxicity of the product coded for by the nucleotide sequence, their secretion characteristics, their ability to fold the polypeptide variant correctly, their fermentation or culture requirements, and the ease of purification of the products coded for by the nucleotide sequence.

[0248] The recombinant vector may be an autonomously replicating vector, i.e. a vector, which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector is one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

[0249] The vector is preferably an expression vector, in which the nucleotide sequence encoding the polypeptide

variant of the invention is operably linked to additional segments required for transcription of the nucleotide sequence. The vector is typically derived from plasmid or viral DNA. A number of suitable expression vectors for expression in the host cells mentioned herein are commercially available or described in the literature. Useful expression vectors for eukaryotic hosts, include, for example, vectors comprising expression control sequences from SV40, bovine papilloma virus, adenovirus and cytomegalovirus. Specific vectors are, e.g., pCDNA3.1(+)\Hyg (Invitrogen, Carlsbad, Calif., USA) and pCI-neo (Stratagene, La Jola, Calif., USA). Useful expression vectors for yeast cells include the 2μ plasmid and derivatives thereof, the POT1 vector (U.S. Pat. No. 4,931,373), the pJSO37 vector described in Okkels, Ann. New York Acad. Sci. 782, 202-207, 1996, and pPICZ A, B or C (Invitrogen). Useful vectors for insect cells include pVL941, pBG311 (Cate et al., "Isolation of the Bovine and Human Genes for Mullerian Inhibiting Substance And Expression of the Human Gene In Animal Cells", Cell, 45, pp. 685-98 (1986), pBluebac 4.5 and pMelbac (both available from Invitrogen). Useful expression vectors for bacterial hosts include known bacterial plasmids, such as plasmids from *E. coli*, including pBR322, pET3a and pET12a (both from Novagen Inc., WI, USA), wider host range plasmids, such as RP4, phage DNAs, e.g., the numerous derivatives of phage lambda, e.g., NM989, and other DNA phages, such as M13 and filamentous single stranded DNA phages.

[0250] Other vectors for use in this invention include those that allow the nucleotide sequence encoding the polypeptide variant to be amplified in copy number. Such amplifiable vectors are well known in the art. They include, for example, vectors able to be amplified by DHFR amplification (see, e.g., Kaufman, U.S. Pat. No. 4,470,461, Kaufman and Sharp, "Construction Of A Modular Dihydrafolate Reductase cDNA Gene: Analysis Of Signals Utilized For Efficient Expression", Mol. Cell. Biol., 2, pp. 1304-19 (1982)) and glutamine synthetase ("GS") amplification (see, e.g., U.S. Pat. No. 5,122,464 and EP 338,841).

[0251] The recombinant vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. An example of such a sequence (when the host cell is a mammalian cell) is the SV40 origin of replication. When the host cell is a yeast cell, suitable sequences enabling the vector to replicate are the yeast plasmid 2, replication genes REP 1-3 and origin of replication.

[0252] The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or the *Schizosaccharomyces pombe* TPI gene (described by P. R. Russell, Gene 40, 1985, pp. 125-130), or one which confers resistance to a drug, e.g. ampicillin, kanamycin, tetracyclin, chloramphenicol, neomycin, hygromycin or methotrexate. For *Saccharomyces cerevisiae*, selectable markers include ura3 and leu2. For filamentous fungi, selectable markers include amdS, pyrG, arcB, niaD and sC.

[0253] The term "control sequences" is defined herein to include all components, which are necessary or advantageous for the expression of the polypeptide variant of the invention. Each control sequence may be native or foreign to the nucleic acid sequence encoding the polypeptide variant. Such control sequences include, but are not limited to, a leader sequence, polyadenylation sequence, propeptide sequence, promoter, enhancer or upstream activating sequence, signal peptide sequence, and transcription terminator. At a minimum, the control sequences include a promoter.

[0254] A wide variety of expression control sequences may be used in the present invention. Such useful expression control sequences include the expression control sequences associated with structural genes of the foregoing expression vectors as well as any sequence known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof.

[0255] Examples of suitable control sequences for directing transcription in mammalian cells include the early and late promoters of SV40 and adenovirus, e.g. the adenovirus 2 major late promoter, the MT-1 (metallothionein gene) promoter, the human cytomegalovirus immediate-early gene promoter (CMV), the human elongation factor 1α (EF-1α) promoter, the *Drosophila* minimal heat shock protein 70 promoter, the Rous Sarcoma Virus (RSV) promoter, the human ubiquitin C (UbC) promoter, the human growth hormone terminator, SV40 or adenovirus E1b region polyadenylation signals and the Kozak consensus sequence (Kozak, M. *J Mol Biol* 1987 Aug. 20;196(4):947-50).

[0256] In order to improve expression in mammalian cells a synthetic intron may be inserted in the 5' untranslated region of the nucleotide sequence encoding the polypeptide. An example of a synthetic intron is the synthetic intron from the plasmid pCI-Neo (available from Promega Corporation, WI, USA).

[0257] Examples of suitable control sequences for directing transcription in insect cells include the polyhedrin promoter, the P10 promoter, the *Autographa californica* polyhedrosis virus basic protein promoter, the baculovirus immediate early gene 1 promoter and the baculovirus 39K delayed-early gene promoter, and the SV40 polyadenylation sequence. Examples of suitable control sequences for use in yeast host cells include the promoters of the yeast α-mating system, the yeast triose phosphate isomerase (TPI) promoter, promoters from yeast glycolytic genes or alcohol dehydrogenase genes, the ADH2-4c promoter, and the inducible GAL promoter. Examples of suitable control sequences for use in filamentous fungal host cells include the ADH3 promoter and terminator, a promoter derived from the genes encoding *Aspergillus oryzae* TAKA amylase triose phosphate isomerase or alkaline protease, an *A. niger* α-amylase, *A. niger* or *A. nidulans* glucoamylase, *A. nidulans* acetamidase, *Rhizomucor miehei* aspartic proteinase or lipase, the TPI1 terminator and the ADH3 terminator. Examples of suitable control sequences for use in bacterial host cells include promoters of the lac system, the trp system, the TAC or TRC system, and the major promoter regions of phage lambda.

[0258] The presence or absence of a signal peptide will, e.g., depend on the expression host cell used for the production of the polypeptide variant to be expressed (whether it is an intracellular or extracellular polypeptide) and whether it is desirable to obtain secretion. For use in filamentous fungi, the signal peptide may conveniently be derived from a gene encoding an *Aspergillus* sp. amylase or

glucoamylase, a gene encoding a *Rhizomucor miehei* lipase or protease or a *Humicola lanuginosa* lipase. The signal peptide is preferably derived from a gene encoding *A. oryzae* TAKA amylase, *A. niger* neutral α-amylase, *A. niger* acid-stable amylase, or *A. niger* glucoamylase. For use in insect cells, the signal peptide may conveniently be derived from an insect gene (cf. WO 90/05783), such as the *Lepidopteran manduca sexta* adipokinetic hormone precursor, (cf. U.S. Pat. No. 5,023,328), the honeybee melittin (Invitrogen), ecdysteroid UDPglucosyltransferase (egt) (Murphy et al., Protein Expression and Purification 4, 349-357 (1993) or human pancreatic lipase (hpl) (Methods in Enzymology 284, pp. 262-272, 1997). A preferred signal peptide for use in mammalian cells is that of hFVII or the murine Ig kappa light chain signal peptide (Coloma, M (1992) J. Imm. Methods 152:89-104). For use in yeast cells suitable signal peptides have been found to be the α-factor signal peptide from *S. cereviciae* (cf. U.S. Pat. No. 4,870,008), a modified carboxypeptidase signal peptide (cf. L. A. Valls et al., Cell 48, 1987, pp. 887-897), the yeast BAR1 signal peptide (cf. WO 87/02670), the yeast aspartic protease 3 (YAP3) signal peptide (cf. M. Egel-Mitani et al., Yeast 6, 1990, pp. 127-137), and the synthetic leader sequence TA57 (WO98/32867). For use in *E. coli* cells a suitable signal peptide have been found to be the signal peptide ompA (EP581821).

[0259] The nucleotide sequence of the invention encoding a polypeptide variant, whether prepared by site-directed mutagenesis, synthesis, PCR or other methods, may optionally include a nucleotide sequence that encode a signal peptide. The signal peptide is present when the polypeptide variant is to be secreted from the cells in which it is expressed. Such signal peptide, if present, should be one recognized by the cell chosen for expression of the polypeptide variant. The signal peptide may, e.g. be that normally associated with hFVII) or, alternatively, the signal peptide may be from another source than hFVII, such as any of those normally associated with other human wild-type vitamin K-dependent polypeptides. Furthermore, the signal peptide may be a signal peptide normally expressed from the host cell or one which is not normally expressed from the host cell. Accordingly, the signal peptide may be prokaryotic, e.g. derived from a bacterium such as *E. coli*, or eukaryotic, e.g. derived from a mammalian, or insect or yeast cell.

[0260] Any suitable host may be used to produce the polypeptide variant, including bacteria (although not particularly preferred), fungi (including yeasts), plant, insect, mammal, or other appropriate animal cells or cell lines, as well as transgenic animals or plants. Examples of bacterial host cells include grampositive bacteria such as strains of *Bacillus*, e.g. *B. brevis* or *B. subtilis, Pseudomonas* or *Streptomyces*, or gramnegative bacteria, such as strains of *E. coli*. The introduction of a vector into a bacterial host cell may, for instance, be effected by protoplast transformation (see, e.g., Chang and Cohen, 1979, *Molecular General Genetics* 168: 111-115), using competent cells (see, e.g., Young and Spizizin, 1961, *Journal of Bacteriology* 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, *Journal of Molecular Biology* 56: 209-221), electroporation (see, e.g., Shigekawa and Dower, 1988, *Biotechniques* 6: 742-751), or conjugation (see, e.g., Koehler and Thorne, 1987, *Journal of Bacteriology* 169: 5771-5278). Examples of suitable filamentous fungal host cells include strains of *Aspergillus*, e.g. *A. oryzae, A. niger*, or *A. nidulans, Fusarium* or *Trichoderma*. Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known per se. Suitable procedures for transformation of *Aspergillus* host cells are described in EP 238 023 and U.S. Pat. No. 5,679,543. Suitable methods for transforming *Fusarium* species are described by Malardier et al., 1989, *Gene* 78: 147-156 and WO 96/00787. Examples of suitable yeast host cells include strains of *Saccharomyces*, e.g. *S. cerevisiae, Schizosaccharomyces, Klyveroinyces, Pichia*, such as *P. pastoris* or *P. methzanolica, Hansenula*, such as *H. Polymorpha* or *Yarrowia*. Yeast may be transformed using the procedures described by Becker and Guarente, In Abelson, J. N. and Simon, M. I., editors, *Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology*, Volume 194, pp 182-187, Academic Press, Inc., New York; Ito et al., 1983, *Journal of Bacteriology* 153: 163; Hinnen et al., 1978, *Proceedings of the National Academy of Sciences USA* 75: 1920: and as disclosed by Clontech Laboratories, Inc, Palo Alto, Calif., USA (in the product protocol for the Yeastmaker™ Yeast Transformation System Kit). Examples of suitable insect host cells include a *Lepidoptora* cell line, such as *Spodoptera frugiperda* (Sf9 or Sf21) or *Trichoplusioa ni* cells (High Five) (U.S. Pat. No. 5,077,214). Transformation of insect cells and production of heterologous polypeptides therein may be performed as described by Invitrogen. Examples of suitable mammalian host cells include Chinese hamster ovary (CHO) cell lines, (e.g. CHO-K1; ATCC CCL-61), Green Monkey cell lines (COS) (e.g. COS 1 (ATCC CRL-1650), COS 7 (ATCC CRL-1651)); mouse cells (e.g. NS/0), Baby Hamster Kidney (BHK) cell lines (e.g. ATCC CRL-1632 or ATCC CCL-10), and human cells (e.g. HEK 293 (ATCC CRL-1573)), as well as plant cells in tissue culture. Additional suitable cell lines are known in the art and available from public depositories such as the American Type Culture Collection, Rockville, Md. Also, the mammalian cell, such as a CHO cell, may be modified to express sialyltransferase, e.g. 1,6-sialyltransferase, e.g. as described in U.S. Pat. No. 5,047,335, in order to provide improved glycosylation of the polypeptide variant.

[0261] In order to increase secretion it may be of particular interest to produce the polypeptide variant of the invention together with an endoprotease, in particular a PACE (paired basic amino acid converting enzyme) (e.g. as described in U.S. Pat. No. 5,986,079), such as a Kex2 endoprotease (e.g. as described in WO 00/28065).

[0262] Methods for introducing exogeneous DNA into mammalian host cells include calcium phosphate-mediated transfection, electroporation, DEAE-dextran mediated transfection, liposome-mediated transfection, viral vectors and the transfection method described by Life Technologies Ltd, Paisley, UK using Lipofectamin 2000. These methods are well known in the art and e.g. described by Ausbel et al. (eds.), 1996, Current Protocols in Molecular Biology, John Wiley & Sons, New York, USA. The cultivation of mammalian cells are conducted according to established methods, e.g. as disclosed in (Animal Cell Biotechnology, Methods and Protocols, Edited by Nigel Jenkins, 1999, Human Press Inc, Totowa, N.J., USA and Harrison M A and Rae I F, General Techniques of Cell Culture, Cambridge University Press 1997).

[0263] In the production methods of the present invention, the cells are cultivated in a nutrient medium suitable for

production of the polypeptide variant using methods known in the art. For example, the cell may be cultivated by shake flask cultivation, small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermenters performed in a suitable medium and under conditions allowing the polypeptide to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or may be prepared according to published compositions (e.g., in catalogues of the American Type Culture Collection). If the polypeptide variant is secreted into the nutrient medium, the polypeptide can be recovered directly from the medium. If the polypeptide variant is not secreted, it can be recovered from cell lysates.

[0264] The resulting polypeptide variant may be recovered by methods known in the art. For example, the polypeptide variant may be recovered from the nutrient medium by conventional procedures including, but not limited to, centrifugation, filtration, extraction, spray drying, evaporation, or precipitation.

[0265] The polypeptides may be purified by a variety of procedures known in the art including, but not limited to, chromatography (e.g., ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (e.g., preparative isoelectric focusing), differential solubility (e.g., ammonium sulfate precipitation), HPLC, or extraction (see, e.g., *Protein Purification*, J.-C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989).

[0266] Single chain polypeptide variants of the invention can be purified and activated to two-chain polypeptide variants by a number of methods as described in the literature (Broze and Majerus, 1980, J. Biol. Chem. 255:1242-47 and Hedner and Kisiel, 1983, J. Clin. Invest. 71:1836-41). Another method whereby single chain polypeptide variant can be purified is by incorporation of Zn ions during purification as described in U.S. Pat. No. 5,700,914. In a preferred embodiment the polypeptide variant is purified as a single chain polypeptide variant. The single chain polypeptide variant is activated by either use of an immobilized enzyme (e.g. factors Ia, IXa, Xa and XIIa) or by autoactivation using a positively charged ion exchange matrix or the like.

[0267] It is advantageous to first purify the polypeptide variant in its single chain form, then PEGylate (if desired) and last activate by one of the methods described above or by autoactivation as described by Pedersen et al, 1989, Biochemistry 28: 9331-36. The advantage of carrying out PEGylation before activation is that PEGylation of the new aminoterminal formed by cleavage of R152-I153 is avoided. PEGylation of this new amino terminal would render the molecule inactive since the formation of a hydrogen bond between D242 and the amino terminal of I153 is necessary for activity.

Pharmaceutical Composition of the Invention and its Use

[0268] In a further aspect, the present invention relates to a composition, in particular to a pharmaceutical composition, comprising a polypeptide variant of the invention and a pharmaceutically acceptable carrier or excipient.

[0269] The polypeptide variant or the pharmaceutical composition according to the invention may be used as a medicament.

[0270] Due to the improved properties mentioned hereinbefore, the polypeptide variants of the invention, or the pharmaceutical composition of the invention, are particular useful for the treatment of uncontrollable bleeding events in trauma patients, thrombocytopenic patients, patients in anticoagulant treatment, and cirrhosis patients with variceal bleeds, or other upper gastrointestinal bleedings, and in patients undergoing orthotopic liver transplantation, or liver resection (allowing for transfusion free surgery).

[0271] Trauma is defined as an injury to living tissue caused by an extrinsic agent. It is the 4th leading cause of death in the US and places a large financial burden on the economy.

[0272] Trauma is classified as either blunt or penetrative. Blunt trauma results in internal compression, organ damage and internal haemorrhage whereas penetrative trauma (as the consequence of an agent penetrating the body and destroying tissue, vessels and organs) results in external haemorrhage.

[0273] Haemorrhage, as a result of trauma, can start a cascade of problems. For example physiological compensation mechanisms are initiated with initial peripheral and mesenteric vasoconstriction to shunt blood to the central circulation. If circulation is not restored, hypovolemia shock (multiple organ failure due to inadequate perfusion) ensues. Since tissues throughout the body become starved for oxygen, anaerobic metabolism begins. However, the concomitant lactic acid leads the blood pH to drop and metabolic acidosis develops. If acidosis is severe and uncorrected, the patient may develop multisystem failure and die.

[0274] Although the majority of trauma patients are hypothermic on arrival in the emergency room due to the environmental conditions at the scene, inadequate protection, intravenous fluid administration and ongoing blood loss worsen the hypothermic state. Deficiencies in coagulation factors can result from blood loss or transfusions. Meanwhile, acidosis and hypothermia interfere with blood clotting mechanisms. Thus coagulopathy develops, which in turn, may mask surgical bleeding sites and hamper the control of mechanical bleeding. Hypothermia, coagulopathy and acidosis are often characterised as the "trauma triad of death"

[0275] Trauma may be caused by several events. For example, road traffic accidents result in many different types of trauma. Whilst some road traffic accidents are likely to result in penetrative trauma, many road traffic accidents are likely to inflict blunt trauma to both head and body. However, these various types of trauma can all result in coagulopathy in the patient. Road traffic accidents are the leading cause of accidental death in the US. There are over 42,000 deaths from them in the US each year. Many trauma patients die at the location of the accident either whilst being treated by the paramedics, before they arrive or in transit to the ER.

[0276] Another example includes gunshot wounds. Gunshot wounds are traumas that can result in massive bleeding. They are penetrative and destroy tissue as the bullet passes through the body, whether it be in the torso or a limb. In the US about 40,000 people a year die from gunshot wounds

[0277] A further example includes falls. Falls result in a similar profile of trauma type to road traffic accidents. By falling onto a solid object or the ground from height can cause both penetrative and decelerative blunt trauma. In the US, falls are a common cause of accidental death, numbering about 13,000.

[0278] A still further example includes machinery accidents. A smaller number of people die in the US from machinery accident related deaths, whether struck by, or entangled in machinery. The figures are small but significant—around 2,000.

[0279] A still further example includes stab wounds. Stab wounds are penetrative injuries that can also cause massive bleeding. The organs most likely to be damaged in a stab wound are the liver, small intestine and the colon.

[0280] Cirrhosis of the liver is the terminal sequel of prolonged repeated injury to the hepatic parenchyma. The end result is the formation of broad bands of fibrous tissue separating regenerative nodules that do not maintain the normal organization of liver lobules and thus cause deteriorated liver function. Patients have prolonged prothrombin times as a result of the depletion of vitamin K-dependent coagulation factors. Pathogenetically, liver cirrhosis should be regarded as the final common pathway of chronic liver injury, which can result from any form of intense repeated prolonged liver cell injury. Cirrhosis of the liver may be caused by direct liver injury, including chronic alcoholism, chronic viral hepatitis (types B, C, and D), and auto immune hepatitis as well as by indirect injury by way of bile duct damage, including primary biliary cirrhosis, primary sclerosing cholangitis and biliary atresia. Less common causes of cirrhosis include direct liver injury from inherited disease such as cystic fibrosis, alpha-1-antitrypsin deficiency, hemochromatosis, Wilson's disease, galactosemia, and glycogen storage disease.

[0281] Transplantation is primarily reserved for late stage cirrhotic patients, where it is the key intervention for treating the disease. To be eligible for transplantation, a patient must be classified as Child's B or C, as well as meet additional criteria for selection. Last year, in the US alone, 4,954 transplants were performed.

[0282] It has been estimated that there are 6,000 bleeding episodes associated with patients undergoing resection each year. This correlates with the reserved position of this procedure although seems slightly high in comparison with transplantation numbers.

[0283] Accurate data on the incidence of variceal bleeding is hard to obtain. The key facts known are that at the time of diagnosis, varices are present in about 60% of decompensated and 30% of compensated patients and that about 30% of these patients with varices will experience a bleed and that each episode of variceal bleeding is associated with a 30% risk of mortality.

[0284] Thus, in a further aspect the present invention relates to a polypeptide variant of the invention for the manufacture of a medicament for the treatment of diseases or disorder wherein clot formation is desirable. A still further aspect of the present invention relates to a method for treating a mammal having a disease or disorder wherein clot formation is desirable, comprising administering to a mammal in need thereof an effective amount of the polypeptide variant or the pharmaceutical composition of the invention.

[0285] Thrombocytopenia is caused by one of three mechanisms-decreased bone marrow production, increased splenic sequestration, or accelerated destruction of platelets. Thrombocytopenia is a risk factor for hemorrhage, and platelet transfusion reduces the incidence of bleeding. The threshold for prophylactic platelet transfusion is 10,000/μl. In patients without fever or infections, a threshold of 5000/μl may be sufficient to prevent spontaneous hemorrhage. For invasive procedures, 50,000/μl platelets is the usual target level. In patients who develop antibodies to platelets following repeated transfusions, bleeding can be extremely difficult to control.

[0286] Examples of diseases/disorders wherein increased clot formation is desirable include, but is not limited to, hemorrhages, including brain hemorrhages, as well as patient with severe uncontrolled bleedings, such as trauma. Further examples include patients undergoing living transplantations, patients undergoing resection and patients with variceal bleedings.

[0287] The polypeptide variants of the invention is administered to patients in a therapeutically effective dose, normally one approximately paralleling that employed in therapy with rFVII such as NovoSeven®, or at lower dosage. By "therapeutically effective dose" herein is meant a dose that is sufficient to produce the desired effects in relation to the condition for which it is administered. The exact dose will depend on the circumstances, and will be ascertainable by one skilled in the art using known techniques. Normally, the dose should be capable of preventing or lessening the severity or spread of the condition or indication being treated. It will be apparent to those of skill in the art that an effective amount of a polypeptide variant or composition of the invention depends, inter alia, upon the disease, the dose, the administration schedule, whether the polypeptide variant or composition is administered alone or in conjunction with other therapeutic agents, the plasma half-life of the compositions, and the general health of the patient. Preferably, the polypeptide variant or composition of the invention is administered in an effective dose, in particular a dose which is sufficient to normalize the coagulation disorder.

[0288] The polypeptide variant of the invention is preferably administered in a composition including a pharmaceutically acceptable carrier or excipient. "Pharmaceutically acceptable" means a carrier or excipient that does not cause any untoward effects in patients to whom it is administered. Such pharmaceutically acceptable carriers and excipients are well known in the art (see, for example, Remington's Pharmaceutical Sciences, 18th edition, A. R. Gennaro, Ed., Mack Publishing Company [1990]; Pharmaceutical Formulation Development of Peptides and Proteins, S. Frokjaer and L. Hovgaard, Eds., Taylor & Francis [2000]; and Handbook of Pharmaceutical Excipients, 3rd edition, A. Kibbe, Ed., Pharmaceutical Press [2000]).

[0289] The polypeptide variant of the invention can be formulated into pharmaceutical compositions by well-known methods. Suitable formulations are described by Remington's Pharmaceutical Sciences by E. W. Martin (Mark Publ. Co., 16th Ed., 1980).

[0290] The polypeptide variant of the invention can be used "as is" and/or in a salt form thereof. Suitable salts

include, but are not limited to, salts with alkali metals or alkaline earth metals, such as sodium, potassium, calcium and magnesium, as well as e.g. zinc salts. These salts or complexes may by present as a crystalline and/or amorphous structure.

[0291] The pharmaceutical composition of the invention may be administered alone or in conjunction with other therapeutic agents. These agents may be incorporated as part of the same pharmaceutical composition or may be administered separately from the polypeptide variant of the invention, either concurrently or in accordance with another treatment schedule. In addition, the polypeptide variant or pharmaceutical composition of the invention may be used as an adjuvant to other therapies.

[0292] A "patient" for the purposes of the present invention includes both humans and other mammals. Thus, the methods are applicable to both human therapy and veterinary applications. The pharmaceutical composition comprising the polypeptide variant of the invention may be formulated in a variety of forms, e.g. as a liquid, gel, lyophilized, or as a compressed solid. The preferred form will depend upon the particular indication being treated and will be apparent to one skilled in the art.

[0293] In particular, the pharmaceutical composition comprising the polypeptide variant of the invention may be formulated in lyophilised or stable soluble form. The polypeptide variant may be lyophilised by a variety of procedures known in the art. The polypeptide variant may be in a stable soluble form by the removal or shielding of proteolytic degradation sites as described herein. The advantage of obtaining a stable soluble preparation lies in easier handling for the patient and, in the case of emergencies, quicker action, which potentially can become life saving. The preferred form will depend upon the particular indication being treated and will be apparent to one of skill in the art.

[0294] The administration of the formulations of the present invention can be performed in a variety of ways, including, but not limited to, orally, subcutaneously, intravenously, intracerebrally, intranasally, transdermally, intraperitoneally, intramuscularly, intrapulmonary, vaginally, rectally, intraocularly, or in any other acceptable manner. The formulations can be administered continuously by infusion, although bolus injection is acceptable, using techniques well known in the art, such as pumps or implantation. In some instances the formulations may be directly applied as a solution or spray.

Parentals

[0295] A preferred example of a pharmaceutical composition is a solution designed for parenteral administration. Although in many cases pharmaceutical solution formulations are provided in liquid form, appropriate for immediate use, such parenteral formulations may also be provided in frozen or in lyophilized form. In the former case, the composition must be thawed prior to use. The latter form is often used to enhance the stability of the active compound contained in the composition under a wider variety of storage conditions, as it is recognized by those skilled in the art that lyophilized preparations are generally more stable than their liquid counterparts. Such lyophilized preparations are reconstituted prior to use by the addition of one or more suitable pharmaceutically acceptable diluents such as sterile water for injection or sterile physiological saline solution.

[0296] In case of parenterals, they are prepared for storage as lyophilized formulations or aqueous solutions by mixing, as appropriate, the polypeptide variant having the desired degree of purity with one or more pharmaceutically acceptable carriers, excipients or stabilizers typically employed in the art (all of which are termed "excipients"), for example buffering agents, stabilizing agents, preservatives, isotonifiers, non-ionic surfactants or detergents, antioxidants and/or other miscellaneous additives.

[0297] Buffering agents help to maintain the pH in the range which approximates physiological conditions. They are typically present at a concentration ranging from about 2 mM to about 50 mM. Suitable buffering agents for use in the present invention include both organic and inorganic acids and salts thereof such as citrate buffers (e.g., monosodium citrate-disodium citrate mixture, citric acid-trisodium citrate mixture, citric acid-monosodium citrate mixture, etc.), succinate buffers (e.g., succinic acid-monosodium succinate mixture, succinic acid-sodium hydroxide mixture, succinic acid-disodium succinate mixture, etc.), tartrate buffers (e.g., tartaric acid-sodium tartrate mixture, tartaric acid-potassium tartrate mixture, tartaric acid-sodium hydroxide mixture, etc.), fumarate buffers (e.g., fumaric acid-monosodium fumarate mixture, fumaric acid-disodium fumarate mixture, monosodium fumarate-disodium fumarate mixture, etc.), gluconate buffers (e.g., gluconic acid-sodium glyconate mixture, gluconic acid-sodium hydroxide mixture, gluconic acid-potassium glyconate mixture, etc.), oxalate buffer (e.g., oxalic acid-sodium oxalate mixture, oxalic acid-sodium hydroxide mixture, oxalic acid-potassium oxalate mixture, etc.), lactate buffers (e.g., lactic acid-sodium lactate mixture, lactic acid-sodium hydroxide mixture, lactic acid-potassium lactate mixture, etc.) and acetate buffers (e.g., acetic acid-sodium acetate mixture, acetic acid-sodium hydroxide mixture, etc.). Additional possibilities are phosphate buffers, histidine buffers and trimethylamine salts such as Tris.

[0298] Stabilizers refer to a broad category of excipients, which can range in function from a bulking agent to an additive which solubilizes the therapeutic agent or helps to prevent denaturation or adherence to the container wall. Typical stabilizers can be polyhydric sugar alcohols (enumerated above); amino acids such as arginine, lysine, glycine, glutamine, asparagine, histidine, alanine, ornithine, L-leucine, 2-phenylalanine, glutamic acid, threonine, etc., organic sugars or sugar alcohols, such as lactose, trehalose, stachyose, mannitol, sorbitol, xylitol, ribitol, myoinisitol, galactitol, glycerol and the like, including cyclitols such as inositol; polyethylene glycol; amino acid polymers; sulfur-containing reducing agents, such as urea, glutathione, thioctic acid, sodium thioglycolate, thioglycerol, α-monothioglycerol and sodium thiosulfate; low molecular weight polypeptides (i.e. <10 residues); proteins such as human serum albumin, bovine serum albumin, gelatin or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; monosaccharides such as xylose, mannose, fructose and glucose; disaccharides such as lactose, maltose and sucrose; trisaccharides such as raffinose, and polysaccharides such as dextran. Stabilizers are typically present in the range of from 0.1 to 10,000 parts by weight based on the active protein weight.

[0299] Preservatives are added to retard microbial growth, and are typically added in amounts of about 0.2%-1% (w/v). Suitable preservatives for use with the present invention include phenol, benzyl alcohol, meta-cresol, methyl paraben, propyl paraben, octadecyldimethylbenzyl ammonium chloride, benzalkonium halides (e.g. benzalkonium chloride, bromide or iodide), hexamethonium chloride, alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol and 3-pentanol.

[0300] Isotonicifiers are added to ensure isotonicity of liquid compositions and include polyhydric sugar alcohols, preferably trihydric or higher sugar alcohols, such as glycerin, erythritol, arabitol, xylitol, sorbitol and mannitol. Polyhydric alcohols can be present in an amount between 0.1% and 25% by weight, typically 1% to 5%, taking into account the relative amounts of the other ingredients.

[0301] Non-ionic surfactants or detergents (also known as "wetting agents") may be present to help solubilizing the therapeutic agent as well as to protect the therapeutic polypeptide against agitation-induced aggregation, which also permits the formulation to be exposed to shear surface stress without causing denaturation of the polypeptide. Suitable non-ionic surfactants include polysorbates (20, 80, etc.), polyoxamers (184, 188 etc.), Pluronic® polyols, polyoxyethylene sorbitan monoethers (Tween®-20, Tween®-80, etc.).

[0302] Additional miscellaneous excipients include bulking agents or fillers (e.g. starch), chelating agents (e.g. EDTA), antioxidants (e.g., ascorbic acid, methionine, vitamin E) and cosolvents.

[0303] The active ingredient may also be entrapped in microcapsules prepared, for example, by coascervation techniques or by interfacial polymerization, for example hydroxymethylcellulose, gelatin or poly-(methylmethacylate) microcapsules, in colloidal drug delivery systems (for example liposomes, albumin micro spheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, supra.

[0304] Parenteral formulations to be used for in vivo administration must be sterile. This is readily accomplished, for example, by filtration through sterile filtration membranes.

Sustained Release Preparations

[0305] Examples of sustained-release preparations include semi-permeable matrices of solid hydrophobic polymers containing the polypeptide variant, the matrices having a suitable form such as a film or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate) or poly(vinylalcohol)), polylactides, copolymers of L-glutamic acid and ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the ProLease® technology or Lupron Depot® (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(−)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for long periods such as up to or over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated polypeptides remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37° C., resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization is depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S—S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

[0306] The invention is further described in the following non-limiting examples.

Materials and Methods

Accessible Surface Area (ASA)

[0307] The computer program Access (B. Lee and F. M. Richards, J. Mol. Biol. 55: 379-400 (1971)) version 2 (© 1983 Yale University) is used to compute the accessible surface area (ASA) of the individual atoms in the structure. This method typically uses a probe-size of 1.4 Å and defines the Accessible Surface Area (ASA) as the area formed by the center of the probe. Prior to this calculation all water molecules and all hydrogen atoms should be removed from the coordinate set, as should other atoms not directly related to the protein.

Fractional ASA of Side Chain

[0308] The fractional ASA of the side chain atoms is computed by division of the sum of the ASA of the atoms in the side chain with a value representing the ASA of the side chain atoms of that residue type in an extended Ala-x-Ala tripeptide (See Hubbard, Campbell & Thornton (1991) J. Mol. Biol. 220,507-530). For this example the CA atom is regarded as a part of the side chain of Glycine residues but not for the remaining residues. The following table is used as standard 100% ASA for the side chain:

Ala	69.23 Å^2
Arg	200.35 Å^2
Asn	106.25 Å^2
Asp	102.06 Å^2
Cys	96.69 Å^2
Gln	140.58 Å^2
Glu	134.61 Å^2
Gly	32.28 Å^2
His	147.00 Å^2
Ile	137.91 Å^2
Leu	140.76 Å^2
Lys	162.50 Å^2
Met	156.08 Å^2
Phe	163.90 Å^2
Pro	119.65 Å^2
Ser	78.16 Å^2
Thr	101.67 Å^2
Trp	210.89 Å^2
Tyr	176.61 Å^2
Val	114.14 Å^2

[0309] Residues not detected in the structure are defined as having 100% exposure as they are thought to reside in flexible regions. The gamma-carboxy glutamic acids at positions 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35 are all defined as being 100% exposed.

Determining Distances Between Atoms

[0310] The distance between atoms is most easily determined using molecular graphics software e.g. InsightII® v. 98.0, MSI INC.

Active Site Region

[0311] The active site region is defined as any residues having at least one atom within 10 Å of any atom in the catalytic triad (residues H193, D242, S344).

Determination of Tissue Factor Binding Site

[0312] The TF binding site is defined as comprising of all residues having their accessible surface area changed upon TF binding. This is determined by at least two ASA calculations; one on the isolated ligand(s) in the ligand(s)/receptor(s) complex and one on the complete ligand(s)/receptor(s) complex.

Measurement of Reduced Sensitivity to Proteolytic Degradation

[0313] Proteolytic degradation can be measured using the assay described in U.S. Pat. No. 5,580,560, Example 5, where proteolysis is autoproteolysis.

[0314] Furthermore, reduced proteolysis can be tested in an in vivo model using radiolabelled samples and comparing proteolysis of rhFVIIa and the polypeptide variant of the invention by withdrawing blood samples and subjecting these to SDS-PAGE and autoradiography.

[0315] Irrespectively of the assay used for determining proteolytic degradation, "reduced proteolytic degradation" is intended to mean a measurable reduction in cleavage compared to is that obtained by rhFVIIa as measured by gel scanning of Coomassie stained SDS-PAGE gels, HPLC or as measured by conserved catalytic activity in comparison to wild type using the tissue factor independent activity assay decribed below.

Determination of the Molecular Weight of Polypeptide Variants

[0316] The molecular weight of polypeptide variants is determined by either SDS-PAGE, get filtration, Western Blots, matrix assisted laser desorption mass spectrometry or equilibrium centrifugation, e.g. SDS-PAGE according to Laemmli, U.K., Nature Vol 227 (1970), pp. 680-85.

TF-Independent Factor X Activation Assay

[0317] This assay has been described in detail on page 39826 in Nelsestuen et al., J Biol Chem, 2001; 276:39825-39831.

[0318] Briefly, the molecule to be assayed (either hFVIIa, rhFVIIa or the polypeptide variant of the invention in its activated form) is mixed with a source of phospholipid (phosphatidylcholine and phosphatidylserine in a ratio of 8:2 or phosphatidyleholine, phosphatidylserine and phosphatidylethanol in a ratio of 4:2:4) and Factor X in Tris buffer containing BSA. After a specified incubation time the reaction is stopped by addition of excess EDTA. The concentration of factor Xa is then measured from absorbance change at 405 nm after addition of a chromogenic substrate (S-2222, Chromogenix). After correction from background the tissue factor independent activity of rhFVIIa (a_{wt}) is determined as the absorbance change after 10 minutes and the tissue factor independent activity of the polypeptide variant of the invention ($a_{variant}$) is also determined as the absorbance change after 10 minutes. The ratio between the activity of the polypeptide variant, in its activated form, and the activity of rhFVIIa is defined as $a_{variant}/a_{wt}$.

Clotting Assay

[0319] Clotting activity is measured in one-stage assays and clotting times are recorded on a Thrombotrack IV coagulometer (MEDINOR). FVII depleted human plasma (American Diagnostica) is reconstituted and equilibrated at room temperature for 15-20 minutes. 50 µl of plasma is then transferred to the coagulometer cups.

[0320] hFVIIa, rhFVIIa or variants are diluted in Glyoxaline Buffer (5.7 mM barbiturate, 4.3 mM sodium citrate, 117 mM NaCl, 1 mg/mL BSA, pH 7.35). The samples are added to the cup in 50 µl and incubated at 37° C. for 2 minutes.

[0321] Thromboplastin (MEDINOR) is reconstituted with water and $CaCl_2$ is added. The reaction is initiated by adding 0.1 ml thromboplastin containing 4.5 mM $CaCl_2$.

[0322] Data are analysed using PRISM software.

Tf-Independent Clotting Assay

[0323] This assay is performed as described above under "Clotting Assay" but without addition of thromboplastin.

Amidolytic Assay

[0324] The ability of the variants to cleave small peptide substrates can be measured using the chromogenic substrate S-2288 (D-Ile-Pro-Arg-p-nitroanilide). The amidolytic activity may be measured both in the presence and absence of antithrombin III (ATIII).

[0325] HFVIIa, rhFVIIa or variant is diluted to 90 nM in assay buffer (50 mM Na-Hepes pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 0.1% BSA, 1 U/ml Heparin). Furthermore, soluble TF (sTF) is diluted to 450 nM in assay buffer. ATIII is diluted to 900 nM in assay buffer. 120 µl of assay buffer is mixed with 20 µl of the FVIIa sample, 20 µl sTF and 20 µl ATIII or assay buffer. The final concentrations of FVIIa, sTF and ATIII are 10, 50 and 100 nM, respectively. After 5 min incubation at room temperature with gentle shaking, followed by 10 min incubation at 37° C., the reaction is started by addition of the S-2288 substrate to 1 mM and the absorption at 405 nm is determined at several time points.

Thrombogram Assay

[0326] The effect of hFVIIa, rhFVIIa or variant on thrombin generation in human plasma is tested in a modified version of the assay described on page 589 in Hemker et al. in Thromb Haemost 2000; 83: 589-91. Briefly, the molecule to be assayed (either hFVIIa, rhFVIIa or variant) is mixed with normal platelet rich plasma (PRP), normal platelet poor plasma (PPP) or FVII depleted PPP with or without the addition of recombinant human tissue factor (rTF), relipidated rTF or another source of TF (such as thromboplastin). A source of phospholipid (phosphatidylcholine and phosphatidylserine in a ratio of 8:2 or phosphatidylcholine, phosphatidylserine and phosphatidylethanol in a ratio of 4:2:4) can be added.

[0327] The reaction is started by addition of a fluoregenic thrombin substrate and calcium chloride. The fluorescence is measured continuously and the thrombin amidolytic activity

is calculated by calculating the slope of the fluorescence curve (the increase in fluorescence over time). In this way the time until maximum thrombin amidolytic activity is obtained (T_{max}) and total thrombin work (area under the curve (AUC)) can be calculated.

[0328] The following procedure is used: PRP is obtained by centrifuging freshly drawn blood at 250 g, 15° C. for 10 min. Blood coagulation is inhibited either by using citrate (13 mM tri-sodium citrate), corn trypsin inhibitor (50-100 μg/ml blood) or a combination of citrate and corn trypsin inhibitor. The platelet count is adjusted to 3×10^8/mL using buffer or autologous platelet poor plasma (PPP). PPP is obtained by double centrifugation of PRP at 1000 g, 15° C. for 10 min. FVII depletion is done by incubating PPP with a FVII specific monoclonal antibody coupled to a solid phase.

[0329] Per well of a 96-well microtiter plate 80 μl PRP is added and 20 μL buffer containing rhFVII or variant to be tested in final concentrations between 0.1 and 100 nM. rTF is added in 5 μL assay buffer to a final concentration of 1 pM. The assay buffer consists of 20 mM Hepes, 150 mM NaCl and 60 mg/ml BSA in distilled water. The reaction is started by adding 20 μL of the substrate solution containing 0.1 M calcium chloride. The assay plate and reagents are pre-warmed to 37° C. and the reaction takes place at this temperature. The fluorimeter used is a BMG Fluormeter with a excitation filter at 390 nm and an emission filter at 460 nm. The fluorescence is measured in each well of 96-well clear bottom plates in 20-40 second interval over 30-180 minutes. Data are analyzed using PRISM Software.

ELISA Assay

[0330] FVII/FVIIa (or variant) concentrations are determined by ELISA. Wells of a microtiter plate are coated with an antibody directed against the protease domain using a solution of 2 μg/ml in PBS (100 μl per well). After 2 hours coating at R.T., the wells are washed 4 times with THT buffer (100 mM NaCl, 50 mM Tris-HCl pH 7.2 0.05% Tween-20). Subsequently, 200 μl of 1% Casein (diluted from 2.5% stock using 100 mM NaCl, 50 mM Tris-HCl pH 7.2) is added per well for blocking. After 1 hr incubation at R.T., the wells are emptied, and 100 μl of sample (optionally diluted in dilution buffer (THT+0.1% Casein)) is added. After another incubation of 1 hr at room temperature, the wells are washed 4 times with THT buffer, and 100 μl of a biotin-labelled antibody directed against the EGF-like domain (1 μg/ml) is added. After another 1 hr incubation at R.T., followed by 4 more washes with THT buffer, 100 μl of streptavidin-horse radish peroxidase (DAKO A/S, Glostrup, Denmark, 1/10000 diluted) is added. After another 1 hr incubation at R.T., followed by 4 more washes with THT buffer, 100 μl of TMB (3,3',5,5'-tetramethylbenzidine, Kemen-Tech A/S, Denmark) is added. After 30 min incubation at R.T. in the dark, 100 μl of 1 M H_2SO_4 is added and OD_{450nm} is determined. A standard curve is prepared using rhFVIIa (NovoSeven®).

[0331] Alternatively, FVII/FVIIa or variants may be quantified through the Gla domain rather than through the protease domain. In this ELISA set-up, wells are coated overnight with an antibody directed against the EGF-like domain and for detection, a calcium-dependent biotin-labelled monoclonal anti-Gla domain antibody is used (2 μg/ml, 100 μl per well). In this set-up, 5 mM $CaCl_2$ is added to the THT and dilution buffers.

Whole Blood Assay

[0332] The whole blood clotting assay is performed as described by Elg et al. Thrombosis Res. 2001, 101(3):159-170.

Reconstituted Coagulation Assay

[0333] The reconstituted coagulation assay is performed as described by Van't Veer et al. Blood 2000, 95(4), 1330-1335.

EXAMPLES

Example 1

[0334] The X-ray structure of hFVIIa in complex with soluble tissue factor by Banner et. al., J Mol Biol, 1996; 285:2089 is used for this example. It is noted that the numbering of residues in the reference does not follow the sequence. Here we have used the sequential numbering according to SEQ ID NO:2. The gamma-carboxy glutamic acids at positions 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35 are all here named GLU (three letter abbreviation) or E (one letter abbreviation). Residues 143-152 are not present in the structure.

Surface Exposure

[0335] Performing fractional ASA calculations on FVII fragments alone combined with the definition of accessibilities of non standard and/or missing residues described in the methods resulted in the following residues having more than 25% of their side chain exposed to the surface: A1, N2, A3, F4, L5, E6, E7, L8, R9, P10, S12, L13, E14, E16, K18, E19, E20, Q21, S23, F24, E25, E26, R8, E29, F31, K32, D33, A34, E35, R36, K38, L39, W41, I42, S43, S45, G47, D48, Q49, A51, S52, S53, Q56, G58, S60, K62, D63, Q64, L65, Q66, S67, I69, F71, L73, P74, A75, E77, G78, R79, E82, T83, H84, K85, D86, D87, Q88, L89, I90, V92, N93, E94, G97, E99, S103, D104, H105, T106, G107, T108, K109, S111, R113, E116, G117, S119, L120, L121, A122, D123, G124, V125, S126, T128, P129, T130, V131, E132, I140, L141, E142, K143, R144, N145, A146, S147, K148, P149, Q150, G151, R152, G155, K157, V158, P160, K161, E163, L171, N173, G174, A175, N184, T185, I186, H193, K197, K199, N200, R202, N203, I205, S214, E215, H216, D217, G218, D219, S222, R224, S232, T233, V235, P236, G237, T238, T239, N240, H249, Q250, P251, V253, T255, D256, E265, R266, T267, E270, R271, F275, V276, R277, F278, L280, L287, L288, D289, R290, G291, A292, T293, L295, E296, N301, M306, T307, Q308, D309, L311, Q312, Q313, R315, K316, V317, G318, D319, S320, P321, N322, T324, E325, Y326, Y332, S333, D334, S336, K337, K341, G342, H351, R353, G354, Q366, G367, T370, V371, G372, R379, E385, Q388, K389, R392, S393, E394, P395, R396, P397, G398, V399, L400, L401, R402, P404 and P406 (A1-S45 are located in the Gla domain, the remaining positions are located outside the Gla domain).

[0336] The following residues had more than 50% of their side chain exposed to the surface: A1, A3, F4, L5, E6, E7, L8, R9, P10, E14, E16, K18, E19, E20, Q21, S23, E25, E26, E29, K32, A34, E35, R36, K38, L39, I42, S43, G47, D48, A51, S52, S53, Q56, G58, S60, K62, L65, Q66, S67, I69, F71, L73, P74, A75, E77, G78, R79, E82, H84, K85, D86, D87, Q88, L89, I90, V92, N93, E94, G97, T106, G107, T108, K109, S111, E116, S119, L121, A122, D123, G124,

V131, E132, L141, E142, K143, R144, N145, A146, S147, K148, P149, Q150, G151, R152, G155, K157, P160, N173, G174, A175, K197, K199, N200, R202, S214, E215, H216, G218, R224, V235, P236, G237, T238, H249, Q250, V253, D256, T267, F275, R277, F278, L288, D289, R290, G291, A292, T293, L295, N301, M306, Q308, D309, L311, Q312, Q313, R315, K316, G318, D319, N322, E325, D334, K341, G354, G367, V371, E385, K389, R392, E394, R396, P397, G398, R402, P404 and P406 (A1-S43 are located in the Gla domain, the remaining positions are located outside the Gla domain).

Tissue Factor Binding Site

[0337] Performing ASA calculations the following residues in human FVII change their ASA in the complex. These residues were defined as constituting the receptor binding site: L13, K18, F31, E35, R36, L39, F40, I42, S43, S60, K62, D63, Q64, L65, I69, C70, F71, C72, L73, P74, F76, E77, G78, R79, E82, K85, Q88, I90, V92, N93, E94, R271, A274, F275, V276, R277, F278, R304, L305, M306, T307, Q308, D309, Q312, Q313, E325 and R379.

Active Site Region

[0338] The active site region is defined as any residue having at least one atom within a distance of 10 Å from any atom in the catalytic triad (residues H193, D242, S344): I153, Q167, V168, L169, L170, L171, Q176, L177, C178, G179, G180, T181, V188, V189, S190, A191, A192, H193, C194, F195, D196, K197, I198, W201, V228, I229, I230, P231, S232, T233, Y234, V235, P236, G237, T238, T239, N240, H241, D242, I243, A244, L245, L246, V281, S282, G283, W284, G285, Q286, T293, T324, E325, Y326, M327, F328, D338, S339, C340, K41, G342, D343, S344, G345, (3346, P347, H348, L358, T359, G360, I361, V362, S363, W364, G365, C368, V376, Y377, T378, R379, V380, Q382, Y383, W386, L387, L400 and F405.

The Ridge of the Active Site Binding Cleft

[0339] The ridge of the active site binding cleft region was defined by visual inspection of the FVIIa structure 1FAK-.pdb as: N173, A175, K199, N200, N203, D289, R290, G291, A292, P321 and T370.

Example 2

Design of an Expression Cassette for Expression of rhFVII in Mammalian Cells

[0340] The DNA sequence shown in SEQ ID NO:1, encompassing the short form of the full length cDNA encoding hFVII with its native short signal peptide (Hagen et al., 1986. PNAS 83:2412), was synthesized in order to facilitate high expression in mammalian cells. First the ATG start codon context was modified according to the Kozak consensus sequence (Kozak, M. *J Mol Biol* 1987 Aug. 20;196(4):947-50), so that there is a perfect match to the consensus sequence upstream of the ATG start codon. Secondly the open reading frame of the native cDNA was modified by making a bias in the codon usage towards the codons frequently used in highly expressed human genes. Further, two translational stop codons were inserted at the end of the open reading frame in order to facilitate efficient translational stop. The fully synthetic and expression optimized hFVII gene was assembled from 70-mer DNA oligonucleotides and finally amplified using end primers inserting BamHI and HindIII sites at the 5' and 3, ends respectively using standard PCR techniques, which resulted in the following sequence:

```
ggatcccgccaccatggtcagccaggccctccgcctcctgtgcctgctcc

tggggctgcagggctgcctggctgccgtcttcgtcacccaggaggaagcc

catggcgtcctgcatcgccggcgccgggccaatgcctttctggaagagct

ccgccctggctccctggaacgcgaatgcaaagaggaacagtgcagctttg

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attagctatagcgatggcgatcagtgcgcctccagcccttgccagaacgg

gggctcctgcaaagaccagctgcagagctatatctgcttctgcctgcctg

cctttgaggggcgcaattgcgaaacccataaggatgaccagctgatttgc

gtcaacgaaaacgggggctgcgagcagtactgcagcgatcacacgggcac

gaagcggagctgccgctgccacgaaggctatagcctcctggctgacgggg

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gggactccggcgggccccatgccacgcactatcgcgggacctggtacctc

accgggatcgtcagctggggccagggctgcgccacggtggggcactttgg

cgtctacacgcgcgtcagccagtacattgagtggctgcagaagctcatgc

ggagcgaaccccggcccggggtgctcctgcgggcccctttcccttgataa

aagctt
```

[0341] A vector for the cloning of the generated PCR product encompassing the expression cassette for hFVII was prepared by cloning the intron from pCINeo (Promega). The synthetic intron from pCI-Neo was amplified using standard PCR conditions and the primers:

```
CBProFpr174: 5'-AGCTGGCTAGCCACTGGGCAGGTAAGTATCA-3'
and

CBProFpr175: 5'-TGGCGGGATCCTTAAGAGCTGTAATTGAACT-3'
```

resulting in a 332 bp PCR fragment. The fragment was cut with NheI and BamHI before cloning into pCDNA3.1/HygR (obtained from Invitrogen) resulting in PF#34.

[0342] The expression cassette for hFVII was cloned between the BamHI and HindIII sites of PF434, resulting in plasmid PF#226.

Example 3

Expression of Polypeptide Variants in CHO K1 Cells

[0343] The cell line CHO K1 (ATCC # CCL-61) is seeded at 50% confluence in T-25 flasks using MEMα, 10% FCS (Gibco/BRL Cat # 10091), P/S and 5 μg/ml phylloquinone and allowed to grow until confluent. The confluent mono cell layer is transfected with 5 μg of the relevant plasmid described above using the Lipofectamine 2000 transfection agent (Life technologies) according to the manufacturer's instructions. Twenty four hours post transfection a sample is drawn and quantified using e.g. an ELISA recognizing the EGF1 domain of hFVII. At this time point relevant selection (e.g. Hygromycin B) may be applied to the cells with the purpose of generating a pool of stable transfectants. When using CHO K1 cells and the Hygromycin B resistance gene as selectable marker on the plasmid, this is usually achieved within one week.

Example 4

Generation of CHO K1 Cells Stably Expressing Polypeptide Variants

[0344] A vial of CHO-K1 transfectant pool is thawed and the cells seeded in a 175 cm^2 tissue flask containing 25 ml of MEMα, 10% FCS, phylloquinone (5 μg/ml), 100 U/l penicillin, 100 μg/l streptomycin and grown for 24 hours. The cells are harvested, diluted and plated in 96 well microtiter plates at a cell density of ½-1 cell/well. After a week of growth, colonies of 20-100 cells are present in the wells and those wells containing only one colony are labelled. After a further two weeks, the media in all wells containing only one colony is substituted with 200 μl fresh medium. After 24 hours, a medium sample is withdrawn and analysed by e.g. ELISA. High producing clones are selected and used to produce FVII or variant on large scale.

Example 5

Purification of Polypeptide Variants and Subsequent Activation

[0345] FVII and FVII variants are purified as follows: The procedure is performed at 4° C. The harvested culture media from large-scale production is ultrafiltered using a Millipore TFF system with 30 KDa cut-off Pellicon membranes. After concentration of the medium, citrate is added to 5 mM and the pH is adjusted to 8.6. If necessary, the conductivity is lowered to below 10 mS/cm. Subsequently, the sample is applied to a Q-sepharose FF column, equilibrated with 50 mM NaCl, 10 mM Tris pH 8.6. After washing the column with 100 mM NaCl, 10 mM Tris pH 8.6, followed by 150 mM NaCl, 10 mM Tris pH 8.6, FVII is eluted using 10 mM Tris, 25 mM NaCl, 35 mM $CaCl_2$, pH 8.6.

[0346] For the second chromatographic step, an affinity column is prepared by coupling of a monoclonal Calcium-dependent antiGla-domain antibody to CNBr-activated Sepharose FF. About 5.5 mg antibody is coupled per ml resin. The column is equilibrated with 10 mM Tris, 100 mM NaCl, 35 mM $CaCl_2$, pH 7.5. NaCl is added to the sample to a concentration of 100 mM NaCl and the pH is adjusted to 7.4-7.6. After O/N application of the sample, the column is washed with 100 mM NaCl, 35 mM $CaCl_2$, 10 mM Tris pH 7.5, and the FVII protein is eluted with 100 mM NaCl, 50 mM citrate, 75 mM Tris pH 7.5.

[0347] For the third chromatographic, the conductivity of the sample is lowered to below 10 mS/cm, if necessary, and the pH is adjusted to 8.6. The sample is then applied to a Q-sepharose column (equilibrated with 50 mM NaCl, 10 mM Tris pH 8.6) at a density around 3-5 mg protein per ml gel to obtain efficient activation. After application, the column is washed with 50 mM NaCl, 10 mM Tris pH 8.6 for about 4 hours with a flow of 3-4 column volumes (cv) per hour. The FVII protein is eluted using a gradient of 0-100% of 500 mM NaCl, 10 mM Tris pH 8.6 over 40 cv. FVII containing fractions are pooled.

[0348] For the final chromatographic step, the conductivity is lowered to below 10 mS/cm. Subsequently, the sample is applied to a Q-sepharose column (equilibrated with 140 mM NaCl, 10 mM glycylglycine pH 8.6) at a concentration of 3-5 mg protein per ml gel. The column is then washed with 140 mM NaCl, 10 mM glycylglycine pH 8.6 and FVII is eluted with 140 mM NaCl, 15 mM $CaCl_2$, 10 mM glycylglycine pH 8.6. The eluate is diluted to 10 mM $CaCl_2$ and the pH is adjusted 6.8-7.2. Finally, Tween-80 is added to 0.01% and the pH is adjusted to 5.5 for storage at −80° C.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 4

<210> SEQ ID NO 1
<211> LENGTH: 1338
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (115)..(1335)

<400> SEQUENCE: 1

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                                                          Ala
                                                           1

aat gcc ttt ctg gaa gag ctc cgc cct ggc tcc ctg gaa cgc gaa tgc      165
Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu Cys
             5                  10                  15

aaa gag gaa cag tgc agc ttt gag gaa gcc cgg gag att ttc aaa gac      213
Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys Asp
         20                  25                  30

gct gag cgg acc aaa ctg ttt tgg att agc tat agc gat ggc gat cag      261
Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp Gln
     35                  40                  45

tgc gcc tcc agc cct tgc cag aac ggg ggc tcc tgc aaa gac cag ctg      309
Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln Leu
 50                  55                  60                  65

cag agc tat atc tgc ttc tgc ctg cct gcc ttt gag ggg cgc aat tgc      357
Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn Cys
                 70                  75                  80

gaa acc cat aag gat gac cag ctg att tgc gtc aac gaa aac ggg ggc      405
Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly Gly
             85                  90                  95

tgc gag cag tac tgc agc gat cac acg ggc acg aag cgg agc tgc cgc      453
Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys Arg
        100                 105                 110

tgc cac gaa ggc tat agc ctc ctg gct gac ggg gtg tcc tgc acg ccc      501
Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr Pro
    115                 120                 125

acg gtg gaa tac cct tgc ggg aag att ccc att cta gaa aag cgg aac      549
Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg Asn
130                 135                 140                 145

gct agc aaa ccc cag ggc cgg atc gtc ggc ggg aag gtc tgc cct aag      597
Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro Lys
                150                 155                 160

ggg gag tgc ccc tgg cag gtc ctg ctc ctg gtc aac ggg gcc cag ctg      645
Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln Leu
            165                 170                 175

tgc ggc ggg acc ctc atc aat acc att tgg gtc gtg tcc gcc gct cac      693
Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala His
        180                 185                 190

tgc ttc gat aag att aag aat tgg cgg aac ctc atc gct gtg ctc ggc      741
Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu Gly
    195                 200                 205

gaa cac gat ctg tcc gag cat gac ggg gac gaa cag tcc cgc cgg gtg      789
Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg Val
210                 215                 220                 225

gct cag gtc atc att ccc tcc acc tat gtg cct ggc acg acc aat cac      837
Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn His
                230                 235                 240

gat atc gct ctg ctc cgc ctc cac cag ccc gtc gtg ctc acc gat cac      885
Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp His
            245                 250                 255

gtc gtg cct ctg tgc ctg cct gag cgg acc ttt agc gaa cgc acg ctg      933
Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr Leu
        260                 265                 270

gct ttc gtc cgc ttt agc ctc gtg tcc ggc tgg ggc cag ctg ctc gac      981
Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu Asp
    275                 280                 285
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Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg Leu
290                 295                 300                 305

atg acc cag gac tgc ctg cag cag tcc cgc aaa gtg ggg gac tcc ccc     1077
Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser Pro
                310                 315                 320

aat atc acg gag tat atg ttt tgc gct ggc tat agc gat ggc tcc aag     1125
Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser Lys
            325                 330                 335

gat agc tgc aag ggg gac tcc ggc ggg ccc cat gcc acg cac tat cgc     1173
Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr Arg
        340                 345                 350

ggg acc tgg tac ctc acc ggg atc gtc agc tgg ggc cag ggc tgc gcc     1221
Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys Ala
    355                 360                 365

acg gtg ggg cac ttt ggc gtc tac acg cgc gtc agc cag tac att gag     1269
Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile Glu
370                 375                 380                 385

tgg ctg cag aag ctc atg cgg agc gaa ccc cgg ccc ggg gtg ctc ctg     1317
Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu Leu
                390                 395                 400

cgg gcc cct ttc cct tga taa                                         1338
Arg Ala Pro Phe Pro
            405


<210> SEQ ID NO 2
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  1               5                  10                  15

Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys
             20                  25                  30

Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp
         35                  40                  45

Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln
     50                  55                  60

Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn
 65                  70                  75                  80

Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly
                 85                  90                  95

Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys
            100                 105                 110

Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr
        115                 120                 125

Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg
    130                 135                 140

Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro
145                 150                 155                 160

Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln
                165                 170                 175

Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala
            180                 185                 190
```

-continued

```
His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu
        195                 200                 205

Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg
    210                 215                 220

Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn
225                 230                 235                 240

His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp
                245                 250                 255

His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr
            260                 265                 270

Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu
        275                 280                 285

Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg
    290                 295                 300

Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser
305                 310                 315                 320

Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser
                325                 330                 335

Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr
            340                 345                 350

Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys
        355                 360                 365

Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile
    370                 375                 380

Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu
385                 390                 395                 400

Leu Arg Ala Pro Phe Pro
                405
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<400> SEQUENCE: 3

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<400> SEQUENCE: 4

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```

1. A Factor VII (FVII) or Factor VIIa (FVIIa) polypeptide variant having an amino acid sequence comprising 3-15 amino acid modifications relative to human Factor VII (hFVII) or human Factor VIIa (hFVIIa) having the amino acid sequence shown in SEQ ID NO:2, wherein said amino acid sequence of the variant comprises an amino acid substitution in position 10 and 32 and wherein a sugar moiety is covalently attached to an introduced in vivo N-glycosylation site located outside the Gla domain.

2. The variant according to claim 1, wherein said substitution in position 10 is P10Q.

3. The variant according to claim 1, wherein said substitution in position 32 is K32E.

4. The variant according to claim 1, wherein said substitution in position 10 is P10Q and said substitution in position 32 is K32E.

5. The variant according to claim 1, wherein said variant comprises at least one further amino acid modification in the Gla domain.

6.-**15**. (canceled)

16. The variant according to claim 5, wherein said further modification in the Gla domain comprises an amino acid substitution in position 34.

17. The variant according to claim 16, wherein a negatively charged amino acid residue is introduced by substitution in position 34.

18. The variant according to claim 17, wherein said substitution is A34E.

19. The variant according to claim 18, wherein said variant comprises the substitutions P10Q+K32E+A34E.

20.-**25**. (canceled)

26. The variant according to claim 1, wherein said in vivo N-glycosylation site is introduced by substitution.

27.-**28**. (canceled)

29. The variant according to claim 26, wherein said in vivo N-glycosylation site is introduced by a substitution selected from the group consisting of A51N, G58N, T106N, K109N, G124N, K143N+N145T, A175T, I205S, I205T, V253N, T267N, T267N+S269T, S314N+K316S, S314N+K316T, R315N+V317S, R315N+V317T, K316N+G318S, K316N+G318T, G318N, D334N and combinations thereof.

30. The variant according to claim 29, wherein said in vivo N-glycosylation site is introduced by a substitution selected from the group consisting of A51N, G58N, T106N, K109N, G124N, K143N+N145T, A175T, J205T, V253N, T267N+S269T, S314N+K316T, R315N+V317T, K316N+G318T, G318N, D334N and combinations thereof.

31. The variant according to claim 30, wherein said in vivo N-glycosylation site is introduced by a substitution selected from the group consisting of T106N, A175T, I205T, V253N, T267N+S269T and combinations thereof.

32. The variant according to claim 16, wherein one in vivo N-glycosylation site has been introduced by substitution.

33. The variant according to claim 16, wherein two or more in vivo N-glycosylation sites have been introduced by substitution.

34.-**40**. (canceled)

41. The variant according to claim 1, wherein said variant is in its activated form.

42. The variant according to claim 1, wherein said variant, in its activated form, has at least 10% of the amidolytic activity of rhFVIIa when assayed in the "Amidolytic Assay" described herein.

43. The variant according to claim 1, wherein said variant, in its activated form, has at least 10% of the clotting activity of rhFVIIa when assayed in the "Clotting Assay" described herein.

44. A nucleotide sequence encoding the variant of claim 1.

45. An expression vector comprising the nucleotide sequences of claim 44.

46. A host cell comprising the nucleotide sequence of claim 44.

47. The host cell according to claim 46, wherein said host cell is a gammacarboxylating cell capable of in vivo glycosylation.

48. A pharmaceutical composition comprising the variant of claim 1, and a pharmaceutical acceptable carrier or excipient.

49.-**54**. (canceled)

55. A method for treating a mammal having a disease or a disorder wherein clot formation is desirable, comprising administering to a mammal in need thereof an effective amount of the pharmaceutical composition of claim 48.

56. The method according to claim 55, wherein said disease or disorder is selected from the group consisting of hemorrhages, including brain hemorrhages, severe uncontrolled bleedings, such as trauma, bleedings in patients undergoing living transplantations, bleeding in patients undergoing resection and variceal bleedings.

57.-**59**. (canceled)

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(54) **FVII OR FVIIA VARIANTS**

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(57) **ABSTRACT**

Variants of FVII or FVIIa comprising at least one amino acid modification in position 196, 237 or 341 relative to hFVII or hFVIIa. The variants exhibit an increased clotting activity, i.e. reduced clotting time, compared to rhFVIIa.

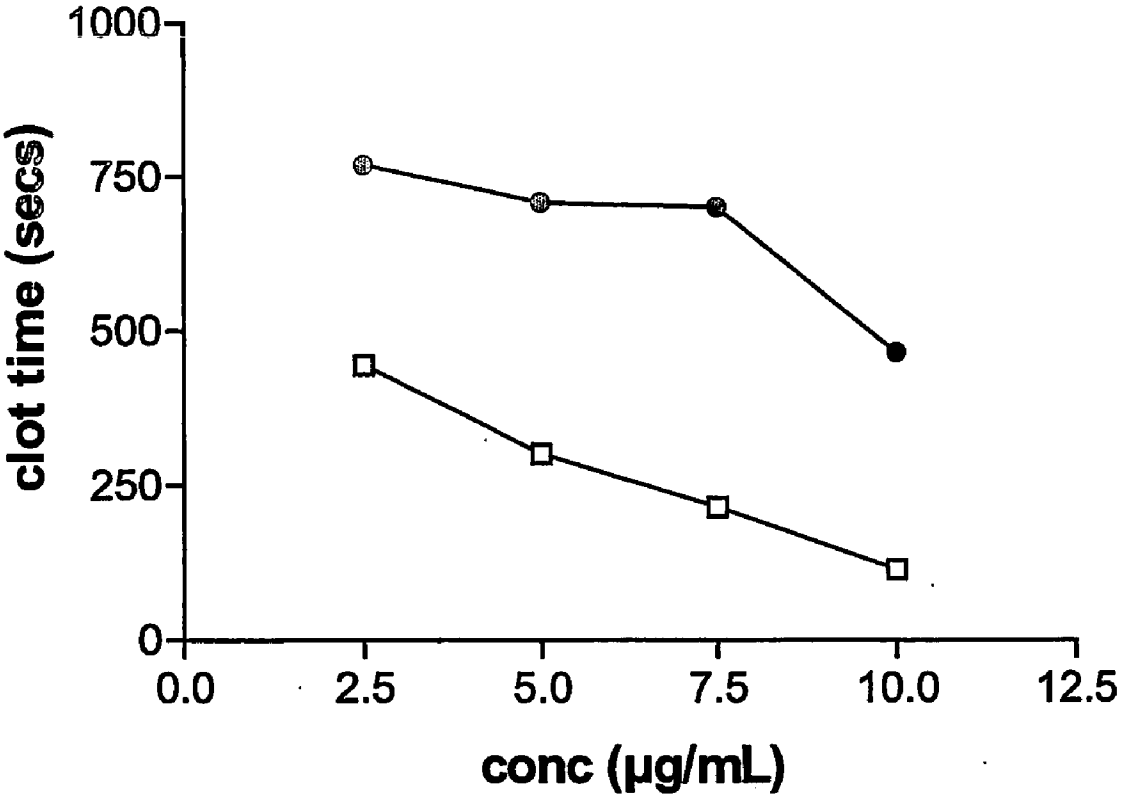

1000
750
500
250
0
clot time (secs)
0.0
2.5
5.0
7.5
10.0
12.5
conc (µg/mL)
rhFVIIa
G237GAA

Fig. 1

FVII OR FVIIA VARIANTS

FIELD OF THE INVENTION

[0001] The present invention relates to novel FVII or FVIIa variants comprising at least one amino acid modification in a position selected from the group consisting of 196, 237 and 341. The present invention also relates to use of such polypeptide variants in therapy, in particular for the treatment of a variety of coagulation-related disorders.

BACKGROUND OF THE INVENTION

[0002] Blood coagulation is a process consisting of a complex interaction of various blood components (or factors) that eventually results in a fibrin clot. Generally, the blood components participating in what is referred to as the "coagulation cascade" are proenzymes or zymogens, i.e. enzymatically inactive proteins that are converted into an active form by the action of an activator. One of these coagulation factors is factor VII (FVII).

[0003] FVII is a vitamin K-dependent plasma protein synthesized in the liver and secreted into the blood as a single-chain glycoprotein with a molecular weight of 53 kDa (Broze & Majerus, *J. Biol. Chem.* 1980; 255:1242-1247). The FVII zymogen is converted into an activated form (FVIIa) by proteolytic cleavage at a single site, R152-I153, resulting in two chains linked by a single disulfide bridge. FVIIa in complex with tissue factor (TF), the FVIIa complex, is able to convert both FIX and FX into their activated forms, followed by reactions leading to rapid thrombin production and fibrin formation (Østerud & Rapaport, *Proc Natl Acad Sci USA* 1977;. 74:5260-5264).

[0004] FVII undergoes post-translational modifications, including vitamin K-dependent carboxylation resulting in ten γ-carboxyglutamic acid residues in the N-terminal region of the molecule. Thus, residues number 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35 shown in SEQ ID NO:2 are γ-carboxyglutamic acids residues in the Gla domain important for FVII activity. Other post-translational modifications include sugar moiety attachment at two naturally occurring N-glycosylation sites at positions 145 and 322, respectively, and at two naturally occurring O-glycosylation sites at positions 52 and 60, respectively.

[0005] The gene coding for human FVII (hFVII) has been mapped to chromosome 13 at q34-qter 9 (de Grouchy et al., *Hum Genet* 1984; 66:230-233). It contains nine exons and spans 12.8 Kb (O'Hara et al., *Proc Natl Acad Sci USA* 1987; 84:5158-5162). The gene organisation and protein structure of FVII are similar to those of other vitamin K-dependent procoagulant proteins, with exons **1***a* and **1***b* encoding for signal sequence; exon **2** the propeptide and Gla domain; exon **3** a short hydrophobic region; exons **4** and **5** the epidermal growth factor-like domains; and exon **6** through **8** the serine protease catalytic domain (Yoshitake et al., *Biochemistry* 1985; 24: 3736-3750).

[0006] Reports exist on experimental three-dimensional structures of hFVIIa (Pike et al., *Proc Natl Acad Sci USA* 1999; 96:8925-30 and Kemball-Cook et al., *J. Struct. Biol.* 1999; 127:213-223), of hFVIIa in complex with soluble tissue factor using X-ray crystallographic methods (Banner et al., *Nature,* 1996; 380:41 and Zhang et al., *J. Mol. Biol.,* 1999; 285: 2089), and of smaller fragments of hFVII (Muranyi et al., *Biochemistry,* 1998; 37:10605 and Kao et al., *Biochemistry,* 1999; 38:7097).

[0007] Relatively few protein-engineered variants of FVII have been reported (Dickinson & Ruf, *J Biol Chem,* 1997;272:19875-19879, Kemball-Cook et al., *J Biol Chem,* 1998; 273:8516-8521, Bharadwaj et al., *J Biol Chem,* 1996; 271:30685-30691, Ruf et al., *Biochemistry,* 1999; 38:1957-1966).

[0008] Reports exist on expression of FVII in BHK or other mammalian cells (WO 92/15686, WO 91/11514 and WO 88/10295) and co-expression of FVII and kex2 endoprotease in eukaryotic cells (WO 00/28065).

[0009] Commercial preparations of recombinant human FVIIa (rhFVIIa) are sold under the tradename NovoSeven®. NovoSeven® is indicated for the treatment of bleeding episodes in hemophilia A or B patients. NovoSeven® is the only rhFVIIa for effective and reliable treatment of bleeding episodes available on the market.

[0010] An inactive form of FVII in which arginine 152 and/or isoleucine 153 are modified has been reported in WO 91/11514. These amino acids are located at the activation site. WO 96/12800 describes inactivation of FVIIa by a serine proteinase inhibitor; inactivation by carbamylation of FVIIa at the α-amino acid group I153 has been described by Petersen et al., *Eur J Biochem,* 1999;261:124-129. The inactivated form is capable of competing with hFVII or hFVIIa for binding to TF and inhibiting clotting activity. The inactivated form of FVIIa is suggested to be used for treatment of patients suffering from hypercoagulable states, such as patients with sepsis, at risk of myocardial infarction or of thrombotic stroke.

[0011] WO 98/32466 suggests that FVII, among many other proteins, may be PEGylated (i.e. attached to one or more polyethylene glycol molecules) but does not contain any further information in this respect.

[0012] WO 01/58935 discloses a new strategy for developing FVII or FVIIa molecules having inter alia an increased half-life by means of directed glycosylation or PEGylation.

[0013] WO 03/093465 discloses FVII or FVIIa variants having certain modifications in the Gla domain and having one or more N-glycosylation sites introduced outside the Gla domain.

[0014] A circulating rhFVIIa half-life of 2.3 hours was reported in "Summary Basis for Approval for NovoSeven®", FDA reference number 96-0597. Relatively high doses and frequent administration are necessary to reach and sustain the desired therapeutic or prophylactic effect. As a consequence, adequate dose regulation is difficult to obtain and the need for frequent intravenous administrations imposes restrictions on the patient's way of living.

[0015] In normal hemostasis, the procoagulant system is in balance with anticoagulant systems involved in the termination of the hemostatic reaction and the fibrinolytic system, which dissolves clots once they are formed. The anticoagulant systems contain several protease inhibitors, e.g., the Tissue Factor Pathway Inhibitor (TFPI), antithrombin-III (AT-III), heparin cofactor-II (HC-II), and the protein C pathway.

[0016] TFPI is a reversible, active site-directed inhibitor of FXa, which regulates coagulation by inhibiting FVIIa-TF in a FXa-dependent manner. The TFPI-FXa complex binds to the FVIIa-TF complex, resulting in the formation of a TF-FVIIa-TFPI-FXa complex.

[0017] The in vivo relevance of TFPI is supported by experiments showing a hemostatic effect of a neutralizing anti-TFPI antibody in a hemophila bleeding model (Erhardtsen et al. *Blood Coagul Fibrinolysis* 1995; 6:388-394). Furthermore, in biochemical reconstitution experiments, TFPI was shown to extend the initiation phase and reduce the rate of thrombin generation during the propagation phase (van't Veer and Mann; *J. Biol. Chem.* 1997; 272: 4367-4377).

[0018] An object of the present invention is to provide FVII or FVIIa variants which exhibit an increased clotting activity as compared to hFVIIa or rhFVIIa. It is contemplated that this may be obtained by way of FVII or FVIIa variants having an altered affinity to TFPI.

[0019] Another problem in current rhFVIIa treatment is the relative instability of the molecule with respect to proteolytic degradation. Proteolytic degradation is a major obstacle for obtaining a preparation in solution as opposed to a lyophilized product. The advantage of obtaining a stable soluble preparation lies in easier handling for the patient, and, in the case of emergencies, quicker action, which potentially can become life saving. Attempts to prevent proteolytic degradation by site directed mutagenesis at major proteolytic sites have been disclosed in WO 88/10295.

[0020] Thus, a further object of the present invention is to provide FVII/FVIIa variants which, in addition to the above-mentioned improved properties, are more stable towards proteolytic degradation, i.e. possess reduced sensitivity to proteolytic degradation.

[0021] A molecule with a longer circulation half-life would decrease the number of necessary administrations. Given the association of current FVIIa product with frequent injections, and the potential for obtaining more optimal therapeutic FVIIa levels with concomitant enhanced therapeutic effect, there is a clear need for improved FVII- or FVIIa-like molecules. One way to increase the circulation half-life of a protein is to ensure that renal clearance of the protein is reduced. This may be achieved by conjugating the protein to a chemical moiety which is capable of conferring reduced renal clearance to the protein. Furthermore, attachment of a chemical moiety to the protein or substitution of amino acids exposed to proteolysis may effectively block a proteolytic enzyme from contact leading to proteolytic degradation of the protein. Polyethylene glycol (PEG) is one such chemical moiety that has been used in the preparation of therapeutic protein products.

[0022] Thus, a further objective of the present invention is to provide FVII/FVIIa variants which, in addition to the above-mentioned improved properties, possess an increased functional in vivo half-life and/or an increased serum half-life.

[0023] The improved FVII/FVIIa variants disclosed herein address these objectives.

BRIEF DISCLOSURE OF THE INVENTION

[0024] The present invention provides improved recombinant FVII or FVIIa variants comprising at least one amino acid modification in a position selected from the group consisting of 196, 237 and 341. These amino acid modifications result in an altered binding of FVIIa to TFPI. As indicated above, the resulting molecules have one or more improved properties as compared to commercially available rhFVIIa, such as NovoSeven®.

[0025] In interesting embodiments, the FVII or FVIIa variant has been further modified so that the resulting variant has an enhanced phospholipid membrane binding affinity, increased functional in vivo half-life, increased plasma half-life and/or an increased Area Under the Curve when administered intravenously (AUC_{iv}). Medical treatment with such a variant is contemplated to offer one or more advantages over the currently available rhFVIIa compound, such as lower dosage, faster action in uncontrolled bleedings and, optionally, longer duration between injections.

[0026] Accordingly, in a first aspect the invention relates to a variant of FVII or FVIIa, wherein said variant comprises at least one amino acid modification in a position selected from the group consisting of 196, 237 and 341 as compared to hFVII or hFVIIa (SEQ ID NO:2).

[0027] Another aspect of the invention relates to a nucleotide sequence encoding the variant of the invention.

[0028] In a further aspect the invention relates to an expression vector comprising the nucleotide sequence of the invention.

[0029] In a still further aspect the invention relates to a host cell comprising the nucleotide sequence of the invention or the expression vector of the invention.

[0030] In an even further aspect the invention relates to a pharmaceutical composition comprising the variant of the invention, and a pharmaceutically acceptable carrier or excipient.

[0031] Still another aspect of the invention relates to a variant of the invention, or a pharmaceutical composition of the invention, for use as a medicament.

[0032] Further aspects of the present invention will be apparent from the below description as well as from the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0033] FIG. **1** shows the clotting time vs. concentration for [G237GAA]rhFVIIa when assayed in the "Whole Blood Assay". For comparison, the result for rhFVIIa is included. rhFVIIa;

[0034] ● rbFVIIa;

[0035] □[G237GAA]rhFVIIa.

DETAILED DISCLOSURE OF THE INVENTION

Definitions

[0036] In the context of the present application and invention the following definitions apply:

[0037] The term "conjugate" (or interchangeably "conjugated polypeptide variant") is intended to indicate a heterogeneous (in the sense of composite or chimeric) molecule formed by the covalent attachment of one or more polypeptide(s) to one or more non-polypeptide moieties such as

polymer molecules, lipophilic compounds, sugar moieties or organic derivatizing agents. Preferably, the conjugate is soluble at relevant concentrations and conditions, i.e. soluble in physiological fluids such as blood. Examples of conjugated polypeptide variants of the invention include glycosylated and/or PEGylated polypeptides.

[0038] The term "covalent attachment" or "covalently attached" means that the polypeptide variant and the non-polypeptide moiety are either directly covalently joined to one another, or else are indirectly covalently joined to one another through an intervening moiety or moieties, such as a bridge, spacer, or linkage moiety or moieties.

[0039] When used herein, the term "non-polypeptide moiety" means a molecule that is capable of conjugating to an attachment group of the polypeptide variant of the invention. Preferred examples of such molecules include polymer molecules, sugar moieties, lipophilic compounds, or organic derivatizing agents, in particular sugar moieties. When used in the context of a polypeptide variant of the invention it will be understood that the non-polypeptide moiety is linked to the polypeptide part of the polypeptide variant through an attachment group of the polypeptide variant. As explained above, the non-polypeptide moiety may be directly covalently joined to the attachment group or it may be indirectly covalently joined to the attachment group through an intervening moiety or moieties, such as a bridge, spacer, or linkage moiety or moieties.

[0040] The "polymer molecule" is a molecule formed by covalent linkage of-two or more monomers, wherein none of the monomers is an amino acid residue, except where the polymer is human albumin or another abundant plasma protein. The term "polymer" may be used interchangeably with the term "polymer molecule". The term is also intended to cover carbohydrate molecules attached by in vitro glycosylation, i.e. a synthetic glycosylation performed in vitro normally involving covalently linking a carbohydrate molecule to an attachment group of the polypeptide variant, optionally using a cross-linking agent. In vitro glycosylation is discussed in detail further below.

[0041] The term "sugar moiety" is intended to indicate a carbohydrate-containing molecule comprising one or more monosaccharide residues, capable of being attached to the polypeptide variant (to produce a polypeptide variant conjugate in the form of a glycosylated polypeptide variant) by way of in vivo glycosylation. The term "in vivo glycosylation" is intended to mean any attachment of a sugar moiety occurring in vivo, i.e. during posttranslational processing in a glycosylating cell used for expression of the polypeptide variant, e.g. by way of N-linked or O-linked glycosylation. The exact oligosaccharide structure depends, to a large extent, on the glycosylating organism in question.

[0042] An "N-glycosylation site" has the sequence N-X-S/T/C, wherein X is any amino acid residue except proline, N is asparagine and S/T/C is either serine, threonine or cysteine, preferably serine or threonine, and most preferably threonine. Preferably, the amino acid residue in position +3 relative to the asparagine residue is not a proline residue.

[0043] An "O-glycosylation site" is the OH-group of a serine or threonine residue.

[0044] The term "attachment group" is intended to indicate a functional group of the polypeptide variant, in particular of an amino acid residue thereof or a carbohydrate moiety, capable of attaching a non-polypeptide moiety such as a polymer molecule, a lipophilic molecule, a sugar moiety or an organic derivatizing agent. Useful attachment groups and their matching non-polypeptide moieties are apparent from the table below.

Attachment group	Amino acid	Examples of non-polypeptide moiety	Conjugation method/-Activated PEG	Reference
—NH_2	N-terminal, Lys	Polymer, e.g. PEG, with amide or imine group	mPEG-SPA Tresylated mPEG	Nektar Therapeutics Delgado et al, Critical reviews in Therapeutic Drug Carrier Systems 9(3, 4): 249–304 (1992)
—COOH	C-terminal, Asp, Glu	Polymer, e.g. PEG, with ester or amide group	mPEG-Hz	Nektar Therapeutics
		Carbohydrate moiety	In vitro coupling	
—SH	Cys	Polymer, e.g. PEG, with disulfide, maleimide or vinylsulfone group	PEG-vinylsulphone PEG-maleimide	Nektar Therapeutics Delgado et al, Critical reviews in Therapeutic Drug Carrier Systems 9(3, 4): 249–304 (1992)
		Carbohydrate moiety	In vitro coupling	
—OH	Ser, Thr, Lys, OH—	Sugar moiety	In vivo O-linked glycosylation	
		PEG with ester, ether, carbamate, carbonate		
—$CONH_2$	Asn as part of an N-glycosylation site	Sugar moiety	In vivo N-glycosylation	
		Polymer, e.g. PEG		

-continued

Attachment group	Amino acid	Examples of non-polypeptide moiety	Conjugation method/-Activated PEG	Reference
Aromatic residue	Phe, Tyr, Trp	Carbohydrate moiety	In vitro coupling	
—$CONH_2$	Gln	Carbohydrate moiety	In vitro coupling	Yan and Wold, Biochemistry, 1984, Jul 31; 23(16): 3759–65
Aldehyde Ketone	Oxidized oligosaccharide	Polymer, e.g. PEG, PEG-hydrazide	PEGylation	Andresz et al., 1978, Macromol. Chem. 179: 301, WO 92/16555, WO 00/23114
Guanidino	Arg	Carbohydrate moiety	In vitro coupling	Lundblad and Noyes, Chemical Reagents for Protein Modification, CRC Press Inc., Florida, USA
Imidazole ring	His	Carbohydrate moiety	In vitro coupling	As for guanidine

[0045] For in vivo N-glycosylation, the term "attachment group" is used in an unconventional way to indicate the amino acid residues constituting a N-glycosylation site (with the sequence N-X-S/T/C, wherein X is any amino acid residue except proline, N is asparagine and S/T/C is either serine, threonine or cysteine, preferably serine or threonine, and most preferably threonine). Although the asparagine residue of the N-glycosylation site is the one to which the sugar moiety is attached during glycosylation, such attachment cannot be achieved unless the other amino acid residues of the N-glycosylation site are present. Accordingly, when the non-polypeptide moiety is a sugar moiety and the conjugation is to be achieved by in vivo N-glycosylation, the term "amino acid residue comprising an attachment group for the-non-polypeptide moiety" as used in connection with alterations of the amino acid sequence of the polypeptide variant is to be understood as meaning that one or more amino acid residues constituting an in vivo N-glycosylation site are to be altered in such a manner that either a functional in vivo N-glycosylation site is introduced into the amino acid sequence or removed from said sequence.

[0046] In the present application, amino acid names and atom names (e.g. CA, CB, CD, CG, SG, NZ, N, O, C, etc) are used as defined by the Protein DataBank (PDB) (www-.pdb.org) based on the IUPAC nomenclature (IUPAC Nomenclature and Symbolism for Amino Acids and Peptides (residue names, atom names, etc.), *Eur. J Biochem.*, 138, 9-37 (1984) together with their corrections in *Eur. J Biochem.*, 152, 1 (1985)).

[0047] The term "amino acid residue" is intended to include any natural or synthetic amino acid residue, and is primarily intended to indicate an amino acid residue contained in the group consisting of the 20 naturally occurring amino acids, i.e. selected from the group consisting of alanine (Ala or A), cysteine (Cys or C), aspartic acid (Asp or D), glutamic acid (Glu or E), phenylalanine (Phe or F), glycine (Gly or G), histidine (His or H), isoleucine (Ile or I), lysine (Lys or K), leucine (Leu or L), methionine (Met or M), asparagine (Asn or N), proline (Pro or P), glutamine (Gln or Q), arginine (Arg or R), serine (Ser or S), threonine (Thr or T), valine (Val or V), tryptophan (Trp or W), and tyrosine (Tyr or Y) residues.

[0048] The terminology used for identifying amino acid positions is illustrated as follows: G124 indicates hat position 124 is occupied by a glycine residue in the amino acid sequence shown in SEQ ID NO:2. G124R indicates that the glycine residue of position 124 has been substituted with an arginine residue. Alternative substitutions are indicated with a "/", e.g. N145S/T means an amino acid sequence in which asparagine in position 145 is substituted with either serine or threonine. Multiple substitutions are indicated with a "+", e.g. K143N+N145S/T means an amino acid sequence which comprises a substitution of the lysine residue in position 143 with an asparagine residue and a substitution of the asparagine residue in position 145 with a serine or a threonine residue. Insertion of an additional amino acid residue, such as insertion of an alanine residue after G124 is indicated by G124GA. Insertion of two additional alanine residues after G124 is indicated by G124GAA, etc. When used herein, the term "inserted in position X" or "inserted at position X" means that the amino acid residue(s) is (are) inserted between amino acid residue X and X+1. A deletion of an amino acid residue is indicated by an asterix. For example, deletion of the glycine residue in position 124 is indicated by G124*. Unless otherwise indicated, the numbering of amino acid residues made herein is made relative to the amino acid sequence of hFVII/hFVIIa (SEQ ID NO:2).

[0049] The term "differs from" as used in connection with specific mutations is intended to allow for additional differences being present apart from the specified amino acid difference. For instance, in addition to the specified modifications in positions 196, 237 and 341, the FVII or FVIIa polypeptide variant may comprise other substitutions. Examples of such additional modifications or differences may include truncation of the N- and/or C-terminus by one or more amino acid residues (e.g. by 1-10 amino acid residues), or addition of one or more extra residues at the N- and/or C-terminus, e.g. addition of a methionine residue at the N-terminus or addition of a cysteine residue near or at

the C-terminus, as well as "conservative amino acid substitutions", i.e. substitutions performed within groups of amino acids with similar characteristics, e.g. small amino acids, acidic amino acids, polar amino acids, basic amino acids, hydrophobic amino acids and aromatic amino acids.

[0050] Examples of such conservative substitutions are shown in the below table.

1	Alanine (A)	Glycine (G)	Serine (S)	Threonine (T)
2	Aspartic acid (D)	Glutamic acid (E)		
3	Asparagine (N)	Glutamine (Q)		
4	Arginine (R)	Histidine (H)	Lysine (K)	
5	Isoleucine (I)	Leucine (L)	Methionine (M)	Valine (V)
6	Phenylalanine (F)	Tyrosine (Y)	Tryptophan (W)	

[0051] Still other examples of additional modifications include modifications giving rise to an increased functional in vivo half-life, an increased serum half-life or an increased AUC_{iv}. Specific examples of such modifications are given further below. Moreover, the polypeptide variant of the invention may contain additional modifications giving rise to an enhanced phospholipid membrane binding affinity. Specific examples of such modifications are also given further below.

[0052] The term "variant" or "polypeptide variant" (of hFVII or hFVIIa) is intended to cover a polypeptide which differs in one or more amino acid residues from SEQ ID NO:2, normally in 1-15 amino acid residues (for example in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acid residues), e.g. in 1-10, 1-8, 1-6, 1-5, 1-4 or 1-3 amino acid residues, e.g. one or two amino acid residues. In the present context, the term "modification" encompasses insertions, deletions, substitutions and combinations thereof. It will be understood that a polypeptide variant according to the present invention will be modified in at least one of the following positions: 196, 237 and/or 314.

[0053] The term "nucleotide sequence" is intended to indicate a consecutive stretch of two or more nucleotide molecules. The nucleotide sequence may be of genomic, cDNA, RNA, semisynthetic, synthetic origin, or any combinations thereof "Cell", "host cell", "cell line" and "cell culture" are used interchangeably herein and all such terms should be understood to include progeny resulting from growth or culturing of a cell.

[0054] "Transformation" and "transfection" are used interchangeably to refer to the process of introducing DNA into a cell.

[0055] "Operably linked" refers to the covalent joining of two or more nucleotide sequences, by means of enzymatic ligation or otherwise, in a configuration relative to one another such that the normal function of the sequences can be performed. Generally, "operably linked" means that the nucleotide sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase.

[0056] The terms "mutation" and "substitution" are used interchangeably herein.

[0057] In the context of the present invention the terms "modification" or "amino acid modification" are intended to cover replacement of an amino acid side chain, substitution of an amino acid residue, deletion of an amino acid residue and insertion of an amino acid residue.

[0058] The term "introduce" refers to introduction of an amino acid residue, in particular by substitution of an existing amino acid residue, or alternatively by insertion of an additional amino acid residue.

[0059] The term "remove" refers to removal of an amino acid residue, in particular by substitution of the amino acid residue to be removed by another amino acid residue, or alternatively by deletion (without substitution) of the amino acid residue to be removed.

[0060] The term "FVII" or "FVII polypeptide" refers to a FVII molecule provided in single chain form.

[0061] The term "FVIIa" or "FVIIa polypeptide" refers to a FVIIa molecule provided in its activated two-chain form. When the amino acid sequence of SEQ ID NO:2 is used to describe the amino acid sequence of FVIIa it will be understood that the peptide bond between R152 and I153 of the single-chain form has been cleaved, and that one of the chains comprises amino acid residues 1-152, the other chain amino acid residues 153-406.

[0062] The terms "rFVII" and "rFVIIa" refer to FVII and FVIIa polypeptides produced by recombinant techniques.

[0063] The terms "hFVII" and "hFVIIa" refer to human wild-type FVII and FVIIa, respectively, having the amino acid sequence shown in SEQ ID NO:2

[0064] The terms "rhFVII" and "rhFVIIa" refer to human wild-type FVII and FVIIa, having the amino acid sequence shown in SEQ ID NO:2, produced by recombinant means. An example of rhFVIIa is NovoSeven®.

[0065] The term "TF" means Tissue Factor.

[0066] The term "TFPI" means Tissue Factor Pathway Inhibitor.

[0067] The term "FX" means Factor X.

[0068] The term "Gla domain" is used about the first about 45 amino acid residues counted from the N-terminus.

[0069] The term "protease domain" is used about residues 153-406 counted from the N-terminus.

[0070] The term "catalytic site" is used to mean the catalytic triad consisting of S344, D242 and H193 of the polypeptide variant.

[0071] The term "amidolytic activity" is intended to mean the activity measured in the "Amidolytic Assay" described herein. In order to exhibit "amidolytic activity" a variant of the invention, in its activated form, should have at least 10% of the amidolytic activity of rhFVIIa when assayed in the "Amidolytic Assay" described herein. In a preferred embodiment of the invention the variant, in its activated form, has at least 20% of the amidolytic activity of rhFVIIa, such at as at least 30%, e.g. at least 40%, more preferably at least 50%, such as at least 60%, e.g. at least 70%, even more preferably at least 80%, such as at least 90% of the amidolytic activity of rhFVIIa when assayed in the "Amidolytic Assay" described herein. In an interesting embodiment the variant, in its activated form, has substantially the same

amidolytic activity as rhFVIIa, such as an amidolytic activity of 75-125% of the amidolytic activity of rhFVIIa.

[0072] The term "clotting activity" is used to mean the activity measured in the "Whole Blood Assay" described herein. It will be understood that the activity measured in the "Whole Blood Assay" is the time needed to obtain clot formation. Thus, a lower clotting time corresponds to a higher clotting activity.

[0073] The term "increased clotting activity" is used to indicate that the clotting time of the polypeptide variant is statistically significantly decreased relative to that generated by rhFVIIa as determined under comparable conditions and when measured in the "Whole Blood Assay" described herein.

[0074] The term "immunogenicity" as used in connection with a given substance is intended to indicate the ability of the substance to induce a response from the immune system. The immune response may be a cell or antibody mediated response (see, e.g., Roitt: Essential Immunology (8th Edition, Blackwell) for further definition of immunogenicity). Normally, reduced antibody reactivity will be an indication of reduced immunogenicity. The reduced immunogenicity may be determined by use of any suitable method known in the art, e.g. in vivo or in vitro.

[0075] The term "functional in vivo half-life" is used in its normal meaning, i.e. the time at which 50% of the biological activity of the polypeptide variant is still present in the body/target organ, or the time at which the amidolytic or clotting activity of the polypeptide variant is 50% of the initial value.

[0076] As an alternative to determining functional in vivo half-life, "serum half-life" may be determined, i.e. the time at which 50% of the polypeptide variant circulates in the plasma or bloodstream prior to being cleared. Determination of serum half-life is often more simple than determining the functional in vivo half-life and the magnitude of serum half-life is usually a good indication of the magnitude of functional in vivo half-life. Alternatively terms to serum half-life include "plasma half-life", "circulating half-life", "serum clearance", "plasma clearance" and "clearance half-life". The polypeptide variant is cleared by the action of one or more of the reticuloendothelial systems (RES), kidney, spleen or liver, by tissue factor, SEC receptor or other receptor mediated elimination, or by specific or unspecific proteolysis. Normally, clearance depends on size (relative to the cutoff for glomerular filtration), charge, attached carbohydrate chains, and the presence of cellular receptors for the protein. The functionality to be retained is normally selected from procoagulant, proteolytic or receptor binding activity. The functional in vivo half-life and the serum half-life may be determined by any suitable method known in the art.

[0077] The term "increased" as used about the functional in vivo half-life or serum half-life is used to indicate that the relevant half-life of the polypeptide variant is statistically i5 significantly increased relative to that of a reference molecule, such as a rhFVIIa as determined under comparable conditions (typically determined in an experimental animal, such as rats, rabbits, pigs or monkeys).

[0078] The term "AUC_{iv}" or "Area Under the Curve when administered intravenously" is used in its normal meaning, i.e. as the area under the activity in serum-time curve, where the polypeptide variant has been administered intravenously, in particular when administered intravenously in rats. Once the experimental activity-time points have been determined, the AUC_{iv} may conveniently be calculated by a computer program, such as GraphPad Prism 3.01.

[0079] The term "reduced sensitivity to proteolytic degradation" is primarily intended to mean that the polypeptide variant has reduced sensitivity to proteolytic degradation in comparison to rhFVIIa as determined under comparable conditions. Preferably, the proteolytic degradation is reduced by at least 10% (e.g. by 10-25% or by 10-50%), such as at least 25% (e.g. by 25-50%, by 25-75% or by 25-100%), more preferably by at least 35%, such as at least 50%, (e.g. by 50-75% or by 50-100%) even more preferably by at least 60%, such as by at least 75% (e.g. by 75-100%) or even at least 90%. Most preferably, the proteolytic degradation is reduced by at least 99%.

[0080] The term "renal clearance" is used in its normal meaning to indicate any clearance taking place by the kidneys, e.g. by glomerular filtration, tubular excretion or degradation in the tubular cells. Renal clearance depends on physical characteristics of the polypeptide, including size (diameter), hydrodynamic volume, symmetry, shape/rigidity, and charge. A molecular weight of about 67 kDa is often considered to be a cut-off-value for renal clearance. Renal clearance may be established by any suitable assay, e.g. an established in vivo assay. Typically, renal clearance is determined by administering a labelled (e.g. radiolabelled or fluorescence labelled) polypeptide to a patient and measuring the label activity in urine collected from the patient. Reduced renal clearance is determined relative to a corresponding reference polypeptide, e.g. rhFVIIa, under comparable conditions. Preferably, the renal clearance rate of the polypeptide variant is reduced by at least 50%, e.g. at least 75% or at least 90% compared to rhFVIIa.

Polypeptide Variants of the Invention

[0081] In its broadest aspect the present invention relates to a variant of FVII or FVIIa, wherein said variant comprises at least one amino acid modification in a position selected from the group consisting of 196, 237 and 341 as compared to hFVII or hFVIIa, preferably as compared to hFVIIa. Although the variant will typically contain a modification in one of these positions, it may also contain a modification in two of these positions, i.e. modifications in 196+237, 196+341 or 237+341, or in all three positions.

[0082] In the following sections preferred modifications in the above-mentioned positions are given.

Position 196

[0083] In one embodiment of the invention, the present invention relates to a variant of FVII or FVIIa, wherein said variant comprises at least one modification in position 196 as compared to hFVII or hFVIIa (SEQ ID NO:2).

[0084] In a preferred embodiment of the invention the modification in position 196 is a substitution, in particular D196N or D196K.

[0085] The variant will generally comprise a total of 1-15 amino acid modifications (e.g. substitutions), such as 1-10 amino acid modifications (e.g. substitutions), e.g. 1-5 amino acid modifications (e.g. substitutions) or 1-3 amino acid modifications (e.g. substitutions).

[0086] For example, the variant may contain at least one further amino acid modification made in the Gla domain as explained in the section entitled "Modifications in the Gla domain" below, and/or at least one further amino acid modification which leads to introduction of an in vivo N-glycosylation site as explained in the section entitled "Introduction of additional sugar moieties" below, and/or at least one further amino acid modification capable of increasing the intrinsic activity and/or at least one further amino acid modification which increases the TF-binding affinity. Examples of the latter modifications are described in the section entitled "Other modifications" below.

Position 237

[0087] In a further embodiment of the invention, the present invention relates to a variant of FVII or FVIIa, wherein said variant comprises at least one modification in position 237 as compared to hFVII or hFVIIa (SEQ ID NO:2).

[0088] In a preferred embodiment of the invention the modification in position 237 is a substitution, in particular G237L.

[0089] The variant will generally comprise a total of 1-15 amino acid modifications (e.g. substitutions), such as 1-10 amino acid modifications (e.g. substitutions), e.g. 1-5 amino acid modifications (e.g. substitutions) or 1-3 amino acid modifications (e.g. substitutions).

[0090] For example, the variant may contain at least one further amino acid modification made in the Gla domain as explained in the section entitled "Modifications in the Gla domain" below, and/or at least one further amino acid modification which leads to introduction of an in vivo N-glycosylation site as explained in the section entitled "Introduction of additional sugar moieties" below, and/or at least one further amino acid modification capable of increasing the intrinsic activity and/or at least one further amino acid modification which increases the TF-binding affinity. Examples of the latter modifications are described in the section entitled "Other modifications" below.

[0091] In still another embodiment of the invention the modification in position 237 is an insertion. In an interesting embodiment the insertion is selected from the-group consisting of G237GXX, G237GXXX and G237GXXXX, wherein X is any amino acid residue. Preferably, X is selected from the group consisting of Ala, Val, Leu, Ile, Gly, Ser and Thr, in particular Ala Specific examples of preferred insertions include G237GAA, G237GAAA and G237GAAAA. Most preferably, the insertions are G237GAA.

Position 341

[0092] In a still further embodiment of the invention, the present invention relates to a variant of FVII or FVIIa, wherein said variant comprises at least one modification in position 341 as compared to hFVII or hFVIIa (SEQ ID NO:2).

[0093] In a preferred embodiment of the invention the modification in position 341 is a substitution, such as K341N or K341Q, in particular K341Q.

[0094] The variant will generally comprise a total of 1-15 amino acid modifications (e.g. substitutions), such as 1-10 amino acid modifications (e.g. substitutions), e.g. 1-5 amino acid modifications (e.g. substitutions) or 1-3 amino acid modifications (e.g. substitutions).

[0095] For example, the variant may contain at least one further amino acid modification made in the Gla domain as explained in the section entitled "Modifications in the Gla domain" below, and/or at least one further amino acid modification which leads to introduction of an in vivo N-glycosylation site as explained in the section entitled "Introduction of additional sugar moieties" below, and/or at least one further amino acid modification capable of increasing the intrinsic activity and/or at least one further amino acid modification which increases the TF-binding affinity. Examples of the latter modifications are described in the section entitled "Other modifications" below.

Properties of the Variants of the Invention

[0096] The variants disclosed may have an altered affinity for TFPI, which may be assessed using the BIAcore® Assays described herein. Using the BIAcore® assays it is possible to estimate various kinetic binding constants, such as the equilibrium dissociation constant, K_D, where $K_D=k_d/k_a$, where k_a is the association rate constant and k_d is the dissociation rate constant. It will be understood that a higher value of K_D corresponds to a decreased affinity for TFPI.

[0097] The variants of the invention possess an increased clotting activity (or a reduced clotting time) as compared to hFVIIa or rhFVIIa. In a preferred embodiment of the invention the ratio between the time to reach clot formation for the variant ($t_{variant}$) and the time to reach clot formation for hFVIIa or rhFVIIa (t_{wt}) is at the most 0.9 when assayed in the "Whole Blood Assay" described herein. More preferably the ratio ($t_{variant}/t_{wt}$) is at the most 0.75, such as 0.7, even more preferably the ratio ($t_{variant/twt}$) is at the most 00.6, most preferably the ratio ($t_{variant}/t_{wt}$) is at the most 0.5 when assayed in the "Whole Blood Assay" described herein.

Further Modifications

[0098] As indicated above the FVII or FVIIa variant of the invention may comprise further modifications aimed at conferring additional advantageous properties to the FVII or FVIIa molecule, e.g. at least one further amino acid substitution.

[0099] In order to avoid too much disruption of the structure and function of the FVII or FVIIa polypeptide, the FVII or FVIIa polypeptide variant of the invention will typically have an amino acid sequence having more than 95% identity with SEQ ID NO:2, preferably more than 96% identity with SEQ ID NO:2, such as more than 97% identity with SEQ ID NO:2, more preferably at least 98% identity with SEQ ID NO:2, such as more than 99% identity with SEQ ID NO:2. Amino acid sequence homology/identity is conveniently determined from aligned sequences, using e.g. the ClustalW program, version 1.8, June 1999, using default parameters (Thompson et al., 1994, ClustalW: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, *Nucleic Acids Res.*, 22: 4673-4680) or from the PFAM families database version 4.0 (http://pfam.wustl.edu/) (*Nucleic Acids Res.* 1999 Jan. 1; 27(1):260-2) by use of GENEDOC version 2.5 (Nicholas et al., 1997 GeneDoc: Analysis and Visualization of Genetic

Variation, EMBNEW.NEWS 4:14; Nicholas, K. B. and Nicholas H. B. Jr. 1997 GeneDoc: Analysis and Visualization of Genetic Variation).

Modifications in the Gla Domain

[0100] In an interesting embodiment of the invention, at least one further amino acid modification is made in the Gla domain, i.e. within the first about 45 amino acid residues counted from the N-terminus of the FVII or FVIIa molecule. Preferably, no modifications are made in residues 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35.

[0101] Without being limited by any particular theory, it is presently believed that an increased clotting activity may be achieved by an enhanced binding affinity of the FVIIa molecule to the phospholipid membranes present on the surface of activated platelets. This enhanced affinity is believed to result in a higher local concentration of the activated FVIIa polypeptide in close proximity to the other coagulation factors, particularly FX. Thus, the rate of activation of FX to FXa will be higher, simply due to a higher molar ratio of the activated FVII polypeptide to FX. The increased activation rate of FX then results in a higher amount of active thrombin, and thus a higher rate of cross-linking of fibrin.

[0102] Thus, in a preferred embodiment according to this aspect of the invention, the polypeptide variant has, in its activated form, an enhanced phospholipid membrane binding affinity relative to the rhFVIIa polypeptide. Phospholipid membrane binding affinity may be measured by methods known in the art, such as by the BIAcore® assays described in K. Nagata and H. Handa (Ads.), Real-Time Analysis of Biomolecular Interactions, Springer-Verlag, Tokyo, 2000, Chapter 6 entitled "Lipid-Protein Interactions".

[0103] A number of modifications in the FVII Gla domain leading to an increased membrane binding affinity have been described in the art (see, for example, WO 99/20767 and WO 00/66753). Particular interesting positions in the Gla domain to be modified are positions P10, K32, D33, A34 as well as insertion of an amino acid residue between A3 and F4. Thus, in a preferred embodiment of the invention, the variant comprises, in addition to one or more of the modifications mentioned above a substitution in a position selected from the group consisting of P10, K32, D33 and A34 and combinations thereof as well as an insertion between A3 and F4. Particularly preferred positions are P10 and K32.

[0104] Preferably, the substitution to be made in position 32 is K32E, the substitution to be made in position 10 is P10Q, the substitution to be made in position 33 is D33F, the substitution to be made in position 34 is A34E and the insertion between A3 and F4 is preferably A3AY. In an interesting embodiment of the invention the variant comprises at least one of the following further modifications: A3AY, P10Q, K32E, D33F, A34E or combinations thereof Most preferably, the variant comprises one of the following further modifications: K32E, P10Q+K32E, A3AY+P10Q+K32E+D33F+A34E.

Introduction of Non-polypeptide Moieties

[0105] In another embodiment, the FVII or FVIIa variant has been further modified so that the resulting polypeptide variant has increased functional in vivo half-life and/or increased plasma half-life and/or increased increased Area Under the Curve when administered intravenously (AUC_{iv}), in particular when administered intravenously in rats, and/or increased bioavailability and/or reduced sensitivity to proteolytic degradation. Medical treatment with a polypeptide variant according to this aspect of the invention may offer a number of advantages over the currently available rFVIIa compound, such as lower dosage and, optionally, longer duration between injections. Numerous examples of relevant amino acid substitutions are given in WO 01/58935.

[0106] The variants disclosed in WO 61/58935 are the result of a generally new strategy for developing improved FVII or FVIIa molecules. This strategy, in which non-polypeptide moieties are attached to FVII/FVIIa variants, may also be used for the FVII or FVIIa variants of the present invention. More specifically, by removing and/or introducing an amino acid residue comprising an attachment group for a non-polypeptide moiety in the FVII or FVIIa polypeptide variant of the invention, it is possible to specifically adapt the polypeptide variant so as to make the molecule more susceptible to conjugation to a non-polypeptide moiety of choice, to optimize the conjugation pattern (e.g. to ensure an optimal distribution and number of non-polypeptide moieties on the surface of the FVII or FVIIa polypeptide variant and to ensure that only the attachment groups intended to be conjugated are present in the molecule) and thereby obtain a new conjugate molecule, which has activity and in addition one or more improved properties as compared to the FVII and FVIIa molecules available today. For instance, when the total number of amino acid residues comprising an attachment group for the non-polypeptide of choice is increased or decreased to an optimized level, the renal clearance of the conjugate is typically significantly reduced due to the altered shape, size and/or charge of the molecule achieved by the conjugation.

[0107] Thus, interesting polypeptide variants according to this aspect of the present invention are such polypeptides wherein at least one amino acid residue comprising an attachment group for a non-polypeptide moiety has been introduced or removed in a FVII or FVIIa polypeptide variant as described elsewhere herein.

[0108] In interesting embodiments of the present invention more than one amino acid residue of the FVII or FVIIa polypeptide variant is altered, e.g. the alteration embraces removal as well as introduction of amino acid residues comprising an attachment group for the non-polypeptide moiety of choice. In addition to the removal and/or introduction of amino acid residues the polypeptide variant may comprise other substitutions or glycosylations that are not related to introduction and/or removal of amino acid residues comprising an attachment group for the non-polypeptide moiety. Also, the polypeptide variant may be attached, e.g., to a serine proteinase inhibitor to inhibit the catalytic site of the polypeptide.

[0109] The amino acid residue comprising an attachment group for a non-polypeptide moiety, whether it be removed or introduced, is selected on the basis of the nature of the non-polypeptide moiety of choice and, in most instances, on the basis of the method in which conjugation between the polypeptide variant and the non-polypeptide moiety is to be achieved. For instance, when the non-polypeptide moiety is a polymer molecule such as a polyethylene glycol or poly-

alkylene oxide derived molecule, amino acid residues comprising an attachment group may be selected from the group consisting of lysine, cysteine, aspartic acid, glutamic acid, histidine, and tyrosine, preferably lysine, cysteine, aspartic acid and glutamic acid, more preferably lysine and cysteine, in particular cysteine.

[0110] Whenever an attachment group for a non-polypeptide moiety is to be introduced into or removed from the FVII or FVIIa polypeptide variant, the position of the amino acid residue to be modified is preferably located at the surface of the FVII or FVIIa polypeptide, and more preferably occupied by an amino acid residue which has more than 25% of its side chain exposed to the surface (as defined in WO 01/58935 and in Example 1 herein), preferably more than 50% of its side chain exposed to the surface (as defined in WO 01/58935 and in Example 1 herein).

[0111] Furthermore, the position is preferably selected from a part of the FVII molecule that is located outside the tissue factor binding site, the Gla domain, the active site region and/or the ridge of the active site binding cleft These sites/regions are identified in Example 1 herein.

[0112] The polypeptide variant of the invention may contain 1-10 non-polypeptide moieties, typically 1-8 or 2-8 non-polypeptide moieties, preferably 1-5 or 2-5 non-polypeptide moieties, such as 1-4 or 1-3 non-polypeptide moieties, e.g. 1, 2 or 3 non-polypeptide moieties, in particular PEG, such as mPEG or sugar moieties.

[0113] Further details regarding introduction of non-polypeptide moieties may be found in WO 01/58935, incorporated by reference.

Introduction of Additional Sugar Moieties

[0114] In an interesting embodiment of the invention the non-polypeptide moiety is a sugar moiety, i.e., the polypeptide variant of the invention is one which, in addition to one or more of the modifications described elsewhere herein comprises at least one sugar moiety covalently attached to an introduced glycosylation site. Preferably said glycosylation site is an in vivo glycosylation site, in particular an in vivo N-glycosylation site, which has been introduced by substitution. Preferably, said glycosylation site is introduced in a position located outside the Gla domain, the tissue factor binding site, the active site region and the ridge of the active site binding cleft.

[0115] When used in the present context, the term "naturally occurring glycosylation site" covers the glycosylation sites at positions N145, N322, S52 and S60. In a similar way, the term "naturally occurring in vivo O-glycosylation site" includes the positions S52 and S60, whereas the term "naturally occurring in vivo N-glycosylation site" includes positions N145 and N322.

[0116] Thus, in a very interesting embodiment of the invention, the non-polypeptide moiety is a sugar moiety and the introduced attachment group is a glycosylation site, preferably an in vivo glycosylation site, such as an in vivo O-glycosylation site or an in vivo N-glycosylation site, in particular an in vivo N-glycosylation site. Typically, 1-10 glycosylation sites, in particular in vivo N-glycosylation sites, have been introduced, preferably 1-8, 1-6, 14 or 1-3 glycosylation sites. In particular, 1, 2 or 3 in vivo N-glycosylation sites have been introduced, preferably by substitution.

[0117] It will be understood that in order to prepare a polypeptide variant wherein the FVII or FVII polypeptide variant comprises one or more glycosylation sites, the polypeptide variant must be expressed in a host cell capable of attaching sugar (oligosaccharide) moieties at the glycosylation site(s). Examples of glycosylating host cells are given in the section further below entitled "Coupling to a sugar moiety".

[0118] Examples of positions wherein the glycosylation sites may be introduced include, but are not limited to, positions comprising an amino acid residue having an amino acid residue having at least 25% of its side chain exposed to the surface (as defined in Example 1 herein), such as in a position comprising an amino acid residue having at least 50% of its side chain exposed to the surface (as defined in Example 1 herein). It should be understood that when the term "at least 25% (or at least 50%) of its side chain exposed to the surface" is used in connection with introduction of an in vivo N-glycosylation site this term refers to the surface accessibility of the amino acid side chain in the position where the sugar moiety is actually attached. In many cases it will be necessary to introduce a serine or a threonine residue in position +2 relative to the asparagine residue to which the sugar moiety is actually attached (unless, of course, this position is already occupied by a serine or a threonine residue) and these positions, where the serine or threonine residues are introduced, are allowed to be buried, i.e. to have less than 25% (or 50%) of their side chains exposed to the surface.

[0119] Specific examples of such substitutions creating an in vivo N-glycosylation site include a substitution selected from the group consisting of A51N, G58N, T106N, K109N, G124N, K143N+N145T, A175T, I205S, I205T, V253N, T267N, T267N+S269T, S314N+k316S, S314N+K316T, R315N+V317S, R315N+V317T, K316N+G318S, K316N+G318T, G318N, D334N and combinations thereof. In a preferred embodiment of the invention the in vivo N-glycosylation site is created by performing a substitution selected from the group consisting of A51N, G58N, T106N, K109N, G124N, K143N+N145T, A175T, I205T, V253N, T267N+S269T, S314N+K316T, R315N+V317T, K316N+G318T, G318N, D334N and combinations thereof, more preferably by a substitution selected from the group consisting of T106N, A175T, I205T, V253N, T267N+S269T and combinations thereof, in particular I205T.

[0120] In one embodiment of the invention, one in vivo N-glycosylation site site has been introduced by substitution. In another embodiment of the invention at least two in vivo N-glycosylation site sites, such as two in vivo N-glycosylation sites, have been introduced by substitution. Specific examples of substitutions creating two in vivo N-glycosylation sites include A51N+G58N, A51N+T106N, A51N+K109N, A51N+G124N, A51N+K143N+N145T, A51N+A175T, A51N+I205T, A51N+V253N, A51N+T267N+S269T, A51N+S314N+K316T, A51N+R315N+V317T, A51N+K316N+G318T, A51N+G318N, A51N+D334N, G58N+T106N, G58N+K109N, G58N+G124N, G58N+K143N+N145T, G58N+A175T, G58N+I205T, G58N+V253N, G58N+T267N+S269T, G58N+S314N+K316T, G58N+R315 N+V317T, G58N+K316N+G318T, G58N+G318N, G58N+D334N, T106N+K109N, T106N+G124N, T106N+K143N+M145T, T106N+A175T, T106N+I205T, T106N+V253N, T106N+T267N+S269T, T106N+

S314N+K316T, T106N+R315N+V317T, T106N+K316N+G318T, T106N+G318N, T106N+D334N, K109N+G124N, K109N+K143N+N145T, K109N+A175T, K109N+I205T, K109N+V253N, K109N+T267N+S269T, K109N+S314N+K316T, K109N+R315N+V317T, K109N+K316N+G318T, K109N+G318N, K109N+D334N, G124N+K143N+N145T, G124N+A175T, G124N+I205T, G124N+V253N, G124N+T267N+S269T, G124N+S314N+K316T, G124N+R315N+V317T, G124N+K316N+G318T, G124N+G318N, G124N+D334N, K143N+N145T+A175T, K143N+N145T+I205T, K143N+N145T+V253N, K143N+N145T+T267N+S269T, K143N+N145T+S314N+K316T, K143N+N145T+R315N+V317T, K143N+N145T+K316N+G318T, K143N+N145T+G318N, K143N+N145T+D334N, A175T+I205T, A175T+V253N, A175T+T267N+S269T, A175T+S314N+K316T, A175T+R315N+V317T, A175T+K316N+G318T, A175T+G318N, A175T+D334N, I205T+V253N, I205T+T267N+S269T, I205T+S314N+K316T, I205T+R315N+V317T, I205T+K316N+G318T, I205T+G318N, I205T+D334N, V253N+T267N+S269T, V253N+S314N+K316T, V253N+R315N+V317T, V253N+K316N+G318T, V253N+G318N, V253N+D334N, T267N+S269T+S314N+K316T, T267N+S269T+R315N+V317T, T267N+S269T+K316N+G318T, T267N+S269T+G318N, T267N+S269T+D334N, S314N+K316T+R315N+V317T, S314N+K316T+G318N, S314N+K316T+D334N, R315N+V317T+K316N+G318T, R315N+V317T+G318N, R315N+V317T+D334N or G318N+D334N, preferably T106N+A175T, T106N+I205T, T106N+V253N, T106N+T267N+S269T, A175T+I205T, A175T+V253N, A175T+T267N+S269T, I205T+V253N, I205T+T267N+S269T or V253N+T267N+S269T, more preferably T106N+I205T, T106N+V253N or I205T+T267N+S269T.

[0121] In a still further embodiment of the invention at least three in vivo N-glycosylation site sites, such as three in vivo N-glycosylation sites, have been introduced by substitution. Specific examples of substitutions creating three in vivo N-glycosylation sites include I205T+V253N+T267N+S269T and T106N+I205T+V253N.

[0122] In addition to a sugar moiety, the polypeptide variant according to the aspect of the invention described in the present section may contain additional non-polypeptide moieties, in particular a polymer molecule, as described in the present application, conjugated to one or more attachment groups present in the FVII or FVIIa variant.

[0123] It will be understood that any of the amino acid changes, in particular substitutions, specified in this section can be combined with any of the amino acid changes, in particular substitutions, specified in the other sections herein disclosing specific amino acid changes. For instance, any of the glycosylated polypeptides variants disclosed in the present section having introduced and/or removed at least one glycosylation site may further be conjugated to a polymer molecule, such as PEG, or any other non-polypeptide moiety.

[0124] Further information on introduction of glycosylation sites may be found in WO 01/58935 and WO 03/093465, incorporated by reference.

Introduction of Non-polypeptide Moieties that have Cysteine as an Attachment Group

[0125] In a further interesting embodiment of the invention the non-polypeptide moiety has cysteine as an attachment group, i.e., the polypeptide variant of the invention is one which, in addition to one or more of the modifications described above comprises at least one non-polypeptide moiety covalently attached to an introduced cysteine. Preferably said cysteine residue is introduced by substitution. Preferably, said cysteine residue is introduced in a position located outside the TF binding site, the Gla domain, the active site region, and the ridge of the active site binding cleft.

[0126] FVII/FVIIa contains 22 cysteine residues located outside the Gla domain and disulfide bridges are established between the following cysteine residues: C50 and C61,C55 and C70,C72 and C81,C91 and C102,C98 and C112,C114 and C127, C135 and C262, C159 and C164,C178 and C194, C310 and C329, and between C340 and C368.

[0127] Thus, in an interesting embodiment of the invention at least one cysteine residue has been introduced, preferably by substitution, in the FVII or FVIIa polypeptide variant. Typically 1-10 cysteine residues have been introduced, preferably 1-8, 1-6, 1-4 or 1-3 cysteine residues have been introduced. In particular 1, 2 or 3 cysteine residues have been introduced, preferably by substitution.

[0128] Examples of positions where the cysteine residues may be introduced include, but are not limited to, positions comprising an amino acid residue having an amino acid residue having at least 25% of its side chain exposed to the surface (as defined in Example 1 herein), such as in a position comprising an amino acid residue having at least 50% of its side chain exposed to the surface (as defined in Example 1 herein).

[0129] In an interesting embodiment of the invention, a cysteine residue is introduced near or at the C-terminus. For example, a cysteine residue may be introduced, either by substitution or insertion, in position 400-406. Specific examples of substitutions include: L400C, L401C, R402C, A403C, P404C, F405C and P406C, in particular P406C. Specific examples of insertions include L400LC, L401LC, R402RC, A403AC, P404PC, F405FC and P406PC, in particular P406PC.

[0130] While the non-polypeptide moiety according to this aspect of the invention may be any molecule which when using the given conjugation method has cysteine as an attachment group, it is preferred that the non-polypeptide moiety is a polymer molecule. The polymer molecule may be any of the molecules mentioned in the section entitled "Conjugation to a polymer molecule", but is preferably selected from the group consisting of linear or branched polyethylene glycol or another polyalkylene oxide. In a particular interesting embodiment the polymer molecule is PEG, such as VS-PEG.

[0131] The conjugation between the polypeptide variant and the polymer may be achieved in any suitable manner, e.g. as described WO 01/58935.

[0132] When the FVII or FVIIa polypeptide variant comprises only one conjugatable cysteine residue, this residue is preferably conjugated to a non-polypeptide moiety with a molecular weight of from about 5 kDa to about 20 kDa, e.g. from about 10 kDa to about 20 kDa, such as a molecular weight of about 5 kDa, about 10 kDa, about 12 kDa, about 15 kDa or about 20 kDa, either directly conjugated or indirectly through a low molecular weight polymer (as

disclosed in WO 99/55377). When the FVII or FVIIa polypeptide variant comprises two or more conjugatable cysteine residues, normally each of the non-polypeptide moieties has a molecular weight of from about 5 to about 10 kDa, such as about 5 kDa or about 10 kDa.

[0133] It will be understood that any of the amino acid changes, in particular substitutions, specified in this section can be combined with any of the amino acid modifications specified in the other sections herein.

Other Modifications

[0134] In a further embodiment of the present invention, the FVII or FVIIa variant may, in addition to the modifications described in the sections above, also contain mutations which are already known to increase the intrinsic activity of the polypeptide, e.g. those described in WO 02/22776.

[0135] Examples of preferred substitutions include substitutions selected from the group consisting of V158D, E296D, M298Q, L305V and K337A. More preferably, said substitutions are selected from the group consisting of

[0136] V158D+E296D+M298Q+L305V+K337A,

[0137] V158D+E296D+M298Q+K337A, V158D+E296D+M298Q+L305V,

[0138] V158D+E296D+M298Q,

[0139] M298Q, L305V+K337A, L305V and K337A.

[0140] Moreover, the variant may contain modifications which increase the TF binding affinity. Examples of such modifications include substitutions selected from the group consisting of L39E, L39Q, L39H, 142R, S43H, S43Q, K62E, K62R, L65Q, L65S, F71D, F71Y, F71E, F71Q, F71N, E82Q, E82N, E82K, F275H and combinations thereof, in particular L65Q, F71Y, K62E, S43Q and combinations thereof.

[0141] As already indicated above, the variant may also contain conservative amino acid substitutions.

The Non-polypeptide Moiety

[0142] As indicated further above the non-polypeptide moiety of the polypeptide variant of the invention is preferably selected from the group consisting of a polymer molecule, a lipophilic compound, a sugar moiety (by way of in vivo glycosylation) and an organic derivatizing agent. All of these agents may confer desirable properties to the polypeptide variant, in particular increased functional in vivo half-life and/or increased plasma half-life.

[0143] The polypeptide variant is normally conjugated to only one type of non-polypeptide moiety, but may also be conjugated to two or more different types of non-polypeptide moieties, e.g. to a polymer molecule and a sugar moiety, to a lipophilic group and a sugar moiety, to an organic derivatizing agent and a sugar moiety, to a lipophilic group and a polymer molecule, etc. The conjugation to two or more different non-polypeptide moieties may be done simultaneously or sequentially. Further information on conjugation to non-polypeptide moieties is found in WO 01/58935 and WO 03/093465, incorporated by reference.

Methods of Preparing a Conjugated Polypeptide Variant of the Invention

[0144] In general, a conjugated polypeptide variant according to the invention may be produced by culturing an appropriate host cell under conditions conducive for the expression of the polypeptide, and recovering the polypeptide variant, wherein a) the polypeptide variant comprises at least one N- or O-glycosylation site and the host cell is a eukaryotic host cell capable of in vivo glycosylation, and/or b) the polypeptide variant is subjected to conjugation to a non-polypeptide moiety in vitro. See e.g. WO 01/58935 and WO 03/093465 for further information on preparation of conjugated variants of FVII.

Conjugation to a Polymer Molecule

[0145] The polymer molecule to be coupled to the polypeptide variant may be any suitable polymer molecule, such as a natural or synthetic homo-polymer or hetero-polymer, typically with a molecular weight in the range of about 300-100,000 Da, such as about 500-20,000 Da, more preferably in the range of about 500-15,000 Da, even more preferably in the range of about 2-12 kDa, such as in the range of about 3-10 kDa When the term "about" is used herein in connection with a certain molecular weight, the word "about" indicates an approximate average molecular weight and reflects the fact that there will normally be a certain molecular weight distribution in a given polymer preparation.

[0146] Examples of homo-polymers include a polyol (i.e. poly-OH), a polyamine (i.e. poly-NH_2) and a polycarboxylic acid (i.e. poly-COOH). A hetero-polymer is a polymer comprising different coupling groups, such as a hydroxyl group and an amine group.

[0147] Examples of suitable polymer molecules include polymer molecules selected from the group consisting of polyalkylene oxide (PAO), including polyalkylene glycol (PAG), such as polyethylene glycol (PEG) and polypropylene glycol (PPG), branched PEGs, poly-vinyl alcohol (PVA), poly-carboxylate, poly-(vinylpyrolidone), polyethylene-co-maleic acid anhydride, polystyrene-co-maleic acid anhydride, dextran, including carboxymethyl-dextran, or any other biopolymer suitable for reducing immunogenicity and/or increasing functional in vivo half-life and/or serum half-life. Another example of a polymer molecule is human albumin or another abundant plasma protein. Generally, polyalkylene glycol-derived polymers are biocompatible, non-toxic, non-antigenic, non-immunogenic, have various water solubility properties, and are easily excreted from living organisms.

[0148] PEG is the preferred polymer molecule, since it has only few reactive groups capable of cross-linking compared to, e.g., polysaccharides such as dextran. In particular, monofunctional PEG, e.g. methoxypolyethylene glycol (mPEG), is of interest since its coupling chemistry is relatively simple (only one reactive group is available for conjugating with attachment groups on the polypeptide). Consequently, the risk of cross-inking is eliminated, the resulting conjugated polypeptide variants are more homogeneous and the reaction of the polymer molecules with the polypeptide variant is easier to control.

[0149] To effect covalent attachment of the polymer molecule(s) to the polypeptide variant, the hydroxyl end groups

of the polymer molecule must be provided in an activated form, i.e. with reactive functional groups (examples of which include primary amino groups, hydrazide (HZ), thiol, succinate (SUC), succinimidyl succinate (SS), succinimidyl succinamide (SSA), succinimidyl proprionate (SPA), succininidy carboxymethylate (SCM), benzotriazole carbonate (BTC), N-hydroxysuccinimide (NHS), aldehyde, nitrophenylcarbonate (NPC), and tresylate (TRES)). Suitable activated polymer molecules are commercially available, e.g. from Nektar Therapeutics, Huntsville, Ala., USA, or from PolyMASC Pharmaceuticals plc, UK. Specific examples of activated linear or branched polymer molecules for use in the present invention are described in the Nektar Molecule Engineering Catalog 2003 (Nektar Therapeutics), incorporated herein by reference.

[0150] In a particular interesting embodiment PEGylation is achieved by conjugating the PEG group(s) to introduced cysteine residues. Specific examples of activated PEG polymers particularly preferred for coupling to cysteine residues, include the following linear PEGs: vinylsulfone-PEG (VS-PEG), preferably vinylsulfone-mPEG (VS-mPEG); maleimide-PEG (MAL-PEG), preferably maleimide-mPEG (MAL-mPEG) and orthopyridyl-disulfide-PEG (OPSS-PEG), preferably orthopyridyl-disulfide-mPEG (OPSS-mPEG). Typically, such PEG or mPEG polymers will have a size of about 5 kDa, about 10 kDa, about 12 kDa or about 20 kDa.

[0151] In another embodiment, a suitable PEG molecule may be attached to the N-terminal.

[0152] Detailed information on methods and polymers that may be used for PEGylation of FVII is found in WO 01/58935, incorporated herein by reference.

Coupling to a Sugar Moiety

[0153] In order to achieve in vivo glycosylation of the polypeptide variant of the invention, the nucleotide sequence encoding the polypeptide variant must be inserted in a glycosylating, eukaryotic expression host. The expression host cell may be selected from fungal (filamentous fungal or yeast), insect or animal cells or from transgenic plant cells. In one embodiment the host cell is a mammalian cell, such as a CHO cell, a COS cell, a BHK cell or a HEK cell, e.g. a HEK 293 cell, or an insect cell, such as an SF9 cell, or a yeast cell, such as *S. cerevisiae* or *Pichia pastoris,* or any of the host cells mentioned hereinafter. Further information on in vivo glycosylation of FVII and FVIIa is found in WO 01/58935 and WO 03/093465, incorporated by reference.

Methods of Preparing a Polypeptide Variant of the Invention

[0154] The polypeptide variants of the present invention, optionally in glycosylated form, may be produced by any suitable method known in the art. Such methods include constructing a nucleotide sequence encoding the polypeptide variant and expressing the sequence in a suitable transformed or transfected host. Preferably, the host cell is a gamma-carboxylating host cell such as a mammalian cell. However, polypeptide variants of the invention may be produced, albeit less efficiently, by chemical synthesis or a combination of chemical synthesis or a combination of chemical synthesis and recombinant DNA technology.

[0155] A nucleotide sequence encoding a polypeptide variant of the invention may be constructed by isolating or synthesizing a nucleotide sequence encoding hFVII and then changing the nucleotide sequence so as to effect introduction (i.e. insertion or substitution) or removal (i.e. deletion or substitution) of the relevant amino acid residue(s).

[0156] The nucleotide sequence is conveniently modified by site-directed mutagenesis in accordance with conventional methods. Alternatively, the nucleotide sequence is prepared by chemical synthesis, e.g. by using an oligonucleotide synthesizer, wherein oligonucleotides are designed based on the amino acid sequence of the desired polypeptide variant, and preferably selecting those codons that are favored in the host cell in which the recombinant polypeptide variant will be produced. For example, several small oligonucleotides coding for portions of the desired polypeptide variant may be synthesized and assembled by PCR (polymerase chain reaction), ligation or ligation chain reaction (LCR) (Barany, *Proc Natl Acad Sci USA* 88:189-193, 1991). The individual oligonucleotides typically contain 5' or 3' overhangs for complementary assembly.

[0157] Once assembled (by synthesis, site-directed mutagenesis or another method), the nucleotide sequence encoding the polypeptide is inserted into a recombinant vector and operably linked to control sequences necessary for expression of the FVII in the desired transformed host cell.

[0158] Persons skilled will be capable of selecting suitable vectors, expression control sequences and hosts for expressing the polypeptide

[0159] The recombinant vector may be an autonomously replicating vector, i.e. a vector, which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector is one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

[0160] The vector is preferably an expression vector in which the nucleotide sequence encoding the polypeptide variant of the invention is operably linked to additional segments required for transcription of the nucleotide sequence. The vector is typically derived from plasmid or viral DNA. A number of suitable expression vectors for expression in the host cells mentioned herein are commercially available or described in the literature. Detailed information on suitable vectors for expressing FVII may be found in WO 01/58935, incorporated by reference.

[0161] The term "control sequences" is defined herein to include all components which are necessary or advantageous for the expression of the polypeptide variant of the invention. Each control sequence may be native or foreign to the nucleic acid sequence encoding the polypeptide variant. Such control sequences include, but are not limited to, a leader sequence, polyadenylation sequence, propeptide sequence, promoter, enhancer or upstream activating sequence, signal peptide sequence, and transcription terminator. At a minimum, the control sequences include a promoter. A wide variety of expression control sequences may be used in the present invention, e.g. any of the control sequences disclosed in WO 01/58935, incorporated by reference.

[0162] The nucleotide sequence of the invention encoding a polypeptide variant, whether prepared by site-directed

mutagenesis, synthesis, PCR or other methods, may optionally include a nucleotide sequence that encodes a signal peptide. The signal peptide is present when the polypeptide variant is to be secreted from the cells in which it is expressed. Such signal peptide, if present, should be one recognized by the cell chosen for expression of the polypeptide variant. The signal peptide may be homologous (i.e. normally associated with hFVII) or heterologous (i.e. originating from another source than hFVII) to the polypeptide or may be homologous or heterologous to the host cell, i.e. be a signal peptide normally expressed from the host cell or one which is not normally expressed from the host cell. For further information on suitable signal peptides, see WO 01/58935.

[0163] Any suitable host may be used to produce the polypeptide variant, including bacteria (although not particularly preferred), fungi (including yeasts), plant, insect, mammal, or other appropriate animal cells or cell lines, as well as transgenic animals or plants. Mammalian cells are preferred. Examples of bacterial host cells include gram-positive bacteria such as strains of *Bacillus,* e.g. *B. brevis* or *B. subtilis,* or *Streptomyces,* or gram-negative bacteria, such as strains of *E. coli.* Examples of suitable filamentous fungal host cells include strains of *Aspergillus,* e.g. *A. oryzae, A. niger,* or *A. nidulans, Fusarium* or *Trichodenna.* Examples of suitable yeast host cells include strains of *Saccharomyces,* e.g. *S. cerevisiae, Schizosaccharomyces, Kluyveromyces, Pichia,* such as *P. pastoris* or *P. methanolica, Hansenula,* such as *H. Polymorpha* or *Yarrowia.* Examples of suitable insect host cells include a *Lepidoptora* cell line, such as *Spodoptera frugiperda* (Sf9 or Sf21) or *Trichoplusioa ni* cells (High Five) (U.S. Pat. No. 5,077,214). Examples of suitable mammalian host cells include Chinese hamster ovary (CHO) cell lines, (e.g. CHO-K1; ATCC CCL-61), Green Monkey cell lines (COS) (e.g. COS 1 (ATCC CRL-1650), COS 7 (ATCC CRL-1651)); mouse cells (e.g. NS/O), Baby Hamster Kidney (BHK) cell lines (e.g. ATCC CRL-1632 or ATCC CCL-10), and human cells (e.g. HEK 293 (ATCC CRL-1573)). Additional suitable cell lines are known in the art and available from public depositories such as the American Type Culture Collection, Rockville, Md. Also, mammalian cells, such as a CHO cell, may be modified to express sialyltransferase, e.g. 1,6-sialyltransferase, e.g. as described in U.S. Pat. No. 5,047,335, in order to provide improved glycosylation of the polypeptide variant.

[0164] Methods for introducing exogeneous DNA into the above cell types, as well as other information regarding expression, production and purification of FVII variants, is found in WO 01/58935, incorporated herein by reference.

Pharmaceutical Composition of the Invention and its Use

[0165] In a further aspect, the present invention relates to a composition, in particular to a pharmaceutical composition, comprising a polypeptide variant of the invention and a pharmaceutically acceptable carrier or excipient.

[0166] The polypeptide variant or the pharmaceutical composition according to the invention may be used as a medicament.

[0167] Due to the high clotting efficiency, the polypeptide variant of the invention, or the pharmaceutical composition of the invention, is particular useful for the treatment of uncontrollable bleeding events in trauma patients, thrombocytopenic patients, patients in anticoagulant treatment, and cirrhosis patients with variceal bleeding, or other upper gastrointestinal bleedings, in patients undergoing orthotopic liver transplantation or liver resection (allowing for transfusion free surgery), or in hemophilia patients.

[0168] Trauma is defined as an injury to living tissue caused by an extrinsic agent. It is the 4th leading cause of death in the US and places a large financial burden on the economy.

[0169] Trauma is classified as either blunt or penetrative. Blunt trauma results in internal compression, organ damage and internal haemorrhage whereas penetrative trauma (as the consequence of an agent penetrating the body and destroying tissue, vessels and organs) results in external haemorrhage.

[0170] Trauma may be caused by numerous events, e.g. traffic accidents, gunshot wounds, falls, machinery accidents, and stab wounds.

[0171] Cirrhosis of the liver may be caused by direct liver injury, including chronic alcoholism, chronic viral hepatitis (types B, C, and D), and autoimmune hepatitis as well as by indirect injury by way of bile duct damage, including primary biliary cirrhosis, primary sclerosing cholangitis and biliary atresia Less common causes of cirrhosis include direct liver injury from inherited disease such as cystic fibrosis, alpha-1-antitrypsin deficiency, hemochromatosis, Wilson's disease, galactosemia, and glycogen storage disease. Transplantation is the key intervention for treating late stage cirrhotic patients.

[0172] Thus, in a further aspect the present invention relates to a polypeptide variant of the invention for the manufacture of a medicament for the treatment of diseases or disorder wherein clot formation is desirable. A still further aspect of the present invention relates to a method for treating a mammal having a disease or disorder wherein clot formation is desirable, comprising administering to a mammal in need thereof an effective amount of the polypeptide variant or the pharmaceutical composition of the invention.

[0173] Examples of diseases/disorders wherein increased clot formation is desirable include, but is not limited to, hemorrhages, including brain hemorrhages, as well as patient with severe uncontrolled bleedings, such as trauma Further examples include patients undergoing transplantations, patients undergoing resection and patients with variceal bleedings. Another widespread disease/disorder in which it is contemplated that the polypeptides of the invention will be useful for increased clot formation is hemophilia, e.g. von Willebrand disease, hemophilia A, hemophilia B or hemophilia C.

[0174] The polypeptide variant of the invention is administered to patients in a therapeutically effective dose, normally one approximately paralleling that employed in therapy with rhFVII such as NovoSeven®, or at lower dosage. By "therapeutically effective dose" herein is meant a dose that is sufficient to produce the desired effects in relation to the condition for which it is administered. The exact dose will depend on the circumstances, and will be ascertainable by one skilled in the art using known techniques. Normally, the dose should be capable of preventing or lessening the severity or spread of the condition or indication being treated. It will be apparent to those of skill

in the art that an effective amount of a polypeptide variant or composition of the invention depends, inter alia, upon the disease, the dose, the administration schedule, whether the polypeptide variant or composition is administered alone or in conjunction with other therapeutic agents, the plasma half-life of the compositions, and the general health of the patient.

[0175] The polypeptide variant of the invention is preferably administered in a composition including a pharmaceutically acceptable carrier or excipient. "Pharmaceutically acceptable" means a carrier or excipient that does not cause any untoward effects in patients to whom it is administered. Such pharmaceutically acceptable carriers and excipients are well known in the art (see, for example, Remington's Pharmaceutical Sciences, 18th edition, A. R. Gennaro, Ed., Mack Publishing Company [1990]; Pharmaceutical Formulation Development of Peptides and Proteins, S. Frokjaer and L. Hovgaard, Eds., Taylor & Francis [2000]; and Handbook of Pharmaceutical Excipients, 3rd edition, A. Kibbe, Ed., Pharmaceutical Press [2000]).

[0176] The polypeptide variants of the invention can be used "as is" and/or in a salt form thereof Suitable salts include, but are not limited to, salts with alkali metals or alkaline earth metals, such as sodium, potassium, calcium and magnesium, as well as e.g. zinc salts. These salts or complexes may by present as a crystalline and/or amorphous structure.

[0177] The pharmaceutical composition of the invention may be administered alone or in conjunction with other therapeutic agents. These agents may be incorporated as part of the same pharmaceutical composition or may be administered separately from the polypeptide variant of the invention, either concurrently or in accordance with another treatment schedule. In addition, the polypeptide variant or pharmaceutical composition of the invention may be used as an adjuvant to other therapies.

[0178] A "patient" for the purposes of the present invention includes both humans and other mammals. Thus, the methods are applicable to both human therapy and veterinary applications.

[0179] The pharmaceutical composition comprising the polypeptide variant of the invention may be formulated in a variety of forms, e.g. as a liquid, gel, lyophilized, or as a compressed solid. The preferred form will depend upon the particular indication being treated and will be apparent to one skilled in the art.

[0180] In particular, the pharmaceutical composition comprising the polypeptide variant of the invention may be formulated in lyophilized or stable soluble form. The polypeptide variant may be lyophilised by a variety of procedures known in the art. The polypeptide variant may be in a stable soluble form by the removal or shielding of proteolytic degradation sites as described herein. The advantage of obtaining a stable soluble preparation lies in easier handling for the patient and, in the case of emergencies, quicker action, which potentially can become life saving. The preferred form will depend upon the particular indication being treated and will be apparent to one of skill in the art.

[0181] The administration of the formulations of the present invention can be performed in a variety of ways, including, but not limited to, orally, subcutaneously, intravenously, intracerebrally, intranasally, transdermally, intraperitoneally, intramuscularly, intrapulmonary, vaginally, rectally, intraocularly, or in any other acceptable manner. The formulations can be administered continuously by infusion, although bolus injection is acceptable, using techniques well known in the art, such as pumps or implantation. In some instances the formulations may be directly applied as a solution or spray.

Parenterals

[0182] A preferred example of a pharmaceutical composition is a solution, in particular an aqueous solution, designed for parenteral administration. Although in many cases pharmaceutical solution formulations are provided in liquid form, appropriate for immediate use, such parenteral formulations may also be provided in frozen or in lyophilized form. In the former case, the composition must be thawed prior to use. The latter form is often used to enhance the stability of the active compound contained in the composition under a wider variety of storage conditions, as it is recognized by those skilled in the art that lyophilized preparations are generally more stable than their liquid counterparts. Such lyophilized preparations are reconstituted prior to use by the addition of one or more suitable pharmaceutically acceptable diluents such as sterile water for injection or sterile physiological saline solution.

[0183] In case of parenterals, they are prepared for storage as lyophilized formulations or aqueous solutions by mixing, as appropriate, the polypeptide variant having the desired degree of purity with one or more pharmaceutically acceptable carriers, excipients or stabilizers typically employed in the art (all of which are termed "excipients"), for example buffering agents, stabilizing agents, preservatives, isotonifiers, non-ionic surfactants or detergents, antioxidants and/or other miscellaneous additives.

[0184] Detailed information on parental formulations suitable for administration of FVII variants, as well as sustained release preparations, is found in WO 01/58935 and WO 03/093465, incorporated herein by reference.

[0185] The invention is further described in the following non-limiting examples.

MATERIALS AND METHODS

Active Site Region

[0186] The active site region is defined as any residues having at least one atom within 10 Å of any atom in the catalytic triad (residues H193, D242, S344).

Measurement of Reduced Sensitivity to Proteolytic Degradation

[0187] Proteolytic degradation can be measured using the assay described in U.S. Pat. No. 5,580,560, Example 5, where proteolysis is autoproteolysis.

[0188] Furthermore, reduced proteolysis can be tested in an in vivo model using radiolabelled samples and comparing proteolysis of rhFVIIa and the polypeptide variant of the invention by withdrawing blood samples and subjecting these to SDS-PAGE and autoradiography.

[0189] Irrespectively of the assay used for determining proteolytic degradation, "reduced proteolytic degradation" is intended to mean a measurable reduction in cleavage compared to that obtained by rhFVIIa as measured by gel

scanning of Coomassie stained SDS-PAGE gels, HPLC or as measured by conserved catalytic activity in comparison to wild type using the tissue factor independent activity assay described below.

Determination of the Molecular Weight of Polypeptide Variants

[0190] The molecular weight of polypeptide variants is determined by either SDS-PAGE, gel filtration, Western Blots, matrix assisted laser desorption mass spectrometry or equilibrium centrifugation, e.g. SDS-PAGE according to Laemmli, U. K., *Nature* Vol 227 (1970), pp. 680-85.

Determination of TFPI Inhibition

[0191] FVII inhibition by TFPI can be monitored in the amidolytic assay described in Chang et al. *Biochemistry* 1999, 38: 10940-10948.

Determination of TFPI Affinity

[0192] The capacity of variants to bind to TFPI is evaluated using one or more of the three BIAcore® assays described in Dickinson et al. *Proc. Natl. Acad. Sci. USA* 1996, 93: 14379-14384; Roberge et al. *Biochemistry* 2001, 40: 9522-9531; and Ruf et al. *Biochemistry* 1999, 38(7): 1957-1966.

TF-independent Factor X Activation Assay

[0193] This assay has been described in detail on page 39826 in Nelsestuen et al., *J Biol Chem,* 2001; 276:39825-39831.

[0194] Briefly, the molecule to be assayed (either hFVIIa, rhFVIIa or the polypeptide variant of the invention in its activated form) is mixed with a source of phospholipid (preferably phosphatidylcholine and phosphatidylserine in a ratio of 8:2) and relipidated Factor X in Tris buffer containing BSA. After a specified incubation time the reaction is stopped by addition of excess EDTA. The concentration of factor Xa is then measured from absorbance change at 405 nm after addition of a chromogenic substrate (S-2222, Chromogenix). After correction from background the tissue factor independent activity of rhFVIIa (a_{wt}) is determined as the absorbance change after 10 minutes and the tissue factor independent activity of the polypeptide variant of the invention ($a_{variant}$) is also determined as the absorbance change after 10 minutes. The ratio between the activity of the polypeptide variant, in its activated form, and the activity of rhFVIIa is defined as $a_{variant}/a_{wt}$.

Clotting Assay

[0195] The clotting activity of the FVIIa and variants thereof were measured in one-stage assays and the clotting times were recorded on a Thrombotrack IV coagulometer (Medinor). Factor VII-depleted human plasma (American Diagnostica) was reconstituted and equilibrated at room temperature for 15-20 minutes. 50 microliters of plasma was then transferred to the coagulometer cups.

[0196] FVIIa and variants thereof were diluted in Glyoxaline Buffer (5.7 mM barbiturate, 4.3 mM sodium citrate, 117 mM NaCl, 1 mg/ml BSA, pH 7.35). The samples were added to the cup in 50 ul and incubated at 37° C. for 2 minutes.

[0197] Technoplastin His (Medinor) was reconstituted with water and $CaCl_2$ was added to a final concentration of 4.5 mM. The mixture was equilibrated at 37° C. for 15-20 min. The reaction was initiated by adding 100 μl Technoplastin His.

[0198] To measure the clotting activity in the absence of TF the same assay was used without addition of Technoplastin His. Data was analysed using PRISM software.

Whole Blood Assay

[0199] The clotting activity of FVIIa and variants thereof were measured in one-stage assays and the clotting times were recorded on a Thrombotrack IV coagulometer (Medinor). 100 μl of FVIIa or variants thereof were diluted in a buffer containing 10 mM glycylglycine, 50 mM NaCl, 37.5 mM $CaCl_2$, pH 7.35 and transferred to the reaction cup. The clotting reaction was initiated by addition of 50 μl blood containing 10% 0.13 M tri-sodium citrate as anticoagulant. Data was analysed using Excel or PRISM software.

Amidolytic Assay

[0200] The ability of the variants to cleave small peptide substrates can be measured using the chromogenic substrate S-2288 (D-Ile-Pro-Arg-p-nitroanilide). FVIIa is diluted to about 10-90 nM in assay buffer (50 mM Na-Hepes pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 0.1% BSA, 1 U/ml Heparin). Furthermore, soluble TF (sTF) is diluted to 50-450 nM in assay buffer. 120 μl of assay buffer is mixed with 20 μl of the FVIIa sample and 20 μl sTF. After 5 min incubation at room temperature with gentle shaking, followed by 10 min incubation at 37° C., the reaction is started by addition of the S-2288 substrate to 1 mM and the absorption at 405 nm is determined at several time points.

ELISA Assay

[0201] FVII/FVIIa (or variant) concentrations are determined by ELISA. Wells of a microtiter plate are coated with an antibody directed against the protease domain using a solution of 2 μg/ml in PBS (100 μl per well). After overnight coating at R.T. (room temperature), the wells are washed 4 times with THT buffer (100 mM NaCl, 50 mM Tris-HCl pH 7.2 0.05% Tween-20). Subsequently, 200 μl of 1% Casein (diluted from 2.5% stock using 100 mM NaCl, 50 mM Tris-HCl pH 7.2) is added per well for blocking. After 1 hr incubation at R.T., the wells are emptied, and 100 μl of sample (optionally diluted in dilution buffer (THT+0.1% Casein)) is added. After another incubation of 1 hr at room temperature, the wells are washed 4 times with THT buffer, and 100 μl of a biotin-labelled antibody directed against the EGF-like domain (1 μg/ml) is added. After another 1 hr incubation at R.T., followed by 4 more washes with THT buffer, 100 μl of streptavidin-horse radish peroxidase (DAKO A/S, Glostrup, Denmark, diluted 1/10000) is added. After another 1 hr incubation at R.T., followed by 4 more washes with THT buffer, 100 μl of TMB (3,3',5,5'-tetramethylbenzidine, Kem-en-Tech A/S, Denmark) is added. After 30 min incubation at R.T. in the dark, 100 μl of 1 M H_2SO_4 is added and OD_{450nm} is determined. A standard curve is prepared using rhFVIIa (NovoSeven®).

[0202] Alternatively, FVII/FVIIa or variants may be quantified through the Gla domain rather than through the protease domain. In this ELISA set-up, wells are coated overnight with an antibody directed against the EGF-like domain and for detection, a calcium-dependent biotin-labelled monoclonal anti-Gla domain antibody is used (2

μg/ml, 100 μl per well). In this set-up, 5 mM $CaCl_2$ is added to the THT and dilution buffers.

EXAMPLES

Example 1

[0203] The X-ray structure of hFVIIa in complex with soluble tissue factor by Banner et al., *J Mol Biol,* 1996; 285:2089 is used for this example. It is noted that the numbering of residues in the reference does not follow the sequence. Here, we have used the sequential numbering according to SEQ ID NO:2. The gamma-carboxy glutamic acids at positions 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35 are all here named Glu (three letter abbreviation) or E (one letter abbreviation). Residues 143-152 are not present in the structure. For further information on the calculations in this example, see WO 01/58935.

Surface Exposure

[0204] Performing fractional ASA calculations resulted in the following residues being determined to have more than 25% of their side chain exposed to the surface: A1, N2, A3, F4, L5, E6, E7, L8, R9, P10, S12, L13, E14, E16, K18, E19, E20, Q21, S23, F24, E25, E26, R28, E29, F31, K32, D33, A34, E35, R36, K38, L39, W41, I42, S43, S45, G47, D48, Q49, A51, S52, S53, Q56, G58, S60, K62, D63, Q64, L65, Q66, S67, I69, F71, L73, P74, A75, E77, G78, R79, E82, T83, H84, K85, D86, D87, Q88, L89, I90, V92, N93, E94, G97, E99, S103, D104, H105, T106, G107, T108, K109, S111, R113, E116, G117, S119, L120, L121, A122, D123, G124, V125, S126, T128, P129, T130, V131, E132, I140, L141, E142, K143, R144, N145, A146, S147, K148, P149, Q150, G151, R152, G155, K157, V158, P160, K161, E163, L171, N173, G174, A175, N184, T185, I186, H193, K197, K199, N200, R202, N203, I205, S214, E215, H216, D217, G218, D219, S222, R224, S232, T233, V235, P236, G237, T238, T239, N240, H249, Q250, P251, V253, T255, D256, E265, R266, T267, E270, R271, F275, V276, R277, F278, L280, L287, L288, D289, R290, G291, A292, T293, L295, E296, N301, M306, T307, Q308, D309, L311, Q312, Q313, R315, K316, V317, G318, D319, S320, P321, N322, T324, E325, Y326, Y332, S333, D334, S336, K337, K341, G342, H351, R353, G354, Q366, G367, T370, V371, G372, R379, E385, Q388, K389, R392, S393, E394, P395, R396, P397, G398, V399, L400, L401, R402, P404 and P406.

[0205] The following residues were determined to have more than 50% of their side chain exposed to the surface: A1, A3, F4, L5, E6, E7, L8, R9, P10, E14, E16, K18, E19, E20, Q21, S23, E25, E26, E29, K32, A34, E35, R36, K38, L39, I42, S43, G47, D48, A51, S52, S53, Q56, G58, S60, K62, L65, Q66, S67, I69, F71, L73, P74, A75, E77, G78, R79, E82, H84, K85, D86, D87, Q88, L89, I90, V92, N93, E94, G97, T106, G107, T108, K109, S111, E116, S119, L121, A122, D123, G124, V131, E132, L141, E142, K143, R144, N145, A146, S147, K148, P149, Q150, G151, R152, G155, K157, P160, N173, G174, A175, K197, K199, N200, R202, S214, E215, H216, G218, R224, V235, P236, G237, T238, H249, Q250, V253, D256, T267, F275, R277, P278, L288, D289, R290, G291, A292, T293, L295, N301, M306, Q308, D309,L311,Q312,Q313,R315,K316,G318,D319, N322,E325,D334,K341,G354, G367, V371, E385, K389, R392, E394, R396, P397, G398, R402, P404 and P406.

Tissue Factor Binding Site

[0206] It was determined using ASA calculations that the following residues in hFVII change their ASA in the complex. These residues were defined as constituting the tissue factor binding site: L13, K18, F31, E35, R36, L39, F40, I42, S43, S60, K62, D63, Q64, L65, I69, C70, F71, C72, L73, P74, F76, E77, G78, R79, E82, K85, Q88, I90, V92, N93, E94, R271, A274, F275, V276, R277, F278, R304, L305, M306, T307, Q308, D309, Q312, Q313, E325 and R379.

Active Site Region

[0207] The active site region is defined as any residue having at least one atom within a distance of 10 Å from any atom in the catalytic triad (residues H193, D242, S344): I153, Q167, V168, L169, L170, L171, Q176, L177, C178, G179, G180, T181, V188, V189, S190, A191, A192, H193, C194, F195, D196, K197, I198, W201, V228, I229, I230, P231, S232, T233, Y234, V235, P236, G237, T238, T239, N240, H241, D242, I243, A244, L245, L246, V281, S282, G283, W284, G285, Q286, T293, T324, E325, Y326, M327, F328, D338, S339, C340, K341, G342, D343, S344, G345, G346, P347, H348, L358, T359, G360, I361, V362, S363, W364, G365, C368, V376, Y377, T378, R379, V380, Q382, Y383, W386, L387, L400 and F405.

The Ridge of the Active Site Binding Cleft

[0208] The ridge of the active site binding cleft region was defined by visual inspection of the FVIIa structure 1FAK-.pdb as: N173, A175, K199, N200, N203, D289, R290, G291, A292, P321 and T370.

Example 2

Design of an Expression Cassette for Expression of hFVII in Mammalian Cells

[0209] The DNA sequence shown in SEQ ID NO:1, encompassing the short form of the full length cDNA encoding hFVII with its native short signal peptide (Hagen et al., 1986. *Proc Natl Acad Sci USA* 83:2412), was synthesized in order to facilitate high expression in mammalian cells. First the ATG start codon context was modified according to the Kozak consensus sequence (Kozak, M. *J Mol Biol* 1987 Aug 20;196(4):947-50), so that there is a perfect match to the consensus sequence upstream of the ATG start codon. Secondly the open reading frame of the native cDNA was modified by making a bias in the codon usage towards the codons frequently used in highly expressed human genes. Further, two translational stop codons were inserted at the end of the open reading frame in order to facilitate efficient translational stop. The fully synthetic and expression optimized hFVII gene was assembled from 70-mer DNA oligonucleotides and finally amplified using end primers inserting BamHI and HindIII sites at the 5' and 3' ends respectively using standard PCR techniques, which resulted in the following sequence (SEQ ID NO:3):

```
ggatcccgccaccatggtcagccaggccctccgcctcctgtgcctgctcc

tggggctgcagggctgcctggctgccgtcttcgtcacccaggaggaagcc

catggcgtcctgcatcgccggcgccgggccaatgcctttctggaagagct
```

-continued

ccgccctggctccctggaacgcgaatgcaaagaggaacagtgcagctttg

aggaagcccgggagattttcaaagacgctgagcggaccaaactgttttgg

attagctatagcgatggcgatcagtgcgcctccagcccttgccagaacgg

gggctcctgcaaagaccagctgcagagctatatctgcttctgcctgcctg

cctttgaggggcgcaattgcgaaacccataaggatgaccagctgatttgc

gtcaacgaaaacgggggctgcgagcagtactgcagcgatcacacgggcac

gaagcggagctgccgctgccacgaaggctatagcctcctggctgacgggg

tgtcctgcacgcccacggtggaataccttgcgggaagattcccattcta

gaaaagcggaacgctagcaaaccccagggccggatcgtcggcgggaaggt

ctgccctaaggggagtgcccctggcaggtcctgctcctggtcaacgggg

cccagctgtgcggcgggaccctcatcaataccatttgggtcgtgtccgcc

gctcactgcttcgataagattaagaattggcggaacctcatcgctgtgct

cggcgaacacgatctgtccgagcatgacggggacgaacagtcccgccggg

tggctcaggtcatcattccctccacctatgtgcctggcacgaccaatcac

gatatcgctctgctccgcctccaccagcccgtcgtgctcaccgatcacgt

cgtgcctctgtgcctgcctgagcggacctttagcgaacgcacgctggctt

tcgtccgctttagcctcgtgtccggctggggccagctgctcgaccggggc

gctaccgctctcgagctgatggtgctcaacgtcccccggctgatgaccca

ggactgcctgcagcagtcccgcaaagtgggggactcccccaatatcacgg

agtatatgttttgcgctggctatagcgatggctccaaggatagctgcaag

ggggactccggcgggccccatgccacgcactatcgcgggacctggtacct

caccgggatcgtcagctggggccagggctgcgccacggtggggcactttg

gcgtctacacgcgcgtcagccagtacattgagtggctgcagaagctcatg

cggagcgaaccccggcccggggtgctcctgcgggccccttccccttgata

aaagctt

[0210] A vector for the cloning of the generated PCR product encompassing the expression cassette for hFVII was prepared by cloning the intron from pCINeo (Promega). The synthetic intron from pCI-Neo was amplified using standard PCR conditions and the primers:

CBProFpr174:
5'-AGCTGGCTAGCCACTGGGCAGGTAAGTATCA-3' (SEQ ID NO:4)
and

CBProFpr175:
5'-TGGCGGGATCCTTAAGAGCTGTAATTGAACT-3' (SEQ ID NO:5)

resulting in a 332 bp PCR fragment. The fragment was cut with NheI and BamHI before cloning into pCDNA3.1/HygR (obtained from Invitrogen) resulting in PF#34.

[0211] The expression cassette for hFVII was clone d bet ween the BamHI and HindIII sites of PF#341, resulting in plasmid PF#226.

Example 3

Construction of Expression Vectors Encoding Polypeptide Variants of the Invention

[0212] Sequence overhang extension (SOE) PCR was used for generating constructs having variant FVII open reading frames with substituted codons. In the SOE-PCR both the N-terminal part and the C-terminal part of the FVII open reading frame was first amplified in individual primary PCRs.

[0213] In order to change the codon for D196 to the codon for N196 the following primers were used pairwise for the primary PCRs:

(SEQ ID NO:6)
CB499: 5'-CCCATTCTAGAAAAGCGGAACGCCAGCAAACCCCAGGG-3'
and

(SEQ ID NO:7)
CB562: 5'-CCAATTCTTAATCTTGTTGAAGCAGTGAGCGGCG-3',
and

(SEQ ID NO:8)
CB256: 5'-CTCCGTGATATTGGGGGAGTC-3'
and

(SEQ ID NO:9)
CB561: 5'-CGCCGCTCACTGCTTCAACAAGATTAAGAATTGG-3'.

[0214] The primary PCR products were then combined and the terminal primers (CB499 and CB256) were added, allowing for the secondary full-length product encoding the mutated fragment of the desired D196N variant to be made. This PCR product was restricted with XbaI and XhoI and used to substitute the equivalent fragment of the FVII coding region of expression vector PF226 resulting in the expression vector pB0014 encoding the D196N variant.

[0215] With the exception of the constructs for position 341 variants, the constructs were made in the same way as for D196N. Constructs for position 341 variants were made using the end primers

CB220: 5'-CGCTCTCGAGCTGATGGTGCTC-3' (SEQ ID NO:10)
and

CB362: 5'-CAAACAACAGATGGCTGGCAAC-3' (SEQ ID NO:11)

[0216] allowing for directional cloning between XhoI and HindIII. The central primer used in the SOE-PCR reactions for the substitution variants were:

D196K
CB563:
CGCCGCTCACTGCTTCAAGAAGATTAAGAATTGG (SEQ ID NO:12)

CB564:
CCAATTCTTAATCTTCTTGAAGCAGTGAGCGGCG (SEQ ID NO:13)

G237L
CB565:
CTCCACCTATGTGCCTCTGACGACCAATCACGA (SEQ ID NO:14)

CB566:
TCGTGATTGGTCGTCAGAGGCACATAGGTGGAG (SEQ ID NO:15)

-continued

K341Q
CB569:
CCAAGGATGCCAGGGGGACTCCGGCGGGC (SEQ ID NO:16)

CB570:
GCCCGCCGGAGTCCCCCTGGCAGCTATCCTTGG (SEQ ID NO:17)

[0217] For the insertion variant the central primers were:

G237GAA
CB597:
ACCTATGTGCCTGGCGCTGCCACGACCAATCACGAT (SEQ ID NO:18)

CB598:
ATCGTGATTGGTCGTGGCAGCGCCAGGCACATAGGT (SEQ ID NO:19)

Example 4

Expression of FVII or FVII variants in CHO K1 Cells

[0218] The cell line CHO K1 (ATCC # CCL-61) is seeded at 50% confluence in T-25 flasks using MEMα, 10% FCS (Gibco/BRL Cat # 10091), P/S and 5 μg/ml phylloquinone and allowed to grow until confluent. The confluent mono cell layer is transfected with 5 μg of the relevant plasmid described above using the Lipofectamine 2000 transfection agent (Life Technologies) according to the manufacturer's instructions. Twenty four hours post transfection a sample is drawn and quantified using e.g. an ELISA recognizing the EGF1 domain of hFVII. At this time point relevant selection (e.g. Hygromycin B) may be applied to the cells for the purpose of generating a pool of stable transfectants. When using CHO K1 cells and the Hygromycin B resistance gene as a selectable marker on the plasmid, this is usually achieved within one week.

Example 5

Generation of CHO-K1 Cells Stably Expressing Polypeptide Variants

[0219] A vial of CHO-K1 transfectant pool is thawed and the cells seeded in a 175 cm^2 tissue flask containing 25 ml of MEMα, 10% FCS, phylloquinone (5 μg/ml), 100 U/l penicillin, 100 μg/l streptomycin and grown for 24 hours. The cells are harvested, diluted and plated in 96 well microtiter plates at a cell density of ½-1 cell/well. After a week of growth, colonies of 20-100 cells are present in the wells and those wells containing only one colony are labelled. After a further two weeks, the media in all wells containing only one colony is substituted with 200 μl fresh medium. After 24 hours, a medium sample is withdrawn and analysed by e.g. ELISA. High producing clones are selected and used to produce FVII or variant on large scale.

Example 6

Purification of Polypeptide Variants and Subsequent Activation

[0220] FVII and FVII variants are purified as follows: The procedure is performed at 4° C. The harvested culture media from large-scale production is ultrafiltered using a Millipore TFF system with 30 KDa cut-off Pellicon membranes. After concentration of the medium, citrate is added to 5 mM and the pH is adjusted to 8.6. If necessary, the conductivity is lowered to below 10 mS/cm. Subsequently, the sample is applied to a Q-sepharose FF column, equilibrated with 50 mM NaCl, 10 mM Tris pH 8.6. After washing the column with 100 mM NaCl, 10 mM Tris pH 8.6, followed by 150 mM NaCl, 10 mM Tris pH 8.6, FVII is eluted using 10 mM Tris, 25 mM NaCl, 35 mM $CaCl_2$, pH 8.6.

[0221] For the second chromatographic step, an affinity column is prepared by coupling of a monoclonal Calcium-dependent antiGla-domain antibody to CNBr-activated Sepharose FF. About 5.5 mg antibody is coupled per ml resin. The column is equilibrated with 10 mM Tris, 100 mM NaCl, 35 mM $CaCl_2$, pH 7.5. NaCl is added to the sample to a concentration of 100 mM NaCl and the pH is adjusted to 7.4-7.6. After O/N application of the sample, the column is washed with 100 mM NaCl, 35 mM $CaCl_2$, 10 mM Tris pH 7.5, and the FVII protein is eluted with 100 mM NaCl, 50 mM citrate, 75 mM Tris pH 7.5.

[0222] For the third chromatographic, the conductivity of the sample is lowered to below 10 mS/cm, if necessary, and the pH is adjusted to 8.6. The sample is then applied to a Q-sepharose column (equilibrated with 50 mM NaCl, 10 mM Tris pH 8.6) at a density around 3-5 mg protein per ml gel to obtain efficient activation. After application, the column is washed with 50 mM NaCl, 10 mM Tris pH 8.6 for about 4 hours with a flow of 3-4 column volumes (cv) per hour. The FVII protein is eluted using a gradient of 0-100% of 500 mM NaCl, 10 mM Tris pH 8.6 over 40 cv. FVII containing fractions are pooled.

[0223] For the final chromatographic step, the conductivity is lowered to below 10 mS/cm. Subsequently, the sample is applied to a Q-sepharose column (equilibrated with 140 mM NaCl, 10 mM glycylglycine pH 8.6) at a concentration of 3-5 mg protein per ml gel. The column is then washed with 140 mM NaCl, 10 mM glycylglycine pH 8.6 and FVII is eluted with 140 mM NaCl, 15 mM $CaCl_2$, 10 mM glycylglycine pH 8.6. The eluate is diluted to 10 mM $CaCl_2$ and the pH is adjusted 6.8-7.2. Finally, Tween-80 is added to 0.01% and the pH is adjusted to 5.5 for storage at −80° C.

Example 7

Experimental Results

[0224] Subjecting the variants of the invention to the "Whole Blood Assay" revealed that the variants exhibit a significantly increased clotting activity (reduced clotting time) as compared to rhFVIIa. The experimental results are compiled in Table 1 below. The results are in addition illustrated in the appended FIG. **1**, which shows the clotting time vs. concentration for the G237GAA variant versus rhFVIIa.

TABLE 1

Variant	Clotting time (Whole Blood Assay) $t_{variant}/t_{wt}$
rhFVIIa (reference)	1
D196K	0.4
D196N	0.4
K341Q	0.4
G237L	0.3
G237GAA	0.3

[0225]

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 19

<210> SEQ ID NO 1
<211> LENGTH: 1338
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (115)..(1338)

<400> SEQUENCE: 1

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atggtcagcc aggccctccg cctcctgtgc ctgctcctgg ggctgcaggg ctgcctggct     60

gccgtcttcg tcacccagga ggaagcccat ggcgtcctgc atcgccggcg ccgg gcc      117
                                                              Ala
                                                              1

aat gcc ttt ctg gaa gag ctc cgc cct ggc tcc ctg gaa cgc gaa tgc      165
Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu Cys
            5                  10                  15

aaa gag gaa cag tgc agc ttt gag gaa gcc cgg gag att ttc aaa gac      213
Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys Asp
        20                  25                  30

gct gag cgg acc aaa ctg ttt tgg att agc tat agc gat ggc gat cag      261
Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp Gln
    35                  40                  45

tgc gcc tcc agc cct tgc cag aac ggg ggc tcc tgc aaa gac cag ctg      309
Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln Leu
50                  55                  60                  65

cag agc tat atc tgc ttc tgc ctg cct gcc ttt gag ggg cgc aat tgc      357
Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn Cys
                70                  75                  80

gaa acc cat aag gat gac cag ctg att tgc gtc aac gaa aac ggg ggc      405
Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly Gly
            85                  90                  95

tgc gag cag tac tgc agc gat cac acg ggc acg aag cgg agc tgc cgc      453
Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys Arg
        100                 105                 110

tgc cac gaa ggc tat agc ctc ctg gct gac ggg gtg tcc tgc acg ccc      501
Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr Pro
    115                 120                 125

acg gtg gaa tac cct tgc ggg aag att ccc att cta gaa aag cgg aac      549
Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg Asn
130                 135                 140                 145

gct agc aaa ccc cag ggc cgg atc gtc ggc ggg aag gtc tgc cct aag      597
Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro Lys
                150                 155                 160

ggg gag tgc ccc tgg cag gtc ctg ctc ctg gtc aac ggg gcc cag ctg      645
Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln Leu
            165                 170                 175

tgc ggc ggg acc ctc atc aat acc att tgg gtc gtg tcc gcc gct cac      693
Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala His
        180                 185                 190

tgc ttc gat aag att aag aat tgg cgg aac ctc atc gct gtg ctc ggc      741
Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu Gly
    195                 200                 205

gaa cac gat ctg tcc gag cat gac ggg gac gaa cag tcc cgc cgg gtg      789
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Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg Val
210                 215                 220                 225

gct cag gtc atc att ccc tcc acc tat gtg cct ggc acg acc aat cac      837
Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn His
                230                 235                 240

gat atc gct ctg ctc cgc ctc cac cag ccc gtc gtg ctc acc gat cac      885
Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp His
            245                 250                 255

gtc gtg cct ctg tgc ctg cct gag cgg acc ttt agc gaa cgc acg ctg      933
Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr Leu
        260                 265                 270

gct ttc gtc cgc ttt agc ctc gtg tcc ggc tgg ggc cag ctg ctc gac      981
Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu Asp
    275                 280                 285

cgg ggc gct acc gct ctc gag ctg atg gtg ctc aac gtc ccc cgg ctg     1029
Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg Leu
290                 295                 300                 305

atg acc cag gac tgc ctg cag cag tcc cgc aaa gtg ggg gac tcc ccc     1077
Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser Pro
                310                 315                 320

aat atc acg gag tat atg ttt tgc gct ggc tat agc gat ggc tcc aag     1125
Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser Lys
            325                 330                 335

gat agc tgc aag ggg gac tcc ggc ggg ccc cat gcc acg cac tat cgc     1173
Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr Arg
        340                 345                 350

ggg acc tgg tac ctc acc ggg atc gtc agc tgg ggc cag ggc tgc gcc     1221
Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys Ala
    355                 360                 365

acg gtg ggg cac ttt ggc gtc tac acg cgc gtc agc cag tac att gag     1269
Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile Glu
370                 375                 380                 385

tgg ctg cag aag ctc atg cgg agc gaa ccc cgg ccc ggg gtg ctc ctg     1317
Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu Leu
                390                 395                 400

cgg gcc cct ttc cct tga taa                                        1338
Arg Ala Pro Phe Pro
            405


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Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys
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Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp
        35                  40                  45

Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln
    50                  55                  60

Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn
65                  70                  75                  80

Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly
                85                  90                  95
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Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys
            100                 105                 110

Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr
        115                 120                 125

Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg
    130                 135                 140

Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro
145                 150                 155                 160

Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln
                165                 170                 175

Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala
            180                 185                 190

His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu
        195                 200                 205

Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg
    210                 215                 220

Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn
225                 230                 235                 240

His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp
                245                 250                 255

His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr
            260                 265                 270

Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu
        275                 280                 285

Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg
    290                 295                 300

Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser
305                 310                 315                 320

Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser
                325                 330                 335

Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr
            340                 345                 350

Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys
        355                 360                 365

Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile
    370                 375                 380

Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu
385                 390                 395                 400

Leu Arg Ala Pro Phe Pro
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<210> SEQ ID NO 3
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gcgccgggcc aatgcctttc tggaagagct ccgccctggc tccctggaac gcgaatgcaa 180

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actgttttgg attagctata gcgatggcga tcagtgcgcc tccagccctt gccagaacgg 300

gggctcctgc aaagaccagc tgcagagcta tatctgcttc tgcctgcctg cctttgaggg 360

gcgcaattgc gaaacccata aggatgacca gctgatttgc gtcaacgaaa acgggggctg 420

cgagcagtac tgcagcgatc acacgggcac gaagcggagc tgccgctgcc acgaaggcta 480

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tcccattcta gaaaagcgga acgctagcaa accccagggc cggatcgtcg gcgggaaggt 600

ctgccctaag ggggagtgcc cctggcaggt cctgctcctg gtcaacgggg cccagctgtg 660

cggcgggacc ctcatcaata ccatttgggt cgtgtccgcc gctcactgct tcgataagat 720

taagaattgg cggaacctca tcgctgtgct cggcgaacac gatctgtccg agcatgacgg 780

ggacgaacag tcccgccggg tggctcaggt catcattccc tccacctatg tgcctggcac 840

gaccaatcac gatatcgctc tgctccgcct ccaccagccc gtcgtgctca ccgatcacgt 900

cgtgcctctg tgcctgcctg agcggacctt tagcgaacgc acgctggctt tcgtccgctt 960

tagcctcgtg tccggctggg gccagctgct cgaccggggc gctaccgctc tcgagctgat 1020

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ggactccccc aatatcacgg agtatatgtt ttgcgctggc tatagcgatg gctccaagga 1140

tagctgcaag ggggactccg gcgggcccca tgccacgcac tatcgcggga cctggtacct 1200

caccgggatc gtcagctggg gccagggctg cgccacggtg gggcactttg gcgtctacac 1260

gcgcgtcagc cagtacattg agtggctgca gaagctcatg cggagcgaac cccggcccgg 1320

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<210> SEQ ID NO 12
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<210> SEQ ID NO 13

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<211> LENGTH: 34
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<210> SEQ ID NO 16
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 16

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<210> SEQ ID NO 17
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<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
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<210> SEQ ID NO 18
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 18

acctatgtgc ctggcgctgc cacgaccaat cacgat 36

<210> SEQ ID NO 19
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:

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<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 19

atcgtgattg gtcgtggcag cgccaggcac ataggt 36

1. A variant of FVII or FVIIa, wherein said variant comprises 1-15 amino acid modifications as compared to hFVII or hFVIIa (SEQ ID NO:2), including at least one amino acid modification in a position selected from the group consisting of positions 196, 237 and 341.

2. (canceled)

3. The variant of claim 1, wherein said variant comprises a modification in position 196 as compared to hFVII or hFVIIa (SEQ ID NO:2).

4. The variant of claim 3, wherein said modification is a substitution.

5. The variant of claim 4, wherein said substitution is D196K or D196N.

6. The variant of claim 1, wherein said variant comprises a modification in position 237 as compared to hFVII or hFVIIa (SEQ ID NO:2).

7. The variant of claim 6, wherein said modification is a substitution.

8. The variant of claim 7, wherein said substitution is G237L.

9. The variant of claim 6, wherein said modification is an insertion.

10. The variant of claim 9, wherein said insertion is selected from the group consisting of G237GXX, G237GXXX and G237GXXXX, wherein X is any amino acid residue.

11. The variant of claim 10, wherein X is selected from the group consisting Ala, Val, Leu, Ile, Gly, Ser and Thr.

12. The variant of claim 11, wherein X is Ala.

13. The variant of claim 12, wherein said insertion is G237GAA.

14. The variant of claim 1, wherein said variant comprises a modification in position 341 as compared to hFVII or hFVIIa (SEQ ID NO:2).

15. The variant of claim 14, wherein said modification is a substitution.

16. The variant of claim 15, wherein said substitution is K341Q.

17. The variant of claim 1, wherein said variant comprises 1-10 further amino acid modifications.

18-53. (canceled)

54. The variant of claim 1, wherein said variant is in its activated form.

55. A nucleotide sequence encoding a variant as defined in claim 1.

56. An expression vector comprising a nucleotide sequence as defined in claim 55.

57. A host cell comprising an expression vector as defined in claim 56.

58. The host cell of claim 57, wherein said host cell is a gamma-carboxylating cell capable of in vivo glycosylation.

59. A composition comprising a variant as defined in claim 1 and at least one pharmaceutically acceptable carrier or excipient.

60-64. (canceled)

65. A method for treating a mammal having a disease or a disorder wherein clot formation is desirable, comprising administering to a mammal in need thereof an effective amount of the variant of claim 1.

66. The method of claim 65, wherein said disease or disorder is selected from the group consisting of hemorrhages, including brain hemorrhages, severe uncontrolled bleedings, such as trauma, bleedings in patients undergoing transplantations or resection, variceal bleedings, and hemophilia.

67. The method of claim 66, wherein said disease or disorder is trauma.

68. The method of claim 66, wherein said disease or disorder is hemophilia.

* * * * *

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Pedersen et al. (43) **Pub. Date: Oct. 18, 2007**

(54) **FACTOR VII OR VIIA - LIKE MOLECULES**

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(73) Assignee: **Maxygen Holdings, Ltd.**

(21) Appl. No.: **11/279,541**

(22) Filed: **Apr. 12, 2006**

Related U.S. Application Data

(62) Division of application No. 10/950,747, filed on Sep. 27, 2004.
Division of application No. 09/782,587, filed on Feb. 12, 2001, now Pat. No. 6,806,063.

(60) Provisional application No. 60/241,916, filed on Oct. 18, 2000. Provisional application No. 60/184,036, filed on Feb. 22, 2000.

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Feb. 11, 2000 (DK)..... PA 2000 00218

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C12N 15/00	(2006.01)
C12N 5/10	(2006.01)

(52) **U.S. Cl.** **435/69.6**; 435/320.1; 435/325; 536/23.5

(57) **ABSTRACT**

Conjugates of Factor VII (FVII) and Factor VIIa (FVIIA) are provided, as are methods for preparing them. Methods for producing novel polypeptides contributing to the production of such conjugates are provided. Methods of treatment by administering a FVII or FVIIa conjugate are provided.

FACTOR VII OR VIIA - LIKE MOLECULES

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] The present application claims priority to and benefit of the following United States and international patent applications: Danish Patent Application Number PA 2000 00218, filed Feb. 11, 2000; U.S. Provisional Patent Application No. 60/184,036, filed Feb. 22, 2000; and, U.S. Provisional Patent Application No. 60/241,916, filed Oct. 18, 2000.

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[0002] Pursuant to 37 C.F.R. 1.71(e), Applicants note that a portion of this disclosure contains material which is subject to copyright protection. The copyright owner has no objection to the facsimile reproduction by anyone of the patent document or patent disclosure, as it appears in the Patent and Trademark Office patent file or records, but otherwise reserves all copyright rights whatsoever.

FIELD OF THE INVENTION

[0003] The present invention relates to novel Factor VII (FVII) or Factor VIIa (FVIIa) polypeptide conjugates, to their preparation and use in therapy, in particular for the treatment of a variety of coagulation-related disorders.

BACKGROUND OF THE INVENTION

[0004] Blood coagulation is a process consisting of a complex interaction of various blood components (or factors) that eventually gives rise to a fibrin clot. Generally, the blood components participating in what has been referred to as the coagulation "cascade" are proenzymes or zymogens, i.e., enzymatically inactive proteins that are converted into an active form by the action of an activator. One of these coagulation factors is FVII.

[0005] FVII is a vitamin K-dependent plasma protein synthesized in the liver and secreted into the blood as a single-chain glycoprotein with a molecular weight of 53 kDa (Broze & Majerus, J. Biol. Chem 1980; 255:1242-1247). The FVII zymogen is converted into an activated form (FVIIa) by proteolytic cleavage at a single site, R152-I153, resulting in two chains linked by a single disulfide bridge. FVIIa in complex with tissue factor (FVIIa complex) is able to convert both factor IX and factor X into their activated forms, followed by reactions leading to rapid thrombin production and fibrin formation (Østerud & Rapaport, Proc Natl Acad Sci USA 1977; 74:5260-5264).

[0006] FVII undergoes post-translational modifications, including vitamin K-dependent carboxylation resulting in ten γ-carboxyglutamic acid residues in the N-terminal region of the molecule. Thus, residue number 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35 shown in SEQ ID NO:1 are γ-carboxyglutamic acids residues in the Gla domain important for FVII activity. Other post-translational modifications include sugar moiety attachment at two naturally occurring N-glycosylation sites at position 145 and 322, respectively, and at two naturally occurring O-glycosylation sites at position 52 and 60, respectively.

[0007] The gene coding for human FVII (hFVII) has been mapped to chromosome 13 at q34-qter 9 (de Grouchy et al., Hum Genet 1984; 66:230-233). It contains nine exons and spans 12.8 Kb (O'Hara et al., Proc Natl Acad Sci USA 1987; 84:5158-5162). The gene organisation and protein structure of FVII are similar to those of other vitamin K-dependent procoagulant proteins, with exons 1a and 1b encoding for signal sequence; exon 2 the propeptide and Gla domain; exon 3 a short hydrophobic region; exons 4 and 5 the epidermal growth factor-like domains; and exon 6 through 8 the serine protease catalytic domain (Yoshitake et al., Biochemistry 1985; 24: 3736-3750).

[0008] Reports exist on experimental three-dimensional structures of hFVIIa (Pike et al., PNAS. U.S.A., 1999; 96:8925-30 and Kemball-Cook et al., J. Struct. Biol, 1999; 127:213-223), of hFVIIa in complex with soluble tissue factor using X-ray crystallographic methods (Banner et al., Nature, 1996; 380:41 and Zhang et al., J. Mol. Biol, 1999; 285: 2089), and of smaller fragments of hFVII (Muranyi et al., Biochemistry, 1998; 37:10605 and Kao et al., Biochemistry, 1999; 38:7097).

[0009] Relatively few protein-engineered variants of FVII have been reported (Dickinson & Ruf, J Bio Chem, 1997; 272:19875-19879, Kemball-Cook et al., J Biol Chem, 1998; 273:8516-8521, Bharadwaj et al., J Biol Chem, 1996; 271:30685-30691, Ruf et al., Biochemistry, 1999; 38:1957-1966).

[0010] Reports exist on expression of FVII in BHK or other mammalian cells (WO92/15686, WO91/11514 and WO88/10295) and co-expression of FVII and kex2 endoprotease in eukaryotic cells (WO 00/28065).

[0011] Commercial preparations of human recombinant FVIIa are sold as NovoSeven®. NovoSeven® is indicated for the treatment of bleeding episodes in hemophilia A or B patients. NovoSeven® is the only rFVIIa for effective and reliable treatment of bleeding episodes available on the market.

[0012] An inactive form of FVII in which arginine 152 and/or isoleucine 153 is/are modified has been reported in WO91/1154. These amino acids are located at the activation site. WO 96/12800 describes inactivation of FVIIa by a serine proteinase inhibitor; inactivation by carbamylation of FVIIa at the α-amino acid group I153 has been described by Petersen et al., Eur J Biochem, 1999; 261:124-129. The inactivated form is capable of competing with wild-type FVII or FVIIa for binding to tissue factor and inhibiting clotting activity. The inactivated form of FVIIa is suggested to be used for treatment of patients being in hypercoagulable states, such as patients with sepsis, in risk of myocardial infarction or of thrombotic stroke.

[0013] A circulating rFVIIa half-life of 2.3 hours was reported in "Summary Basis for Approval for NovoSeven®," FDA reference number 96-0597. Relatively high doses and frequent administration are necessary to reach and sustain the desired therapeutic or prophylactic effect. As a consequence adequate dose regulation is difficult to obtain and the need of frequent intravenous administrations imposes restrictions on the patient's way of living.

[0014] Another problem in current rFVIIa treatment is the relative instability of the molecule with respect to proteolytic degradation. Proteolytic degradation is a major obstacle for obtaining a preparation in solution as opposed to a lyophilised product. The advantage of obtaining a stable

soluble preparation lies in easier handling for the patient, and, in the case of emergencies, quicker action, which potentially can become life saving. Attempts to prevent proteolytic degradation by site directed mutagenesis at major proteolytic sites have been disclosed in WO88/10295.

[0015] A molecule with a longer circulation half-life would decrease the number of necessary administrations. Given the association of current FVIIa product with frequent injections, and the potential for obtaining more optimal therapeutic FVIIa levels with concomitant enhanced therapeutic effect, there is a clear need for improved FVII or FVIIa-like molecules.

[0016] One way to increase the circulation half-life of a protein is to ensure that renal clearance of the protein is reduced. This can be achieved by conjugating the protein to a chemical moiety, which is capable of conferring reduced renal clearance to the protein.

[0017] Furthermore, attachment of a chemical moiety to the protein or substitution of amino acids exposed to proteolysis can effectively block a proteolytic enzyme from contact leading to proteolytic degradation of the protein. Polyethylene glycol (PEG) is one such chemical moiety that has been used in the preparation of therapeutic protein products.

[0018] WO98/32466 suggests that FVII, among many other proteins, can be PEGylated but does not provide any further information in this respect.

SUMMARY OF THE INVENTION

[0019] This application discloses improved FVII and FVIIa molecules, in particular recombinant hFVII and hFVIIa molecules, providing one or more of the aforementioned desired benefits. Thus, the conjugate of the present invention has one or more improved properties as compared to commercially available rFVIIa, including increased functional in vivo half-life and/or increased plasma half-life, and/or increased bioavailability and/or reduced sensitivity to proteolytic degradation. Consequently, medical treatment with a conjugate of the invention offers a number of advantages over the currently available rFVIIa compound, such as longer duration between injections.

[0020] Accordingly, in a first aspect, the invention relates to a conjugate comprising at least one non-polypeptide moiety covalently attached to a polypeptide, wherein the amino acid sequence of the polypeptide differs from that of wild-type FVII or FVIIa shown in SEQ ID NO:1 in that at least one amino acid residue comprising an attachment group for said non-polypeptide moiety has been introduced or removed.

[0021] In another aspect, the invention relates to a polypeptide, wherein the amino acid sequence of the polypeptide differs from that of wild-type FVII or hFVIIa shown in SEQ ID NO:1 in that at least one amino acid residue comprising an attachment group for a non-polypeptide moiety has been introduced or removed. Such novel FVII polypeptides are contemplated to be useful as such for therapeutic, diagnostic or other purposes, but find particular interest as intermediate products for the preparation of a conjugate of the invention.

[0022] In further aspects, the invention relates to: a nucleotide sequence encoding the polypeptide of the invention or the polypeptide part of the conjugate of the invention; an expression vector harbouring the nucleotide sequence of the invention; a host cell comprising the nucleotide sequence of the invention or the expression vector of the invention.

[0023] In still further aspects, the invention relates to pharmaceutical compositions comprising the conjugate of the invention as well as to methods for preparing and using such conjugates.

DETAILED DISCUSSION

Definitions

[0024] In the context of the present application and invention the following definitions apply:

[0025] The term "conjugate" (or interchangeably "conjugated polypeptide") is intended to indicate a heterogeneous (in the sense of composite or chimeric) molecule formed by the covalent attachment of one or more polypeptide(s) to one or more non-polypeptide moieties such as polymer molecules, lipophilic compounds, sugar moieties or organic derivatizing agents. Preferably, the conjugate is soluble at relevant concentrations and conditions, i.e., soluble in physiological fluids such as blood. Examples of conjugated polypeptides of the invention include glycosylated and/or PEGylated polypeptides.

[0026] The term "covalent attachment" means that the polypeptide and the non-polypeptide moiety are either directly covalently joined to one another, or else are indirectly covalently joined to one another through an intervening moiety or moieties, such as a bridge, spacer, or linkage moiety or moieties.

[0027] The term "non-conjugated polypeptide" can be used about the polypeptide part of the conjugate.

[0028] When used herein, the term "non-polypeptide moiety" means a molecule that is capable of conjugating to an attachment group of the polypeptide of the invention. Preferred examples of such molecules include polymer molecules, sugar moieties, lipophilic compounds, or organic derivatizing agents. When used in the context of a conjugate of the invention it will be understood that the non-polypeptide moiety is linked to the polypeptide part of the conjugate through an attachment group of the polypeptide. As explained above, the non-polypeptide moiety can be directly covalently joined to the attachment group or it can be indirectly covalently joined to the attachment group through an intervening moiety or moieties, such as a bridge, spacer, or linkage moiety or moieties.

[0029] The "polymer molecule" is a molecule formed by covalent linkage of two or more monomers, wherein none of the monomers is an amino acid residue, except where the polymer is human albumin or another abundant plasma protein. The term "polymer" can be used interchangeably with the term "polymer molecule." The term is intended to cover carbohydrate molecules attached by in vitro glycosylation, i.e., a synthetic glycosylation performed in vitro normally involving covalently linking a carbohydrate molecule to an attachment group of the polypeptide, optionally using a cross-linking agent.

[0030] Carbohydrate molecules attached by in vivo glycosylation, such as N- or O-glycosylation (as further described below) are referred to herein as a "sugar moiety."

Except where the number of non-polypeptide moieties, such as polymer molecule(s) or sugar moieties in the conjugate is expressly indicated every reference to "a non-polypeptide moiety" contained in a conjugate or otherwise used in the present invention shall be a reference to one or more non-polypeptide moieties, such as polymer molecule(s) or sugar moieties.

[0031] The term "attachment group" is intended to indicate a functional group of the polypeptide, in particular of an amino acid residue thereof or a carbohydrate moiety, capable of attaching a non-polypeptide moiety such as a polymer molecule, a lipophilic molecule, a sugar moiety or an organic derivatizing agent. Useful attachment groups and their matching non-polypeptide moieties are apparent from the table below.

[0032] For in vivo N-glycosylation, the term "attachment group" is used in an unconventional way to indicate the amino acid residues constituting a N-glycosylation site (with the sequence N-X-S/T/C, wherein X is any amino acid residue except proline, N is asparagine and S/T/C is either serine, threonine or cysteine, preferably serine or threonine, and most preferably threonine). Although the asparagine residue of the N-glycosylation site is the one to which the sugar moiety is attached during glycosylation, such attachment cannot be achieved unless the other amino acid residues of the N-glycosylation site is present.

[0033] Accordingly, when the non-polypeptide moiety is a sugar moiety and the conjugation is to be achieved by N-glycosylation, the term "amino acid residue comprising an attachment group for the non-polypeptide moiety" as

Attachment group	Amino acid	Examples of non-polypeptide moiety	Conjugation method/-Activated PEG	Reference
—NH_2	N-terminal, Lys	Polymer, e.g., PEG, with amide or imine group	mPEG-SPA Tresylated mPEG	Shearwater Inc. Delgado et al, critical reviews in Therapeutic Drug Carrier Systems 9(3, 4): 249–304 (1992)
—COOH	C-terminal, Asp, Glu	Polymer, e.g., PEG, with ester or amide group Carbohydrate moiety	mPEG-Hz In vitro coupling	Shearwater Inc.
—SH	Cys	Polymer, e.g., PEG, with disulfide, maleimide or vinyl sulfone group Carbohydrate moiety	PEG-vinylsulphone PEG-maleimide In vitro coupling	Shearwater Inc. Delgado et al, critical reviews in Therapeutic Drug Carrier Systems 9(3, 4): 249–304 (1992)
—OH	Ser, Thr, Lys, OH—	Sugar moiety PEG with ester, ether, carbamate, carbonate	In vivo O-linked glycosylation	
—$CONH_2$	Asn as part of an N-glycosylation site	Sugar moiety Polymer, e.g., PEG	In vivo N-glycosylation	
Aromatic residue	Phe, Tyr, Trp	Carbohydrate moiety	In vitro coupling	
—$CONH_2$	Gln	Carbohydrate moiety	In vitro coupling	Yan and Wold, Biochemistry, 1984, Jul 31; 23(16): 3759–65
Aldehyde Ketone	Oxidized oligo-saccharide	Polymer, e.g., PEG, PEG-hydrazide	PEGylation	Andresz et al., 1978, Makromol. Chem. 179: 301, WO 92/16555, WO 00/23114
Guanidino	Arg	Carbohydrate moiety	In vitro coupling	Lundblad and Noyes, Chimical Reagents for Protein Modification, CRC Press Inc., Florida, USA
Imidazole ring	His	Carbohydrate moiety	In vitro coupling	As for guanidine

used in connection with alterations of the amino acid sequence of the polypeptide of interest is to be understood as meaning that one or more amino acid residues constituting an N-glycosylation site are to be altered in such a manner that either a functional N-glycosylation site is introduced into the amino acid sequence or removed from said sequence.

[0034] In the present application, amino acid names and atom names (e.g., CA, CB, CD, CG, SG, NZ, N, O, C, etc) are used as defined by the Protein DataBank (PDB) (www-.pdb.org) based on the IUPAC nomenclature (IUPAC Nomenclature and Symbolism for Amino Acids and Peptides (residue names, atom names, etc.), *Eur. J. Biochem.,* 138, 9-37 (1984) together with their corrections in *Eur. J. Biochem.,* 152, 1 (1985)).

[0035] The term "amino acid residue" is intended to indicate an amino acid residue contained in the group consisting of alanine (Ala or A), cysteine (Cys or C), aspartic acid (Asp or D), glutamic acid (Glu or E), phenylalanine (Phe or F), glycine (Gly or G), histidine (H is or H), isoleucine (Ile or I), lysine (Lys or K), leucine (Leu or L), methionine (Met or M), asparagine (Asn or N), proline (Pro or P), glutamine (Gln or Q), arginine (Arg or R), serine (Ser or S), threonine (Thr or T), valine (Val or V), tryptophan (Trp or W), and tyrosine (Tyr or Y) residues.

[0036] The terminology used for identifying amino acid positions is illustrated as follows: G124 indicates that position 124 is occupied by a glycine residue in the amino acid sequence shown in SEQ ID NO:1. G124R indicates that the glycine residue of position 124 has been substituted with an arginine residue. Alternative substitutions are indicated with a "/," e.g., K32D/E means an amino acid sequence in which lysine in position 32 is substituted with either aspartic acid or glutamic acid. Multiple substitutions are indicated with a "+," e.g., K143N+N145S/T means an amino acid sequence which comprises a substitution of the lysine residue in position 143 with an asparagine residue and a substitution of the asparagine residue in position 145 with a serine or a threonine residue. The insertion of an additional amino acid residue, such as insertion of an alanine residue after G124 is indicated by G124GA. A deletion of an amino acid residue is indicated by an asterix. For example, deletion of a glycine in position 124 is indicated by G124*. Unless otherwise indicated, the numbering of amino acid residues made herein is made relative to the amino acid sequence of wild-type FVII/FVIIa shown in SEQ ID NO:1.

[0037] The term "differs from" as used in connection with specific mutations is intended to allow for additional differences being present apart from the specified amino acid difference. For instance, in addition to the removal and/or introduction of amino acid residues comprising an attachment group for the non-polypeptide moiety the FVII or FVIIa polypeptide can comprise other substitutions that are not related to introduction and/or removal of such amino acid residues. Thus, in addition to the amino acid alterations disclosed herein aimed at removing and/or introducing attachment sites for the non-polypeptide moiety, it will be understood that the amino acid sequence of the polypeptide of the invention can, if desired, contain other alterations that need not be related to introduction or removal of attachment sites, i.e., other substitutions, insertions or deletions. These can, for example, include truncation of the N- and/or C-terminus by one or more amino acid residues, or addition of one or more extra residues at the N- and/or C-terminus, e.g., addition of a methionine residue at the N-terminus as well as "conservative amino acid substitutions," i.e., substitutions performed within groups of amino acids with similar characteristics, e.g., small amino acids, acidic amino acids, polar amino acids, basic amino acids, hydrophobic amino acids and aromatic amino acids.

[0038] Preferred substitutions in the present invention can in particular be selected from the conservative substitution groups listed in the table below.

1	Alanine (A)	Glycine (G)	Serine (S)	Threonine (T)
2	Aspartic acid (D)	Glutamic acid (E)		
3	Asparagine (N)	Glutamine (Q)		
4	Arginine (R)	Histidine (H)	Lysine (K)	
5	Isoleucine (I)	Leucine (L)	Methionine (M)	Valine (V)
6	Phenylalanine (F)	Tyrosine (Y)	Tryptophan (W)	

[0039] The terms "mutation" and "substitution" are used interchangeably herein.

[0040] The term "nucleotide sequence" is intended to indicate a consecutive stretch of two or more nucleotide molecules. The nucleotide sequence can be of genomic, cDNA, RNA, semisynthetic, synthetic origin, or any combinations thereof.

[0041] The term "polymerase chain reaction" or "PCR" generally refers to a method for amplification of a desired nucleotide sequence in vitro, as described, for example, in U.S. Pat. No. 4,683,195. In general, the PCR method involves repeated cycles of primer extension synthesis, using oligonucleotide primers capable of hybridising preferentially to a template nucleic acid.

[0042] "Cell,""host cell,""cell line" and "cell culture" are used interchangeably herein and all such terms should be understood to include progeny resulting from growth or culturing of a cell. "Transformation" and "transfection" are used interchangeably to refer to the process of introducing DNA into a cell.

[0043] "Operably linked" refers to the covalent joining of two or more nucleotide sequences, by means of enzymatic ligation or otherwise, in a configuration relative to one another such that the normal function of the sequences can be performed. For example, the nucleotide sequence encoding a presequence or secretory leader is operably linked to a nucleotide sequence coding for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide: a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the nucleotide sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, then synthetic oligonucleotide adaptors or linkers are used, in conjunction with standard recombinant DNA methods.

[0044] The term "introduce" refers to introduction of an amino acid residue comprising an attachment group for a non-polypeptide moiety, in particular by substitution of an existing amino acid residue, or alternatively by insertion of an additional amino acid residue.

[0045] The term "remove" refers to removal of an amino acid residue comprising an attachment group for a non-polypeptide moiety, in particular by substitution of the amino acid residue to be removed by another amino acid residue, or alternatively by deletion (without substitution) of the amino acid residue to be removed

[0046] The term "FVII" or "FVII polypeptide" refers to a FVII molecule provided in single chain form.

[0047] The term "FVIIa" or "FVIIa polypeptide" refers to a FVIIa molecule provided in its activated two-chain form, wherein the peptide bond between R152 and I153 of the single-chain form has been cleaved. When the amino acid sequence of SEQ ID NO;1 is used herein to describe the amino acid sequence of FVIIa it will be understood to that one of the chains comprises amino acid residues 1-152, the other chain amino acid residues 153-406.

[0048] The terms "rFVII" and "rFVIIa" refer to FVII and FVIIa molecules produced by recombinant techniques, respectively.

[0049] The terms "hFVII" and "hFVIIa" refer to wild-type human FVII and FVIIa, respectively.

[0050] The term "catalytic site" is used to mean the catalytic triad consisting of S344, D242 and H193 of the FVII polypeptide.

[0051] The term "active FVIIa,""active FVIIa polypeptide,""active FVIIa conjugate" or "active conjugate" is used to mean a FVIIa polypeptide or conjugate that possesses at least 10% of the catalytic activity of wild-type hFVIIa. Catalytic acitivity, as used herein, can suitably be determined in the assay described in the section entitled *Method of measuring the catalytic activity* or in the assay entitled *Method of measuring low levels of catalytic activity* (see the Materials and Methods section herein). Preferably, an active conjugate has at least 15%, such as at least 20%, e.g., at least 25%, more preferably at least 30%, such as at least 40%, most preferably at least 50%, e.g., at least 60% of the catalytic acitivity of wild-type hFVIIa, when tested in the assays described above.

[0052] Preferably, an active conjugate is able to bind to tissue factor and further activate plasma factor X and/or IX. Thus, in a preferred embodiment, the active FVIIa polypeptide or a conjugate thereof has a clotting activity of at least 25% as compared to wild type FVIIa, such as a clotting activity of at least 50% as compared to wild type FVIIa, e.g., a clotting activity of at least 75% as compared to wild type FVII. Thus, the clotting activity of the active FVIIa polypeptide or conjugate thereof is preferably in the range of 25-200% as compared to the wild type FVIIa. In particular, it is preferred that the FVIIa polypeptide or conjugate has a clotting activity in the range of 30-150% as compared to wild type FVIIa, such as a clotting activity in the range of 30-100% as compared to wild type FVIIa. The clotting activity can be determined by any method known in the art as further discussed in the Materials and Methods section hereinafter. It is particularly preferred, however, that the clotting activity is determined in accordance with the method described in the section entitled "*Method of measuring the clotting activity*" (see the Materials and Method section).

[0053] The term "immunogenicity" as used in connection with a given substance is intended to indicate the ability of the substance to induce a response from the immune system. The immune response can be a cell or antibody mediated response (see, e.g., Roitt: Essential Immunology (8th Edition, Blackwell) for further definition of immunogenicity). Normally, reduced antibody reactivity will be an indication of reduced immunogenicity. The reduced immunogenicity can be determined by use of any suitable method known in the art, e.g., in vivo or in vitro.

[0054] The term "inactive FVIIa,""inactive FVIIa polypeptide,""inactive FVIIa conjugate" or "inactive conjugate" is used to mean a FVIIa polypeptide or conjugate that possesses less than 10% of the catalytic activity of wild-type hFVIIa. Catalytic acitivity, as used herein, can suitably be determined in the assay described in the section entitled *Method of measuring the catalytic activity* or in the assay entitled *Method of measuring low levels of catalytic activity* (see the Materials and Methods section herein). Preferably, an inactive conjugate has less than 8%, such as less than 6%, e.g., less than 5%, more preferably less than 4%, such as less than 3%, most preferably less than 2%, e.g., less than 1% of the catalytic activity of wild-type hFVIIa, when tested in the assays described above.

[0055] Typically, an inactive conjugate has significantly reduced in vitro or in vivo clotting activity as compared to wild-type hFVIIa. The inactive FVII or FVIIa polypeptide or conjugate can be capable of competing with wild-type FVII or FVIIa for binding tissue factor, thereby inhibiting clotting activity. Preferably, the inactive FVII or FVIIa polypeptide or conjugate has less than 1% clotting activity compared to wild-type hFVII or hFVIIa. More preferably the inactive FVII or FVIIa polypeptide or conjugate has less than 0.05% clotting activity compared to wild type hFVII or hFVIIa. Most preferably the inactive FVII or FVIIa polypeptide or conjugate has less than 0.01% clotting activity as compared to wild type hFVII or hFVIIa. In a similar way as described above, the clotting activity can be determined by any method known in the art as further discussed in the Materials and Method section hereinafter, but is preferably determined in accordance with the method described in the section entitled "*Method of measuring the clotting activity.*"

[0056] The term "functional in vivo half-life" is used in its normal meaning, i.e., the time at which 50% of the biological activity of the polypeptide or conjugate is still present in the body/target organ, or the time at which the activity of the polypeptide or conjugate is 50% of the initial value. As an alternative to determining functional in vivo half-life, "serum half-life" can be determined, i.e., the time at which 50% of the polypeptide or conjugate molecules circulate in the plasma or bloodstream prior to being cleared. Determination of serum half-life is often more simple than determining the functional in vivo half-life and the magnitude of serum half-life is usually a good indication of the magnitude of functional in vivo half-life. Alternatively terms to serum half-life include "plasma half-life,""circulating half-life, ""serum clearance,""plasma clearance" and "clearance half-life." The polypeptide or conjugate is cleared by the action

of one or more of the reticuloendothelial systems (RES), kidney, spleen or liver, by tissue factor, SEC receptor or other receptor mediated elimination, or by specific or unspecific proteolysis. Normally, clearance depends on size (relative to the cutoff for glomerular filtration), charge, attached carbohydrate chains, and the presence of cellular receptors for the protein. The functionality to be retained is normally selected from procoagulant, proteolytic or receptor binding activity. The functional in vivo half-life and the serum half-life can be determined by any suitable method known in the art as further discussed in the Materials Methods section below.

[0057] The term "increased" as used about the functional in vivo half-life or plasma half-life is used to indicate that the relevant half-life of the conjugate or polypeptide is statistically significantly increased relative to that of a reference molecule, such as a non-conjugated rFVIIa (e.g., NovoSeven®) as determined under comparable conditions. For instance, the relevant half-life can increased by at least about 25%, such as by at least about 50%, e.g., by at least about 100%, 150%, 200%, 250%, 300%, 500% or 1000%.

[0058] The term "renal clearance" is used in its normal meaning to indicate any clearance taking place by the kidneys, e.g., by glomerular filtration, tubular excretion or degradation in the tubular cells. Renal clearance depends on physical characteristics of the conjugate, including size (diameter), hydrodynamic volume, symmetry, shape/rigidity, and charge. Normally, a molecular weight of about 67 kDa is considered to be a cut-off-value for renal clearance. Renal clearance can be established by any suitable assay, e.g., an established in vivo assay. Typically, renal clearance is determined by administering a labelled (e.g., radiolabelled or fluorescence labelled) polypeptide conjugate to a patient and measuring the label activity in urine collected from the patient. Reduced renal clearance is determined relative to a corresponding reference polypeptide, e.g., the corresponding non-conjugated polypeptide, a non-conjugated corresponding wild-type polypeptide or another conjugated polypeptide (such as a conjugated polypeptide not according to the invention), under comparable conditions. Preferably, the renal clearance rate of the conjugate is reduced by at least 50%, preferably by at least 75%, and most preferably by at least 90% compared to a relevant reference polypeptide.

[0059] The ability of the conjugates of the invention to exhibit a reduced sensitivity to proteolytic degradation is of utmost importance; Compositions comprising degradation products will typically have less specific activity as compared to compositions in which none or only a minor part of the conjugate has been degraded. Furthermore, a content of non-physiological degradation products in the composition to be administered can trigger the immune system of the patient.

[0060] The term "reduced sensitivity to proteolytic degradation" is primarily intended to mean that the conjugate has reduced sensitivity to proteolytic degradation in comparison to non-conjugated wild type FVIIa as determined under comparable conditions. Preferably, the proteolytic degradation is reduced by at least 10%, such as at least 25% (e.g., by 10-25%), more preferably by at least 35%, such as at least 50%, (e.g., by 10-50%, such as 25-50%) even more preferably by at least 60%, such as by at least 75% or even at least 90%. Most preferably, the proteolytic degradation is reduced by 100%. Thus, preferably the conjugate of the invention is subjected to proteolytic degradation to a lesser extent than wild-type FVIIa, i.e., compared to non-conjugated wild type FVIIa the proteolytic degradation of the conjugate of the invention is preferably reduced by 10-100%, such as by 25-100%, more preferably by 50-100%, and most preferably by 75-100%.

[0061] The present inventors have developed a suitable preliminary in vitro test, which can be employed in the assessment of whether such conjugates possess reduced sensitivity to proteolytic cleavage (reduced autoproteolysis). Thus, in a preferred embodiment, of the invention, the conjugate of the invention has a reduced sensitivity to proteolytic degradation (as defined above) as compared to wild type FVIIa when determined by the method described in the section entitled "*Measurement of reduced sensitivity to proteolytic degradation*," when determined by the method described in the section entitled *Method of measuring the catalytic activity* or when determined by the method described in the section entitled *Method of measuring low levels of catalytic activity* (see the Materials and Methods section herein).

[0062] The term "parent FVII" or "parent polypeptide" is intended to indicate the molecule to be modified in accordance with the present invention. A typical parent FVII is the hFVII or hFVIIa (including rFVIIa (NovoSeven®)) with the amino acid sequence shown in SEQ ID NO:1.

[0063] A "variant" is a polypeptide, which differs in one or more amino acid residues from a parent polypeptide, normally in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acid residues.

[0064] Conjugate of the Invention

[0065] The conjugates of the invention are the result of a generally new strategy for developing improved FVII or FVIIa molecules. More specifically, by removing and/or introducing an amino acid residue comprising an attachment group for the non-polypeptide moiety it is possible to specifically adapt the polypeptide so as to make the molecule more susceptible to conjugation to the non-polypeptide moiety of choice, to optimize the conjugation pattern (e.g., to ensure an optimal distribution and number of non-polypeptide moieties on the surface of the FVII or FVIIa molecule and to ensure that only the attachment groups intended to be conjugated is present in the molecule) and thereby obtain a new conjugate molecule, which has or has not FVII activity and in addition one or more improved properties as compared to FVII and FVIIa molecules available today. For instance, when the total number of amino acid residues comprising an attachment group for the non-polypeptide of choice is increased or decreased to an optimized level, the renal clearance of the conjugate is typically significantly reduced due to the altered shape, size and/or charge of the molecule achieved by the conjugation.

[0066] In preferred embodiments of the present invention more than one amino acid residue of the FVII or FVIIa polypeptide is altered, e.g., the alteration embraces removal as well as introduction of amino acid residues comprising an attachment group for the non-polypeptide moiety of choice. In addition to the removal and/or introduction of amino acid residues the polypeptide can comprise other substitutions or

glycosylations that are not related to introduction and/or removal of amino acid residues comprising an attachment group for the non-polypeptide moiety. Also, the polypeptide can be attached, e.g., to a serine proteinase inhibitor to inhibit the catalytic site of the polypeptide.

[0067] The amino acid residue comprising an attachment group for a non-polypeptide moiety, either it be removed or introduced, is selected on the basis of the nature of the non-polypeptide moiety of choice and, in most instances, on the basis of the method in which conjugation between the polypeptide and the non-polypeptide moiety is to be achieved. For instance, when the non-polypeptide moiety is a polymer molecule such as a polyethylene glycol or polyalkylene oxide derived molecule amino acid residues comprising an attachment group can be selected from the group consisting of lysine, cysteine, aspartic acid, glutamic acid, histidine, and tyrosine, preferably cysteine and lysine, in particular lysine.

[0068] Whenever an attachment group for a non-polypeptide moiety is to be introduced into or removed from the FVII or FVIIa polypeptide in accordance with the present invention, the position of the polypeptide to be modified is preferably located at the surface of the poly-peptide, and more preferably occupied by an amino acid residue which has more than 25% of its side chain exposed to the solvent, preferably more than 50% of its side chain exposed to the solvent. Such positions have been identified on the basis of an analysis of a 3D structure of the human FVII or FVIIa molecule as described in the Materials and Methods section herein. Furthermore, the position is preferably selected from a part of the FVII molecule that is located outside a tissue factor binding site region and/or an active site region. These regions are identified in the Materials and Methods section hereinafter. It should be emphasized, however, that in certain situations, e.g., in case an inactivated conjugate is desired, it can be advantageous to perform modifications in or close to such regions. For example, it is contemplated that one or more attachment groups for the non-polypeptide moieties, such as attachment groups for in vivo N-glycosylation sites, can advantageously be inserted in the active site region or at the ridge of the active site binding cleft of the FVII molecule. The active site region and the ridge of the active site binding cleft are defined in the Materials and Methods section herein and is constituted by the following residues:

[0069] I153, Q167, V168, L169, L170, L171, Q176, L177, C178, G179, G180, T181, V188, V189, S190, A191, A192, H193, C194, F195, D196, K197, I198, W201, V228, I229, I230, P231, S232, T233, Y234, V235, P236, G237, T238, T239, N240, H241, D242, I243, A244, L245, L246, V281, S282, G283, W284, G285, Q286, T293, T324, E325, Y326, M327, F328, D338, S339, C340, K341, G342, D343, S344, G345, G346, P347, H348, L358, T359, G360, I361, V362, S363, W364, G365, C368, V376, Y377, T378, R379, V380, Q382, Y383, W386, L387, L400 and F405 (active site region); and N173, A175, K199, N200, N203, D289, R290, G291, A292, P321 and T370 (the ridge of the active site binding cleft).

[0070] In order to determine an optimal distribution of attachment groups, the distance between amino acid residues located at the surface of the FVII or FVUa molecule is calculated on the basis of a 3D structure of the polypeptide. More specifically, the distance between the CB's of the amino acid residues comprising such attachment groups, or the distance between the functional group (NZ for lysine, CG for aspartic acid, CD for glutamic acid, SG for cysteine) of one and the CB of another amino acid residue comprising an attachment group are determined. In case of glycine, CA is used instead of CB. In the FVII or FVIIa polypeptide part of a conjugate of the invention, any of said distances is preferably more than 8 Å, in particular more than 10 Å in order to avoid or reduce heterogeneous conjugation.

[0071] In case of removal of an attachment group, the relevant amino acid residue comprising such group and occupying a position as defined above is preferably substituted with a different amino acid residue that does not comprise an attachment group for the non-polypeptide moiety in question. Normally, the amino acid residue to be removed is one to which conjugation is disadvantageous, e.g., an amino acid residue located at or near a functional site of the polypeptide (since conjugation at such a site can result in inactivation or reduced FVII or FVIIa activity of the resulting conjugate due to impaired receptor recognition). In the present context the term "functional site" is intended to indicate one or more amino acid residues which is/are essential for or otherwise involved in the function or performance of FVII or FVIIa. Such amino acid residues are a part of the functional site. The functional site can be determined by methods known in the art and is preferably identified by analysis of a structure of the FVIIa-tissue factor complex (See Banner et al., Nature 1996; 380:41-46).

[0072] In case of introduction of an attachment group, an amino acid residue comprising such group is introduced into the position, preferably by substitution of the amino acid residue occupying such position.

[0073] The exact number of attachment groups present and available for conjugation in the FVII or FVIIa polypeptide is dependent on the effect desired to be achieved by the conjugation. The effect to be obtained is, e.g., dependent on the nature and degree of conjugation (e.g., the identity of the non-polypeptide moiety, the number of non-polypeptide moieties desirable or possible to conjugate to the polypeptide, where they should be conjugated or where conjugation should be avoided, etc.).

[0074] Functional in vivo half-life is i.a. dependent on the molecular weight of the conjugate, and the number of attachment groups needed for providing increased half-life thus depends on the molecular weight of the non-polypeptide moiety in question. In one embodiment, the conjugate of the invention has a molecular weight of at least 67 kDa, in particular at least 70 kDa, e.g., as measured by SDS-PAGE according to Laemmli, U.K., Nature Vol 227 (1970), p 680-85. FVII has a molecular weight of about 53 kDa, and therefore additional 10-20 kDa is required to obtain the desired effect. This can, e.g., be provided by conjugating 2-4 10 kDa PEG molecules or as otherwise described herein.

[0075] In order to avoid too much disruption of the structure and function of the parent molecule the polypeptide part of the conjugate will typically have an amino acid sequence having more than 90% identity with SEQ ID NO:1, preferably more than 95%, such as more than 96%. In particular, the polypeptide part of the conjugate will typically have an amino acid sequence having more than 97% identity with SEQ ID NO:1, such as more than 98%, more than 99%, more than 99.25%, more than 99.25% or more than 99.5%.

[0076] Amino acid sequence homology/identity is conveniently determined from aligned sequences, using e.g., the ClustalW program, version 1.8, June 1999, using default parameters (Thompson et al., 1994, ClustalW: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, Nucleic Acids Research, 22: 4673-4680) or from the PFAM families database version 4.0 (http://pfam.wustl.edu/) (*Nucleic Acids Res.* 1999 Jan. 1; 27(1):260-2) by use of GENEDOC version 2.5 (Nicholas, K. B., Nicholas H. B. Jr., and Deerfield, D. W. II. 1997 GeneDoc: Analysis and Visualization of Genetic Variation, EMBNEW.NEWS 4:14; Nicholas, K. B. and Nicholas H. B. Jr. 1997 GeneDoc: Analysis and Visualization of Genetic Variation).

[0077] Stated differently, the total number of amino acid residues to be altered in accordance with the present invention (as compared to the amino acid sequence shown in SEQ ID NO:1) will typically not exceed 15. Preferably, the FVII or FVIIa polypeptide part of the conjugate of the invention or the polypeptide of the invention comprises an amino acid sequence differing in 1-15 amino acid residues from the amino acid sequence shown in SEQ ID NO:1, typically in 1-10 or in 2-10 amino acid residues, e.g., in 1-8 or in 2-8 amino acid residues, such as in 3-7 or in 4-6 amino acid residues from the amino acid sequence shown in SEQ ID NO:1. Thus, normally the polypeptide part of the conjugate or the polypeptide of the invention comprises an amino acid sequence which differs from the amino acid sequence shown in SEQ ID NO:1 in at the most 15 amino acid residues (such as 15 amino acid residues), in at the most 14 amino acid residues (such as 14 amino acid residues), in at the most 13 amino acid residues (e.g., 13 amino acid residues), in at the most 12 amino acid residues (such as 12 amino acid residues), in at the most 11 amino acid residues (such as 11 amino acid residues), in at the most 10 amino acid residues (e.g., 10 amino acid residues), in at the most 9 amino acid residues (such as 9 amino acid residues), in at the most 8 amino acid residues (such as 8 amino acid residues), in at the most 7 amino acid residues (such as 7 amino acid residues), in at the most 6 amino acid residues (such as 6 amino acid residues), in at the most 5 amino acid residues (such as 5 amino acid residues), in at the most 4 amino acid residues (such as 4 amino acid residues), in at the most 3 amino acid residues (such as 3 amino acid residues) or in at the most 2 amino acid residues (such as 2 amino acid residues).

[0078] Analogously, the conjugate of the invention typically contains, e.g., 1-15 non-polypeptide moieties, typically 1-10 non-polypeptide moieties or 2-10 non-polypeptide moieties, such as 1-8 or 2-8 non-polypeptide moieties, e.g., 1-6, 1-4, 3-7 or 4-6 non-polypeptide moieties.

[0079] Preferably, the conjugate of the invention has one or more of the following improved properties: Increased functional in vivo half-life, increased plasma half-life, reduced renal clearance and reduced sensitivity to proteolytic degradation as compared to rFVIIa (e.g. NovoSeven®).

[0080] It is known from, inter alia, WO 88/10295 that proteolytic degradation of FVII/FVIIa primarily takes place at various proteolytic sites in the molecule, namely at the positions K32, K38, I42, Y44, K143, R290, R315, K341, R392, R396 and R402 (or rather between the positions K32-D33, K38-L39, I42-S43, Y44-S45, K143-R144, R290-G291, R315-K316, K341-G342, R392-S393, R396-P397 and R402-A403). Thus, one preferred strategy for increasing e.g., the functional in vivo half-life, the increased plasma half-life or for reducing the sensitivity to proteolytic degradation is to modify the parent polypeptide at and/or around one or more of these proteolytic degradation sites, e.g., by introducing non-polypeptide moieties at and/or around these sites. Thus, in a preferred embodiment, of the invention, an attachment group has been introduced in one or more of the above-mentioned identified positions or at position −4, −3, −2, −1, 1, 2, 3, 4, preferably position −2, −1, 1, 2, such as position −1, 1, relative to the position directly involved in proteolytic degradation.

[0081] More specifically, it is preferred that a non-naturally occurring in vivo glycosylation site, in particular a non-naturally occurring in vivo N-glycosylation site (see further below) has been introduced in positions selected from the group consisting of 28-48, 139-147, 286-294, 311-319, 338-345, and 388-406. In particular, it is preferred that the in vivo glycosylation site, such as an in vivo N-glycosylation site (see further below) has been introduced in positions selected from the group consisting of 30-34, 36-46, 141-144, 288-292, 313-317, 341-343, 390-398 and 400-404. More preferably, the in vivo glycosylation site, such as an in vivo N-glycosylation site (see further below) has been introduced in positions selected from the group consisting of 31-33 (e.g., positions 31, 32 or 33), 37-39 (e.g., positions 37, 38 or 39), 41-46 (e.g., positions 41, 42, 43, 44, 45 or 46), 142-144 (e.g., positions 142, 143 or 144), 289-291 (e.g., positions 289, 290 or 291), 314-316, (e.g., positions 314, 315 or 316), 341-342 (e.g., positions 341, 342 or 343), 391-393 (e.g., positions 391, 392 or 393), 395-397 (e.g., positions 395, 396 or 397) and 401-403 (e.g., positions 401, 402 or 403). The introduction is preferably performed by substitution.

[0082] Conjugate of the Invention Wherein the Non-Polypeptide Moiety is a Molecule that has Lysine as an Attachment Group

[0083] In another embodiment of the invention, the non-polypeptide moiety has lysine as an attachment group, i.e., the conjugate of the invention is one which comprises at least one non-polypeptide moiety covalently attached to a polypeptide, wherein the amino acid sequence of the polypeptide differs from that of wild-type FVII or FVIIa shown in SEQ ID NO:1 in that at least one lysine residue has been introduced or removed.

[0084] FVII/FVIIa contains 17 lysine residues. Three lysine residues (K18, K62 and K85) are located in the tissue factor binding domain and two lysine residues (K197 and K341) are located in the active site region.

[0085] Due to the relative high amount of lysine residues in the parent polypeptide it is envisaged that at least one lysine residue should preferably be removed, in particular by substitution of a lysine residue with a non-lysine residue, in order to avoid excessive conjugation to non-polypeptide moieties.

[0086] Thus, in one embodiment, the amino acid sequence of the FVII or FVIIa polypeptide part of the conjugate differs from that shown in SEQ ID NO:1 in that at least one lysine residue, such as 1-15 lysine residues, in particular 1-10, 1-6

or 2-4 lysine residues, has been removed, preferably by substitution. For example, the lysine residue(s) to be removed, preferably by substitution, is selected from the group consisting of K18, K32, K38, K62, K85, K109, K137, K143, K148, K157, K161, K197, K199, K316, K337, K341, K389 and combinations thereof. In particular, it is preferred to remove one or more lysine residues, which constitute part of tissue factor binding site and/or the active site region, i.e., the residues K18, K62, K85, K197, K341 or combinations thereof. The lysine residue can be substituted with any other amino acid residue, but is preferably substituted with R, Q, N or H, more preferably R.

[0087] In another embodiment, the amino acid sequence of the FVII or FVIIa polypeptide part of the conjugate differs from that shown in SEQ ID NO:1 in that at least one lysine residue, such as 1-15 lysine residues, in particular 1-10, 1-6 or 2-4 lysine residues, has been introduced, preferably by substitution. It will be understood that it is particularly preferred that at least one of the lysines residues, which are introduced at a predetermined site in the parent molecule, is combined with at least one of the above-mentioned removals of lysine residues. Thus, in a preferred embodiment of the invention, the amino acid sequence of the FVII or FVIIa polypeptide part of the conjugate differs from that shown in SEQ ID NO:1 in that at least one lysine residue has been removed and at least one lysine residue has been introduced.

[0088] Examples of positions, wherein lysine residues can be introduced include, but is not limited to, positions at or in the vicinity of the proteolytic degradation sites described above. Thus, in a preferred embodiment, the substitution of a non-lysine residue with a lysine residue is selected from the group consisting of I42K, Y44K, L288K, D289K, R290K, G291K, A292K, T293K, Q313K, S314K, R315K, V317K, L390K, M391K, R392K, S393K, E394K, P395K, R396K, P397K, G398K, V399K, L400K, L401K, R402K, A403K, P404K, F405K and combinations thereof, in particular selected from the group consisting of R290K, R315K, R392K, R396K, R402K and combinations thereof.

[0089] While the non-polypeptide moiety of the conjugate according to this aspect of the invention can be any molecule which, when using the given conjugation method has lysine as an attachment group it is preferred that the non-polypeptide moiety is a polymer molecule. The polymer molecule can be any of the molecules mentioned in the section entitled "Conjugation to a polymer molecule," but is preferably selected from the group consisting of linear or branched polyethylene glycol or another polyalkylene oxide. Examples of preferred polymer molecules are, e.g., SS-PEG, NPC-PEG, aldehyde-PEG, mPEG-SPA, mPEG-SCM, mPEG-BTC from Shearwater Polymers, Inc, SC-PEG from Enzon, Inc., tresylated mPEG as described in U.S. Pat. No. 5,880,255, or oxycarbonyl-oxy-N-dicarboxylmide-PEG (U.S. Pat. No. 5,122,614).

[0090] Normally, for conjugation to a lysine residue the non-polypeptide moiety has a molecular weight of from about 5 to about 20 kDa, such as from about 5 to about 10 kDa, e.g., about 5 kDa or about 10 kDa

[0091] It will be understood that any of the amino acid changes, in particular substitutions, specified in this section can be combined with any of the amino acid changes, preferably substitutions specified in the other sections herein disclosing specific amino acid modifications, including introduction and/or removal of glycosylation sites.

[0092] Conjugate of the Invention Wherein the Non-Polypeptide Moiety is a Molecule that has Cysteine as an Attachment Group

[0093] In a further embodiment of the invention, the non-polypeptide moiety has cysteine as an attachment group, i.e., the conjugate of the invention is one which comprises at least one non-polypeptide moiety covalently attached to a polypeptide, wherein the amino acid sequence of the polypeptide differs from that of wild-type FVII or FVIIa shown in SEQ ID NO:1 in that at least one cysteine residue has been introduced or removed.

[0094] FVII/FVIIa contains 24 cysteine residues and disulfide bridges are established between the following cysteine residues: C17 and C22, C50 and C61, C55 and C70, C72 and C81, C91 and C102, C98 and C112, C114 and C127, C135 and C262, C159 and C164, C178 and C194, C310 and C329, and between C340 and C368.

[0095] In a further interesting embodiment, the amino acid sequence of the FVII or FVIIa polypeptide differs from that shown in SEQ ID NO:1 in that at least one cysteine residue, such as 1-15 cysteine residues, in particular 1-10, 1-6 or 2-4 cysteine residues, has been introduced, preferably by substitution.

[0096] Examples of positions, wherein cysteine residues can be introduced include, but is not limited to, positions at or in the vicinity of the proteolytic degradation sites described above.

[0097] Thus, in an interesting embodiment of the invention, the lysine residue(s) to be introduced, preferably by substitution, is selected from the group consisting of I30C, K32C, D33C, A34C, T37C, K38C, W41C, Y44C, S45C, D46C, L141C, E142C, K143C, R144C, L288C, D289C, R290C, G291C, A292C, S314C, R315C, K316C, V317C, L390C, M391C, R392C, S393C, E394C, P395C, R396C, P397C, G398C, V399C, L401C, R402C, A403C, P404C and combinations thereof, in particular selected from the group consisting of K32C, Y44C, K143C, R290C, R315C, K341C, R392C, R396C, R402C and combinations thereof.

[0098] In a further embodiment of the invention, the cysteine residue(s) is/are introduced into a position that in wildtype hFVII is occupied by a threonine or serine residue having at least 25% of its side chain exposed to the surface. For instance, in the FVII or FVIIa polypeptide a cysteine residue is introduced, preferably by substitution, into at least one position selected from the group consisting of S12, S23, S43, S45, S52, S53, S60, S67, T83, S103, T106, T108, S111, S119, S126, T128, T130, S147, T185, S214, S222, S232, T233, T238, T239, T255, T267, T293, T307, S320, T324, S333, S336, T370 and S393. Even more preferably the cysteine residue is introduced into at least one position of hFVII containing an S residue, the position being selected from the group consisting of S12, S23, S43, S45, S52, S53, S60, S67, S103, S111, S119, S126, S147, S214, S222, S232, S320, S333, S336 and S393.

[0099] In a further embodiment, the cysteine residue(s) is/are introduced into a position that in wildtype hFVII is occupied by a threonine or serine residue having at least 50% of its side chain exposed to the surface. For instance, in the FVII or FVIIa polypeptide a cysteine residue is introduced, preferably by substitution, into at least one position selected from the group consisting of S23, S43,

S52, S53, S60, S67, T106, T108, S111, S119, S147, S214, T238, T267 and T293, even more preferably a position selected from the group consisting of S23, S43, S52, S53, S60, S67, S111, S119, S147 and S214.

[0100] In a still further embodiment, a cysteine residue is introduced into at least one position selected from any of the above-mentioned positions, which is not located in an active site region. Preferably, the position is one occupied by a T or an S residue. As an example, the FVII polypeptide comprises a cysteine residue introduced into at least one position selected from the group consisting of S12, S23, S43, S45, S52, S53, S60, S67, T83, S103, T106, T108, S111, S119, S126, T128, T130, S147, T185, S214, S222, T255, T267, T307, S320, S333, S336, T370 and S393 (having more than 25% of its side chain exposed to the surface), in particular selected from the group consisting of S12, S23, S43, S45, S52, S53, S60, S67, S103, S111, S119, S126, S147, S214, S222, S320, S333, S336 and S393 (occupied by S residue), and more preferably from the group consisting of S23, S43, S52, S53, S60, S67, T106, T108, S111, S119, S147, S214 and T267 (having more than 50% of its side chain exposed to the surface), in particular from the group consisting of S23, S43, S52, S53, S60, S67, S111, S119, S147 and S214 (occupied by an S residue).

[0101] In an even further embodiment, a cysteine residue is introduced into at least one position selected from any of the above lists, which is not located in a tissue factor binding site region. Preferably, the position is one occupied by a T or an S residue. As an example, the FVII polypeptide comprises a cysteine residue introduced into at least one position selected from the group consisting of S12, S23, S45, S52, S53, S67, T83, S103, T106, T108, S111, S119, S126, T128, T130, S147, T185, S214, S222, S232, T233, T238, T239, T255, T267, T293, S320, T324, S333, S336, T370 and S393 (having more than 25% of its side chain exposed to the surface), in particular selected from the group consisting of S12, S23, S45, S52, S53, S67, S103, S111, S119, S126, S147, S214, S222, S232, S320, S333, S336 and S393 (occupied by S residue), and more preferably from the group consisting of S23, S52, S53, S67, T106, T108, S111, S119, S147, S214, T238, T267 and T293 (having more than 50% of its side chain exposed to the surface), in particular from the group consisting of S23, S52, S53, S67, S111, S119, S147 and S214 (occupied by an S residue).

[0102] In a still further embodiment, a cysteine residue is introduced into at least one position selected from any of the above lists, which is neither located in a tissue factor binding site region nor in an active site region. Preferably, the position is one occupied by a T or an S residue. As an example, the FVII polypeptide comprises a cysteine residue introduced into at least one position selected from the group consisting of S12, S23, S45, S52, S53, S67, T83, S103, T106, T108, S111, S119, S126, T128, T130, S147, T185, S214, S222, T255, T267, S320, S333, S336, T370 and S393 (having more than 25% of its side chain exposed to the surface), in particular selected from the group consisting of S12, S23, S45, S52, S53, S67, S103, S111, S119, S126, S147, S214, S222, S320, S333, S336 and S393 (occupied by S residue), and more preferably from the group consisting of S23, S52, S53, S67, T106, T108, S111, S119, S147, S214 and T267 (having more than 50% of its side chain exposed to the surface), in particular from the group consisting of S23, S52, S53, S67, S111, S119, S147 and S214 (occupied by an S residue).

[0103] While the non-polypeptide moiety of the conjugate according to this aspect of the invention can be any molecule which, when using the given conjugation method has cysteine as an attachment group it is preferred that the non-polypeptide moiety is a polymer molecule. The polymer molecule can be any of the molecules mentioned in the section entitled "Conjugation to a polymer molecule," but is preferably selected from the group consisting of linear or branched polyethylene glycol or another polyalkylene oxide. In another embodiment, the polymer molecule is PEG, such as VS-PEG. The conjugation between the polypeptide and the polymer can be achieved in any suitable manner, e.g., as described in the section entitled "Conjugation to a polymer molecule," e.g., in using a one step method or in the stepwise manner referred to in said section. When the FVII or FVIIa polypeptide comprises only one conjugatable cysteine residue, this is preferably conjugated to a non-polypeptide moiety with a molecular weight of from about 5 kDa to about 20 kDa, e.g., from about 10 kDa to about 20 kDa, such as a molecular weight of about 5 kDa, about 10 kDa, about 12 kDa, about 15 kDa or about 20 kDa, either directly conjugated or indirectly through a low molecular weight polymer (as disclosed in WO 99/55377). When the conjugate comprises two or more conjugatable cysteine residue, normally each of the non-polypeptide moieties has a molecular weight of from about 5 to about 10 kDa, such as about 5 kDa or about 10 kDa.

[0104] It will be understood that any of the amino acid changes, in particular substitutions, specified in this section can be combined with any of the amino acid changes, preferably substitutions specified in the other sections herein disclosing specific amino acid modifications, including introduction and/or removal of glycosylation sites.

[0105] Conjugate of the Invention Wherein the Non-Polypeptide Moiety is a Molecule that has Aspartic Acid or Glutamic Acid as an Attachment Group.

[0106] In a still further interesting embodiment of the invention, the non-polypeptide moiety has aspartic acid or glutamic acid as an attachment group, i.e., the conjugate of the invention is one which comprises at least one non-polypeptide moiety covalently attached to a polypeptide, wherein the amino acid sequence of the polypeptide differs from that of wild-type FVII or FVIIa shown in SEQ ID NO:1 in that at least one aspartic acid residue and/or at least one glutamic acid residue has been introduced or removed.

[0107] In a further interesting embodiment, the amino acid sequence of the FVII or FVIIa polypeptide differs from that shown in SEQ ID NO:1 in that at least one aspartic acid residue and/or glutamic acid residue, such as 1-15 aspartic acid residues and/or glutamic acid residues, in particular 1-10, 1-6 or 2-4 aspartic acid residues and/or glutamic acid residues, has been introduced, preferably by substitution.

[0108] Examples of positions, wherein aspartic acid residues or glutamic acid residues can be introduced include, but is not limited to, positions at or in the vicinity of the proteolytic degradation sites described above.

[0109] Thus, in an interesting embodiment of the invention, the aspartic acid residue and/or the glutamic acid

residue to be introduced, preferably by substitution, is selected from the group consisting of I30D/E, K32D/E, A34D/E, T37D/E, K38D/E, W41D/E, Y44D/E, S45D/E, D46C, L141D/E, E142D/E, K143D/E, R144D/E, L288D/E, R290D/E, G291D/E, A292D/E, Q313D/E, S314D/E, R315D/E, K316D/E, V317D/E, L390D/E, M391D/E, R392DE, S393D/E, P395DE, R396D/E, P397D/E, G398D/E, V399D/E, L401DE, R402D/E, A403D/E, P404D/E, and combinations thereof, in particular selected from the group consisting of K32D/E, Y44D/E, K143D/E, R290D/E, R315D/E, K341D/E, R392D/E, R396D/E, R402D/E and combinations thereof.

[0110] In addition to the above listed substitution(s), the polypeptide of the conjugate according to the above embodiment can comprise removal, preferably by substitution, of at least one of the aspartic acid residue and/or at least one glutamic acid residue.

[0111] Due to the relative high amount of lysine residues in the parent polypeptide it is envisaged that at least one aspartic acid or glutamic acid residue should preferably be removed, in particular by substitution, in order to avoid excessive conjugation to non-polypeptide moieties.

[0112] Thus, in one embodiment, the amino acid sequence of the FVII or FVIIa polypeptide part of the conjugate differs from that shown in SEQ ID NO:1 in that at least one aspartic acid or glutamic acid residue, such as 1-15 aspartic acid or glutamic acid residues, in particular 1-10, 1-6 or 2-4 aspartic acid or glutamic acid residues, has been removed, preferably by substitution. For example, the aspartic acid and glutamic acid residue(s) to be removed, preferably by substitution, is selected from the group consisting of D33, D46, D48, E77, E82, D86, D87, E94, E99, D104, E116, D123, E132, E142, E163, D196, E210, D212, E215, D217, D219, E220, D256, E265, E270, D289, E296, D309, D319, E325, D334, D338, D343, E385, E394 and combinations thereof.

[0113] It will be understood that it is particularly preferred that at least one of the aspartic acid or glutamic acid residues, which are introduced at a predetermined site in the parent molecule, is combined with at least one of the above-mentioned removals of aspartic acid or glutamic acid residues. Thus, in a preferred embodiment of the invention, the amino acid sequence of the FVII or FVIIa polypeptide part of the conjugate differs from that shown in SEQ ID NO:1 in that at least one aspartic acid or glutamic acid residue has been removed, preferably by substitution, and at least one aspartic acid or glutamic acid residue has been introduced, preferably by substitution.

[0114] While the non-polypeptide moiety of the conjugate according to this aspect of the invention, which has an aspartic acid group or a glutamic acid group as an attachment group, can be any non-polypeptide moiety with such property, it is presently preferred that the non-polypeptide moiety is a polymer molecule or an organic derivatizing agent, in particular a polymer molecule, and the conjugate is prepared, e.g., as described by Sakane and Pardridge, Pharmaceutical Research, Vol. 14, No. 8, 1997, pp 1085-1091.

[0115] It will be understood that any of the amino acid changes, in particular substitutions, specified in this section can be combined with any of the amino acid changes, in particular substitutions specified in the other sections herein disclosing specific amino acid changes, including introduction and/or removal of glycosylation sites.

[0116] Conjugate of the Invention Wherein the Non-Polypeptide Moiety is a Sugar Moiety

[0117] In a further interesting embodiment of the invention, an attachment group for a sugar moiety, such as a glycosylation site, in particular an in vivo glycosylation site, has been inserted and/or removed.

[0118] Preferably, the conjugate of the invention is one which comprises at least one sugar moiety covalently attached to a polypeptide, wherein the amino acid sequence of the polypeptide differs from that of wild-type FVII or FVIIa shown in SEQ ID NO:1 in that at least one non-naturally occurring glycosylation site has been introduced and/or at least one naturally occurring glycosylation site has been removed. In particular, a non-naturally occurring glycosylation site has been introduced, or a non-naturally occurring glycosylation site has been introduced in combination with the removal of a natural occurring glycosylation site. The introduced glycosylation site can be an O-glycosylation site or an N-glycosylation site. Preferably the glycosylation site is an in vivo O-glycosylation site or an in vivo N-glycosylation site, in particular an in vivo N-glycosylation site.

[0119] When used in the present context, the term "naturally occurring glycosylation site" covers the glycosylation sites at postions N145, N322, S52 and S60. In a similar way, the term "naturally occurring in vivo O-glycosylation site" includes the positions S52 and S60, whereas the term "naturally occurring in vivo N-glycosylation site" includes positions N145 and N322.

[0120] Typically, the amino acid sequence of the FVII or FVIIa polypeptide differs from that shown in SEQ ID NO:1 in that at least one non-naturally occurring glycosylation site (e.g., at least one non-naturally occurring in vivo N-glycosylation site), such as 1-15 non-naturally occurring glycosylation sites (e.g., 1-15 non-naturally occurring in vivo N-glycosylation sites), in particular 1-10, 1-6 or 2-4 non-naturally occurring glycosylation sites (e.g., 1-10, 1-6 or 2-4 non-naturally occurring in vivo N-glycosylation sites), has been introduced, preferably by substitution.

[0121] It will be understood that in order to prepare a conjugate, wherein the polypeptide of the conjugate comprises one or more glycosylation sites, the polypeptide must be expressed in a host cell capable of attaching sugar (oligosaccharide) moieties at the glycosylation site(s) or alternatively subjected to in vitro glycosylation. Examples of glycosylating host cells are given in the section further below entitled "Coupling to a sugar moiety."

[0122] In an interesting embodiment of this aspect, an in vivo glycosylation site is introduced into a position of the parent FVII or FVIIa molecule occupied by an amino acid residue exposed to the surface of the molecule, preferably with more than 25% of the side chain exposed to the solvent, in particular more than 50% exposed to the solvent (these positions are identified in the Methods section herein). The N-glycosylation site is then introduced in such a way that the N-residue of said site is located in said position. Analogously, an O-glycosylation site is introduced so that the S or T residue making up such site is located in said position. Examples of such positions include K32S/T, I42S/T, Y44S/T, K143S/T, R290S/T, R315S/T, K341S/T, R392S/T, R396S/T, R402S/T and combinations thereof.

[0123] With respect to N-glycosylation, the in vivo glycosylation site is introduced into a position wherein only one mutation is required to create the site (i.e., where any other amino acid residues required for creating a functional glycosylation site is already present in the molecule).

[0124] In other words, the conjugate according to the invention is preferably a conjugate, wherein the amino acid sequence of the polypeptide differs from SEQ ID NO:1 in that at least one naturally occurring N-X'-X sequence is substituted with a N-X'-S or N-X'-T sequence, wherein X' is any amino acid except P, and X is any amino acid except for S and T.

[0125] In a similar way, the conjugate according to the invention is preferably a conjugate, wherein the amino acid sequence of the polypeptide differs from SEQ ID NO:1 in that at least one naturally occurring X-X'-S or X-X'-T sequence naturally present in SEQ ID NO:1 is substituted with a N-X'-S or a N-X'-T sequence, wherein X' is any amino acid except P, and X is any amino acid except for N.

[0126] Specific examples of such substitutions creating an in vivo N-glycosylation site include a substitution selected from the group consisting of F4S/T, P10N, Q21N, W41N, S43N, A51N, G58N, L65N, G59S/T, E82S/T, N95S/T, G97S/T, Y101N, D104N, T106N, K109N, G117N, G124N, S126N, T128N, A175S/T, G179N, I186S/T, V188N, R202S/T, I205S/T, D212N, E220N, I230N, P231N, P236N, G237N, V253N, E265N, T267N, E270N, R277N, L280N, G291N, P303S/T, L305N, Q312N, G318N, G331N, D334N, K337N, G342N, H348N, R353N, Y357N, I361N, V376N, R379N, M391N, and combinations thereof. Preferably, the substitution is selected from the group consisting of F4S/T, P10N, Q21N, W41N, A51N, G58N, G59S/T, N95S/T, G97S/T, Y101N, D104N, T106N, K109N, G117N, G124N, S126N, T128N, A175S/T, I186S/T, V188N, R202S/T, I205S/T, D212N, E220N, V253N, E265N, T267N, E270N, L280N, G291N, P303S/T, G318N, G331N, D334N, K337N, R353N, Y357N, M391N, and combinations thereof.

[0127] Alternatively, the in vivo glycosylation site is introduced in a position occupied by a lysine residue or an arginine residue, preferably occupied by an lysine residue, in particular so that the N-residue of the N-glycosylation site or the S or T residue of an O-glycosylation site substitutes the lysine residue.

[0128] Stated differently, the conjugate according to the invention is preferably a conjugate, wherein the amino acid sequence of the polypeptide differs from SEQ ID NO:1 in that at least one K-X'-X sequence or R-X'-X sequence, preferably a K-X'-X sequence, naturally present in SEQ ID NO:1 is substituted with a N-X'-S or a N-X'-T sequence, wherein X' is any amino acid except P, and X is any amino acid except for S and T.

[0129] Specific examples of such substitutions creating an in vivo N-glycosylation site include a substitution selected from the group consisting of K32N+A34S/T, K38N+F40S/T, K62N+Q64S/T, K85N+D87S/T, K137N+P139S/T, K143N+N145S/T, K148N+Q149S/T, K161N+E163S/T, K197N+K199S/T, K199N+W201S/T, K316N+G318S/T, K337N, K341N+D343S/T, K389N+M391S/T and combinations thereof.

[0130] In a further interesting embodiment of the glycosylation aspect described above, a glycosylation site, in particular an N-glycosylation site, can be introduced in positions at or in the vicinity of the proteolytic degradation sites described above (see the section entitled "*Conjugate of the invention*").

[0131] Thus, specific examples of such substitutions creating an in vivo N-glycosylation site include a substitution selected from the group consisting of K32N+A34S/T, F31N+D33S/T, I30N+K32S/T, A34N+R36S/T, K38N+F40S/T, T37N+L39S/T, R36N+K38S/T, L39N+W41S/T, F40N+142S/T, W41N, I42N+Y44S/T, S43N, Y44N+D46S/T, S45N+G47S/T, D46N+D48S/T, G47N+Q49S/T, K143N+N145S/T, E142N+R144S/T, L141N+K143S/T, I140N+E142S/T, R144N+A146S/T, A146N+K148S/T, S147N+P149S/T, R290N+A292S/T, D289N+G291S/T, L288N+R290S/T, L287N+D289S/T, G291N, A292N+A294S/T, T293N+L295S/T, R315N+V317S/T, S314N+K316S/T, Q313N+R315S/T, Q312N, K316N+G318S/T, V317N+D319S/T, G318N, K341N+D343S/T, S339N+K341S/T, G342N, D343N+G345S/T, R392N+E394S/T, M391N, L390N+R392S/T, K389N+M391S/T, S393N+P395S/T, E394N+R396S/T, P395N+P397S/T, R396N+G398S/T, P397N+V399S/T, G398N+L400S/T, V399N+L4011S/T, L400N+R402S/T, L401N+A403S/T, R402N+P404S/T, A403N+F405S/T, P404N+P406S/T and combinations thereof, such as K143N+N145S/T+R315N+V317S/T. Preferably, the substitution is selected from the group consisting of K32N+A34S/T, K38N+F40S/T, Y44N+D46S/T, K143N+N145S/T, R290N+A292S/T, R315N+V317S/T, K341N+D343S/T, R392N+E394S/T, R396N+G398S/T, R402N+P404S/T and combinations thereof, such as K143N+N145S/T+R315N+V317S/T. More preferably, the substitution is selected from the group consisting of K32N+A34T, K38N+F40T, Y44N+D46T, K143N+N145T, R290N+A292T, R315N+V317T, 341N+D343T, R392N+E394T, R396N+G398T, R402N+P404T and combinations thereof, in particular K143N+N145T+R315N+V317T.

[0132] In a still further interesting embodiment of the invention, it is preferred that the in vivo glycosylation site is introduced in a position which does neither form part of the tissue factor binding nor form part of the active site region and the ridge of the active site binding cleft as defined herein. It is envisaged that such glycosylation variants will primarily belong to the class of active conjugates as defined hereinbefore.

[0133] Thus, specific examples of substitutions creating such an in vivo N-glycosylation site include substitutions selcted from the group consisting of K32N+A34S/T, I30N+K32S/T, A34N+R36S/T, K38N+F40S/T, T37N+L39S/T, W41N, Y44N+D46S/T, S45N+G47S/T, D46N+D48S/T, G47N+Q49S/T, K143N+N145S/T, E142N+R144S/T, L141N+K143S/T, I140N+E142S/T, R144N+A146S/T, A146N+K148S/T, S147N+P149S/T, L288N+R290S/T, L287N+D289S/T, R315N+V317S/T, S314N+K316S/T, K316N+G318S/T, V317N+D319S/T, G318N, R392N+E394S/T, M391N, L390N+R392S/T, K389N+M391S/T, S393N+P395S/T, E394N+R396S/T, P395N+P397S/T, R396N+G398S/T, P397N+V399S/T, G398N+L400S/T, V399N+L401S/T, L401N+A403S/T, R402N+P404S/T, A403N+F405S/T, P404N+P406S/T and combinations thereof, such as K143N+N145S/T+R315N+V317S/T. Preferably, the substitution is selected from the group consisting of K32N+A34S/T, K38N+F40S/T, Y44N+D46S/T, K143N+N145S/T, R315N+V317S/T, R392N+E394S/T, R396N+

G398S/T, R402N+P404S/T and combinations thereof, such as K143N+N145S/T+R315N+V317S/T. More preferably, the substitution is selected from the group consisting of K32N+A34T, K38N+F40T, Y44N+D46T, K143N+N145T, R315N+V317T, R392N+E394T, R396N+G398T, R402N+P404T and combinations thereof, in particular K143N+N145T+R315N+V317T.

[0134] In an even further interesting embodiment of the invention, it is preferred that the in vivo glycosylation site is introduced in a position which does not form part of the tissue factor but which forms part of the active site region and the ridge of the active site binding cleft as defined herein. It is envisaged that such glycosylation variants will primarily belong to the class of inactive conjugates as defined hereinbefore.

[0135] Thus, specific examples of substitutions creating such an in vivo N-glycosylation site include substitutions selected from the group consisting of I153N+G155S/T, Q167N+L169S/T, V168N+L170S/T, L169N+L171S/T, L170N+V172S/T, L171N+N173S/T, A175S/T, A175N+L177S/T, L177N+G179S/T, G179N, G180N+L182S/T, T181N+I183S/T, V188N, V189N+A191S/T, S190N+A192S/T, A191N+H193S/T, H193N+F195S/T, F195N+K197S/T, D196N+I198S/T, K197N+K199S/T, I198N+N200S/T, K199N+W201S/T, W201N+N203S/T, R202S/T, I205S/T, V228N+I230S/T, I229N+P231S/T, I230N, P231N, S232N+Y234S/T, T233N+V235S/T, Y234N+P236S/T, V235N+G237S/T, P236N, G237N, T238N+N240S/T, T239N+H241S/T, H241N+I243S/T, D242S/T, I243N+L245S/T, A244N+L246S/T, L245N+R247S/T, L246N+L246S/T, V281N+G283S/T, S282N+W284S/T, G283N+G285S/T, W284N+Q286S/T, G285N+L287S/T, Q286N+L288S/T, D289N+G291S/T, R290N+A292S/T, G291N, A292N+A294S/T, T293N+L295S/T, P321N+I323S/T, T324N+Y326S/T, E325N+M327S/T, Y326N+F327S/T, F328N+A330S/T, S339N+K341S/T, K341N+D343S/T, G342N+S344S/T, D343N+G345S/T, S344N+G346S/T, G345N+P347S/T, P347N+A349S/T, H348N, L358N+G360S/T, T359N+I361S/T, G360N+V362S/T, I361N, V362N+W364S/T, S363N+G365S/T, W364N+Q366S/T, G365N+G367S/T, T370N+G372S/T, V376N, Y377N+R379S/T, T378N+V380S/T, R379N, V380N+Q382S/T, Q382N+I384S/T, Y383N+E385S/T, W386N+Q388S/T, L387N+K389S/T, L400N+R402S/T and combinations thereof. Preferably, the substitution is selected from the group consisting of D289N+G291S/T, R290N+A292S/T, G291N, A292N+A294S/T, T293N+L295S/T, S339N+K341S/T, K341N+D343S/T, G342N+S344S/T, D343N+G345S/T, and combinations thereof. More preferably, the substitution is selcted from the group consisting of D289N+G291T, R290N+A292T, G291N, A292N+A294T, T293N+L295T, S339N+K341T, K341N+D343T, G342N+S344T, D343N+G345T, and combinations thereof.

[0136] In addition to a sugar moiety, the conjugate according to the aspect of the invention described in the present section can contain additional non-polypeptide moieties, in particular a polymer molecule, as described in the present application, conjugated to one or more attachment groups present in the polypeptide part of the conjugate.

[0137] It will be understood that any of the amino acid changes, in particular substitutions, specified in this section can be combined with any of the amino acid changes, in particular substitutions, specified in the other sections herein disclosing specific amino acid changes.

[0138] For instance, any of the glycosylated variants disclosed in the present section having introduced and/or removed at least one glycosylation site, such as a variant comprising the substitutions R315N+V317T and/or K143N+N145T, can further be conjugated to a polymer molecule, such as PEG, or any other non-polypeptide moiety. For this purpose the conjugation can be achieved by use of attachment groups already present in the FVII or FVIIa polypeptide or attachment groups can have been introduced and/or removed, in particular such that a total of 1-6, in particular 3-4 or 1, 2, 3, 4, 5, or 6 attachment groups are available for conjugation.

[0139] Preferably, in a conjugate of the invention wherein the FVII or FVIIa polypeptide comprises two glycosylation sites, the number and molecular weight of the non-polypeptide moiety is chosen so as that the total molecular weight added by the non-polypeptide moiety is in the range of 5-25 kDa, such as in the range of 10-25 kDa, in particular about 5 kDa, about 12 kDa, about 15 kDa or about 20 kDa.

[0140] An Inactive Conjugate

[0141] The conjugates of the invention can be rendered inactive by removing at least one amino acid residue occupying a position selected from the group consisting of R152, I153, S344, D242 and H193 of SEQ ID NO:1. The removal can be effected by substitution or deletion of one or more of the above-identified amino acid residues. Preferably, the removal is effected by substitution, in particular by conservative substitution. Accordingly, the inactive FVII or FVIIa polypeptide used herein can comprise one or more of the following substitutions: R152X, I153X, S344X, D242X or H193X, wherein X is any amino acid residue, preferably one leading to a conservative substitution. For instance, the inactive FVII or FVIIa polypeptide comprises the mutations R152X, wherein X is any amino acid residue other than lysine (since lysine forms part of a protease cleavage site). Other examples of specific substitutions include I153A/V/L; S344T/AIG/Y; D242E/A and/or H193R/A.

[0142] Alternatively, an active FVII or FVIIa polypeptide can be rendered inactive by carbamylating the α-amino acid group I153 or by complexing the polypeptide to a serine proteinase inhibitor. A suitable serine inhibitor protein is, e.g., selected from the group consisting of an organophosphor compound, a sulfanylfluoride, a peptide halomethylketone, preferably a Dansyl-Phe-Pro-Arg chloromethylketone, Dansyl-Glu-Glu-Arg chlormethylketone, Dansyl-Phe-Phe-Arg chlormethylketone or a Phe-Phe-Arg chlormethylketone, or an azapeptide.

[0143] A conjugate can also be rendered inactive by introducing at least one glycosylation site in a position selected so that the subsequent glycosylation inactivates the conjugate.

[0144] As explained above in the last part of the section entitled "*Conjugate of the invention wherein the non-polypeptide moiety is a sugar moiety*" it is preferred that such glycosylation sites are introduced in a position which does not form part of the tissue factor but which forms part of the active site region and the ridge of the active site binding cleft as defined herein. Specific examples of pre-

ferred substitutions are given above in the section entitled "*Conjugate of the invention wherein the non-polypeptide moiety is a sugar moiety*".

[0145] Non-Polypeptide Moiety of the Conjugate of the Invention

[0146] As indicated further above the non-polypeptide moiety of the conjugate of the invention is preferably selected from the group consisting of a polymer molecule, a lipophilic compound, a sugar moiety (by way of in vivo glycosylation) and an organic derivatizing agent. All of these agents can confer desirable properties to the polypeptide part of the conjugate, in particular increased functional in vivo half-life and/or increased plasma half-life. The polypeptide part of the conjugate is normally conjugated to only one type of non-polypeptide moiety, but can also be conjugated to two or more different types of non-polypeptide moieties, e.g., to a polymer molecule and a sugar moiety, to a lipophilic group and a sugar moiety, to an organic derivatizing agent and a sugar moiety, to a lipophilic group and a polymer molecule, etc. The conjugation to two or more different non-polypeptide moieties can be done simultaneous or sequentially.

[0147] Methods of Preparing a Conjugate of the Invention

[0148] In the following sections "Conjugation to a lipophilic compound,""Conjugation to a polymer molecule, ""Conjugation to a sugar moiety" and "Conjugation to an organic derivatizing agent" conjugation to specific types of non-polypeptide moieties is described. In general, a polypeptide conjugate according to the invention can be produced by culturing an appropriate host cell under conditions conducive for the expression of the polypeptide, and recovering the polypeptide, wherein a) the polypeptide comprises at least one N- or O-glycosylation site and the host cell is a eukaryotic host cell capable of in vivo glycosylation, and/or b) the polypeptide is subjected to conjugation to a non-polypeptide moiety in vitro.

[0149] It will be understood that the conjugation should be designed so as to produce the optimal molecule with respect to the number of non-polypeptide moieties attached, the size and form of such molecules (e.g., whether they are linear or branched), and the attachment site(s) in the polypeptide. The molecular weight of the non-polypeptide moiety to be used can e.g., be chosen on the basis of the desired effect to be achieved. For instance, if the primary purpose of the conjugation is to achieve a conjugate having a high molecular weight (e.g., to reduce renal clearance) it is usually desirable to conjugate as few high molecular weight non-polypeptide moieties as possible to obtain the desired molecular weight. When a high degree of shielding is desirable this can be obtained by use of a sufficiently high number of low molecular weight non-polypeptide moieties (e.g., with a molecular weight of from about 300 Da to about 5 kDa, such as a molecular weight of from 300 Da to 2 kDa) to effectively shield all or most protease cleavage sites or other vulnerable sites of the polypeptide.

[0150] Conjugation to a Lipophilic Compound

[0151] The polypeptide and the lipophilic compound can be conjugated to each other, either directly or by use of a linker. The lipophilic compound can be a natural compound such as a saturated or unsaturated fatty acid, a fatty acid diketone, a terpene, a prostaglandin, a vitamine, a carotenoide or steroide, or a synthetic compound such as a carbon acid, an alcohol, an amine and sulphonic acid with one or more alkyl-, aryl-, alkenyl- or other multiple unsaturated compounds. The conjugation between the polypeptide and the lipophilic compound, optionally through a linker can be done according to methods known in the art, e.g., as described by Bodanszky in Peptide Synthesis, John Wiley, New York, 1976 and in WO 96/12505.

[0152] Conjugation to a Polymer Molecule, Including Conjugation of a Polymer Molecule to the N-Terminal of the Polypeptide

[0153] The polymer molecule to be coupled to the polypeptide can be any suitable polymer molecule, such as a natural or synthetic homo-polymer or hetero-polymer, typically with a molecular weight in the range of about 300-100,000 Da, such as about 500-20,000 Da, more preferably in the range of about 500-15,000 Da, even more preferably in the range of about 2-12 kDa, such as in the range of about 3-10 kDa. When the term "about" is used herein in connection with a certain molecular weight, the word "about" indicates an approximate average molecular weight and reflects the fact that there will normally be a certain molecular weight distribution in a given polymer preparation.

[0154] Examples of homo-polymers include a polyol (i.e., poly-OH), a polyamine (i.e., poly-NH_2) and a polycarboxylic acid (i.e., poly-COOH). A hetero-polymer is a polymer comprising different coupling groups, such as a hydroxyl group and an amine group.

[0155] Examples of suitable polymer molecules include polymer molecules selected from the group consisting of polyalkylene oxide (PAO), including polyalkylene glycol (PAG), such as polyethylene glycol (PEG) and polypropylene glycol (PPG), branched PEGs, poly-vinyl alcohol (PVA), poly-carboxylate, poly-(vinylpyrolidone), polyethylene-co-maleic acid anhydride, polystyrene-co-maleic acid anhydride, dextran, including carboxymethyl-dextran, or any other biopolymer suitable for reducing immunogenicity and/or increasing functional in vivo half-life and/or serum half-life. Another example of a polymer molecule is human albumin or another abundant plasma protein. Generally, polyalkylene glycol-derived polymers are biocompatible, non-toxic, non-antigenic, non-immunogenic, have various water solubility properties, and are easily excreted from living organisms.

[0156] PEG is the preferred polymer molecule, since it has only few reactive groups capable of cross-linking compared to, e.g., polysaccharides such as dextran. In particular, mono-functional PEG, e.g., methoxypolyethylene glycol (mPEG), is of interest since its coupling chemistry is relatively simple (only one reactive group is available for conjugating with attachment groups on the polypeptide). Consequently, the risk of cross-linking is eliminated, the resulting polypeptide conjugates are more homogeneous and the reaction of the polymer molecules with the polypeptide is easier to control.

[0157] To effect covalent attachment of the polymer molecule(s) to the polypeptide, the hydroxyl end groups of the polymer molecule must be provided in activated form, i.e., with reactive functional groups (examples of which include primary amino groups, hydrazide (HZ), thiol, succinate

(SUC), succinimidyl succinate (SS), succinimidyl succinamide (SSA), succinimidyl proprionate (SPA), succinimidy carboxymethylate (SCM), benzotriazole carbonate (BTC), N-hydroxysuccinimide (NHS), aldehyde, nitrophenylcarbonate (NPC), and tresylate (TRES)). Suitable activated polymer molecules are commercially available, e.g., from Shearwater Polymers, Inc., Huntsville, Ala., USA, or from PolyMASC Pharmaceuticals plc, UK.

[0158] Alternatively, the polymer molecules can be activated by conventional methods known in the art, e.g., as disclosed in WO 90/13540. Specific examples of activated linear or branched polymer molecules for use in the present invention are described in the Shearwater Polymers, Inc. 1997 and 2000 Catalogs (Functionalized Biocompatible Polymers for Research and pharmaceuticals, Polyethylene Glycol and Derivatives, incorporated herein by reference).

[0159] Specific examples of activated PEG polymers include the following linear PEGs: NHS-PEG (e.g., SPA-PEG, SSPA-PEG, SBA-PEG, SS-PEG, SSA-PEG, SC-PEG, SG-PEG, and SCM-PEG), and NOR-PEG, BTC-PEG, EPOX-PEG, NCO-PEG, NPC-PEG, CDI-PEG, ALD-PEG, TRES-PEG, VS-PEG, IODO-PEG, and MAL-PEG, and branched PEGs such as PEG2-NHS and those disclosed in U.S. Pat. No. 5,932,462 and U.S. Pat. No. 5,643,575, both of which are incorporated herein by reference. Furthermore, the following publications, incorporated herein by reference, disclose useful polymer molecules and/or PEGylation chemistries: U.S. Pat. No. 5,824,778, U.S. Pat. No. 5,476,653, WO 97/32607, EP 229,108, EP 402,378, U.S. Pat. No. 4,902,502, U.S. Pat. No. 5,281,698, U.S. Pat. No. 5,122,614, U.S. Pat. No. 5,219,564, WO 92/16555, WO 94/04193, WO 94/14758, WO 94/17039, WO 94/18247, WO 94/28024, WO 95/00162, WO 95/11924, WO95/13090, WO 95/33490, WO 96/00080, WO 97/18832, WO 98/41562, WO 98/48837, WO 99/32134, WO 99/32139, WO 99/32140, WO 96/40791, WO 98/32466, WO 95/06058, EP 439 508, WO 97/03106, WO 96/21469, WO 95/13312, EP 921 131, U.S. Pat. No. 5,736,625, WO 98/05363, EP 809 996, U.S. Pat. No. 5,629,384, WO 96/41813, WO 96/07670, U.S. Pat. No. 5,473,034, U.S. Pat. No. 5,516,673, EP 605 963, U.S. Pat. No. 5,382,657, EP 510 356, EP 400 472, EP 183 503 and EP 154 316.

[0160] The conjugation of the polypeptide and the activated polymer molecules is conducted by use of any conventional method, e.g., as described in the following references (which also describe suitable methods for activation of polymer molecules): R. F. Taylor, (1991), "Protein immobilisation. Fundamental and applications," Marcel Dekker, N.Y.; S. S. Wong, (1992), "Chemistry of Protein Conjugation and Crosslinking," CRC Press, Florida, USA; G. T. Hermanson et al., (1993), "Immobilized Affinity Ligand Techniques," Academic Press, N.Y.). The skilled person will be aware that the activation method and/or conjugation chemistry to be used depends on the attachment group(s) of the polypeptide (examples of which are given further above), as well as the functional groups of the polymer (e.g., being amine, hydroxyl, carboxyl, aldehyde, sulfydryl, succinimidyl, maleimide, vinysulfone or haloacetate). The PEGylation can be directed towards conjugation to all available attachment groups on the polypeptide (i.e., such attachment groups that are exposed at the surface of the polypeptide) or can be directed towards one or more specific attachment groups, e.g., the N-terminal amino group as described in U.S. Pat. No. 5,985,265. Furthermore, the conjugation can be achieved in one step or in a stepwise manner (e.g., as described in WO 99/55377).

[0161] It will be understood that the PEGylation is designed so as to produce the optimal molecule with respect to the number of PEG molecules attached, the size and form of such molecules (e.g., whether they are linear or branched), and the attachment site(s) in the polypeptide. The molecular weight of the polymer to be used can e.g., be chosen on the basis of the desired effect to be achieved. For instance, if the primary purpose of the conjugation is to achieve a conjugate having a high molecular weight (e.g., to reduce renal clearance) it is usually desirable to conjugate as few high molecular weight polymer molecules as possible to obtain the desired molecular weight. When a high degree of shielding is desirable this can be obtained by use of a sufficiently high number of low molecular weight polymer molecules (e.g., with a molecular weight of from about 300 Da to about 5 kDa) to effectively shield all or most protease cleavage sites or other vulnerable sites of the polypeptide. For instance, 2-8, such as 3-6 such polymers can be used.

[0162] In connection with conjugation to only a single attachment group on the protein (e.g., the N-terminal amino group), it can be advantageous that the polymer molecule, which can be linear or branched, has a high molecular weight, preferably about 10-25 kDa, such as about 15-25 kDa, e.g., about 20 kDa.

[0163] Normally, the polymer conjugation is performed under conditions aimed at reacting as many of the available polymer attachment groups with polymer molecules. This is achieved by means of a suitable molar excess of the polymer relative to the polypeptide. Typically, the molar ratios of activated polymer molecules to polypeptide are up to about 1000-1, such as up to about 200-1, or up to about 100-1. In some cases the ration can be somewhat lower, however, such as up to about 50-1, 10-1 or 5-1 in order to obtain optimal reaction.

[0164] It is also contemplated according to the invention to couple the polymer molecules to the polypeptide through a linker. Suitable linkers are well known to the skilled person. A preferred example is cyanuric chloride (Abuchowski et al., (1977), J. Biol. Chem., 252, 3578-3581; U.S. Pat. No. 4,179,337; Shafer et al., (1986), J. Polym. Sci. Polym. Chem. Ed., 24, 375-378).

[0165] Subsequent to the conjugation, residual activated polymer molecules are blocked according to methods known in the art, e.g., by addition of primary amine to the reaction mixture, and the resulting inactivated polymer molecules are removed by a suitable method.

[0166] It will be understood that depending on the circumstances, e.g., the amino acid sequence of the polypeptide, the nature of the activated PEG compound being used and the specific PEGylation conditions, including the molar ratio of PEG to polypeptide, varying degrees of PEGylation can be obtained, with a higher degree of PEGylation generally being obtained with a higher ratio of PEG to polypeptide. The PEGylated polypeptides resulting from any given PEGylation process will, however, normally comprise a stochastic distribution of polypeptide conjugates having slightly different degrees of PEGylation.

[0167] In an interesting embodiment of the invention, the polypeptide conjugate of the invention comprises a polymer

molecule covalently attached to the A1 N-terminal of the wild type FVII or FVIIa polypeptide shown in SEQ ID NO:1, where said polymer molecule is the only polymer molecule attached to the polypeptide. Preferably, such polypeptide conjugates are ones, which comprise a single PEG molecule attached to the N-terminal of the polypeptide and no other PEG molecules. In particular, a linear or branched PEG molecule with a molecular weight of at least about 5 kDa, in particular about 10-25 kDa, such as about 15-25 kDa, e.g., about 20 kDa is preferred. The polypeptide conjugate according to this embodiment can further comprise one or more sugar moieties attached to an N-linked or O-linked glycosylation site of the polypeptide or sugar moieties attached by in vitro glycosylation.

[0168] In a further interesting embodiment of the invention the polypeptide conjugate of the invention comprises polymer molecules covalently attached to the A1 N-terminal and to the I153 N-terminal of the wild type FVIIa polypeptide shown in SEQ ID NO:1, where said polymer molecules are the only polymer molecules attached to the polypeptide. Preferably, such polypeptide conjugates are ones, which comprise a PEG molecule attached to both of the N-terminals of FVIIa and no other PEG molecules. In particular, linear or branched PEG molecules with a molecular weight of at least about 5 kDa, in particular about 10-25 kDa, such as about 15-25 kDa, e.g., about 20 kDa are preferred. The polypeptide conjugate according to this embodiment can further comprise one or more sugar moieties attached to an N-linked or O-linked glycosylation site of the polypeptide or sugar moieties attached by in vitro glycosylation.

[0169] One preferred method for selectively coupling polymer molecules, such as PEG molecules, to the N-terminal of the polypeptide is the method disclosed in U.S. Pat. No. 5,985,265. This method involves reductive alkylation (reaction of the N-terminal amino group of the polypeptide with an aldehyde-containing polypeptide, such as aldehylde-PEG, in the presence of a reducing agent, such as $NaCNBH_3$). This method exploits differential reactivity of different types of primary amino groups (lysine versus N-terminal) available for derivatization in the polypeptide, thereby achieving substantially selective derivatization of the polypeptide at the N-terminus with a carbonyl group-containing polymer molecule, such as aldehyde-PEG. The reaction is performed at a pH which allows one to take advantage of the pK_a differences between the ε-amino groups of the lysine residues and that of the α-amino group of the N-terminal residue of the polypeptide. In order to achieve this differential reactivity, the reaction is typically carried at slightly acidic conditions. Specific examples of suitable pH ranges include pH 4.5-7, such as pH 4.5-6, e.g., pH 5-6, in particular about pH 5.

[0170] In another specific embodiment, the polypeptide conjugate of the invention comprises a PEG molecule attached to each of the lysine residues in the polypeptide available for PEGylation, in particular a linear or branched PEG molecule, e.g., with a molecular weight of about 1-15 kDa, typically about 2-12 kDa, such as about 3-10 kDa, e.g., about 5 or 6 kDa.

[0171] In yet another embodiment, the polypeptide conjugate of the invention comprises a PEG molecule attached to each of the lysine residues in the polypeptide available for PEGylation, and in addition to the N-terminal amino acid residue of the polypeptide.

[0172] Covalent in vitro coupling of carbohydrate moieties (such as dextran) to amino acid residues of the polypeptide can also be used, e.g., as described, for example in WO 87/05330 and in Aplin etl al., CRC Crit Rev. Biochem, pp. 259-306, 1981. The in vitro coupling of carbohydrate moieties or PEG to protein- and peptide-bound Gln-residues can be carried out by transglutaminases (TGases). Transglutaminases catalyse the transfer of donor amine-groups to protein- and peptide-bound Gln-residues in a so-called cross-linking reaction. The donor-amine groups can be protein- or peptide-bound, such as the ε-amino-group in Lys-residues or it can be part of a small or large organic molecule. An example of a small organic molecule functioning as amino-donor in TGase-catalysed cross-linking is putrescine (1,4-diaminobutane). An example of a larger organic molecule functioning as amino-donor in TGase-catalysed cross-linking is an amine-containing PEG (Sato et al., 1996, Biochemistry 35, 13072-13080).

[0173] TGases, in general, are highly specific enzymes, and not every Gln-residues exposed on the surface of a protein is accessible to TGase-catalysed cross-linking to amino-containing substances. On the contrary, only few Gln-residues are naturally functioning as TGase substrates but the exact parameters governing which Gln-residues are good TGase substrates remain unknown. Thus, in order to render a protein susceptible to TGase-catalysed cross-linking reactions it is often a prerequisite at convenient positions to add stretches of amino acid sequence known to function very well as TGase substrates. Several amino acid sequences are known to be or to contain excellent natural TGase substrates e.g., substance P, elafin, fibrinogen, fibronectin, α_2-plasmin inhibitor, α-caseins, and β-caseins.

[0174] Coupling to a Sugar Moiety

[0175] In order to achieve in vivo glycosylation of a FVII molecule comprising one or more glycosylation sites the nucleotide sequence encoding the polypeptide must be inserted in a glycosylating, eucaryotic expression host. The expression host cell can be selected from fungal (filamentous fungal or yeast), insect or animal cells or from transgenic plant cells. In one embodiment, the host cell is a mammalian cell, such as a CHO cell, BHK or HEK, e.g., HEK 293, cell, or an insect cell, such as an SF9 cell, or a yeast cell, e.g., *S. cerevisiae* or *Pichia pastoris*, or any of the host cells mentioned hereinafter.

[0176] Coupling to an Organic Derivatizing Agent

[0177] Covalent modification of the polypeptide can be performed by reacting one or more attachment groups of the polypeptide with an organic derivatizing agent. Suitable derivatizing agents and methods are well known in the art. For example, cysteinyl residues most commonly are reacted with α-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, α-bromo-β-(4-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole. Histidyl residues are derivatized by reaction with diethylpyrocarbonateat pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful. The reaction is

preferably performed in 0.1 M sodium cacodylate at pH 6.0. Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing α-amino-containing residues include imidoesters such as methyl picolinimidate, pyridoxal phosphate, pyridoxal, chloroborohydride, trinitrobenzenesulfonic acid, O-methylisourea, 2,4-pentanedione and transaminase-catalyzed reaction with glyoxylate. Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pKa of the guanidine functional group.

[0178] Furthermore, these reagents can react with the groups of lysine as well as the arginine guanidino group. Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R—N═C═N—R'), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

[0179] Blocking of Functional Site

[0180] It has been reported that excessive polymer conjugation can lead to a loss of activity of the polypeptide to which the non-polypeptide moiety is conjugated. This problem can be eliminated, e.g., by removal of attachment groups located at the functional site or by reversible blocking the functional site prior to conjugation so that the functional site is blocked during conjugation. The latter strategy constitutes further embodiments of the invention (the first strategy being exemplified further above, e.g., by removal of lysine residues which can be located close to the functional site). More specifically, according to the second strategy the conjugation between the polypeptide and the non-polypeptide moiety is conducted under conditions where the functional site of the polypeptide is blocked by a helper molecule e.g., tissue factor capable of binding to the functional site of the polypeptide or a serine protease inhibitor.

[0181] Preferably, the helper molecule is one, which specifically recognizes a functional site of the polypeptide, such as a receptor, in particular tissue factor, either full length or a suitably truncated form of tissue factor or two molecules, one being tissue factor the other one being a peptide or peptide inhibitor binding to and thus protecting the area around the catalytic triad (preferably defined as amino acid residues within 10 Å of any atom in the catalytic triad).

[0182] Alternatively, the helper molecule can be an antibody, in particular a monoclonal antibody recognizing the FVII polypeptide. In particular, the helper molecule can be a neutralizing monoclonal antibody.

[0183] The polypeptide is allowed to interact with the helper molecule before effecting conjugation. This ensures that the functional site of the polypeptide is shielded or protected and consequently unavailable for derivatization by the non-polypeptide moiety such, as a polymer. Following its elution from the helper molecule, the conjugate between the non-polypeptide moiety and the polypeptide can be recovered with at least a partially preserved functional site.

[0184] The subsequent conjugation of the polypeptide having a blocked functional site to a polymer, a lipophilic compound, a sugar moiety, an organic derivatizing agent or any other compound is conducted in the normal way, e.g., as described in the sections above entitled "Conjugation to . . . "

[0185] Irrespectively of the nature of the helper molecule to be used to shield the functional site of the polypeptide from conjugation, it is desirable that the helper molecule is free from or comprises only few attachment groups for the non-polypeptide moiety of choice in part(s) of the molecule, where the conjugation to such groups will hamper the desorption of the conjugated polypeptide from the helper molecule. Hereby, selective conjugation to attachment groups present in non-shielded parts of the polypeptide can be obtained and it is possible to reuse the helper molecule for repeated cycles of conjugation. For instance, if the non-polypeptide moiety is a polymer molecule such as PEG, which has the epsilon amino group of a lysine or N-terminal amino acid residue as an attachment group, it is desirable that the helper molecule is substantially free from conjugatable epsilon amino groups, preferably free from any epsilon amino groups. Accordingly, in a preferred embodiment, the helper molecule is a protein or peptide capable of binding to the functional site of the polypeptide, which protein or peptide is free from any conjugatable attachment groups for the non-polypeptide moiety of choice.

[0186] In a further embodiment, the helper molecule is first covalently linked to a solid phase such as column packing materials, for instance Sephadex or agarose beads, or a surface, e.g., reaction vessel. Subsequently, the polypeptide is loaded onto the column material carrying the helper molecule and conjugation carried out according to methods known in the art, e.g., as described in the sections above entitled "Conjugation to . . . " This procedure allows the polypeptide conjugate to be separated from the helper molecule by elution. The polypeptide conjugate is eluted by conventional techniques under physico-chemical conditions that do not lead to a substantive degradation of the polypeptide conjugate. The fluid phase containing the polypeptide conjugate is separated from the solid phase to which the helper molecule remains covalently linked. The separation can be achieved in other ways: For instance, the helper molecule can be derivatised with a second molecule (e.g., biotin) that can be recognized by a specific binder (e.g., streptavidin). The specific binder can be linked to a solid phase thereby allowing the separation of the polypeptide conjugate from the helper molecule-second molecule complex through passage over a second helper-solid phase column which will retain, upon subsequent elution, the helper molecule-second molecule complex, but not the polypeptide conjugate. The polypeptide conjugate can be released from the helper molecule in any appropriate fashion. Deprotection can be achieved by providing conditions in which the helper molecule dissociates from the functional site of the FVII to which it is bound. For instance, a complex between an antibody to which a polymer is conjugated and an anti-idiotypic antibody can be dissociated by adjusting the pH to an acid or alkaline pH. Even more preferred is the use of a conformation specific antibody that recognizes a

Ca^{2+} specific conformation of FVII and consequently can be eluted with EDTA under mild conditions.

Attachment of Serine Protease Inhibitor

[0187] Attachment of a serine protease inhibitor can be performed in accordance with the method described in WO 96/12800.

[0188] Conjugation of a Tagged Polypeptide

[0189] In an alternative embodiment, the polypeptide is expressed as a fusion protein with a tag, i.e., an amino acid sequence or peptide stretch made up of typically 1-30, such as 1-20 amino acid residues. Besides allowing for fast and easy purification, the tag is a convenient tool for achieving conjugation between the tagged polypeptide and the non-polypeptide moiety. In particular, the tag can be used for achieving conjugation in microtiter plates or other carriers, such as paramagnetic beads, to which the tagged polypeptide can be immobilised via the tag. The conjugation to the tagged polypeptide in, e.g., microtiter plates has the advantage that the tagged polypeptide can be immobilised in the microtiter plates directly from the culture broth (in principle without any purification) and subjected to conjugation. Thereby, the total number of process steps (from expression to conjugation) can be reduced. Furthermore, the tag can function as a spacer molecule, ensuring an improved accessibility to the immobilised polypeptide to be conjugated. The conjugation using a tagged polypeptide can be to any of the non-polypeptide moieties disclosed herein, e.g., to a polymer molecule such as PEG.

[0190] The identity of the specific tag to be used is not critical as long as the tag is capable of being expressed with the polypeptide and is capable of being immobilised on a suitable surface or carrier material. A number of suitable tags are commercially available, e.g., from Unizyme Laboratories, Denmark. For instance, the tag can consist of any of the following sequences:

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His-His-His-His-His-His

Met-Lys-His-His-His-His-His-His

Met-Lys-His-His-Ala-His-His-Gln-His-His

Met-Lys-His-Gln-His-Gln-His-Gln-His-Gln-His-Gln-
His-Gln

Met-Lys-His-Gln-His-Gln-His-Gln-His-Gln-His-Gln-
His-Gln-Gln
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[0191] or any of the following:

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EQKLISEEDL
(a C-terminal tag described in Mol. Cell. Biol.
5:3610-16, 1985)

DYKDDDDK
(a C- or N-terminal tag)

YPYDVPDYA
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[0192] Antibodies against the above tags are commercially available, e.g., from ADI, Aves Lab and Research Diagnostics.

[0193] The subsequent cleavage of the tag from the polypeptide can be achieved by use of commercially available enzymes.

[0194] Methods of Preparing a Polypeptide of the Invention or the Polypeptide of the Conjugate of the Invention

[0195] The polypeptide of the present invention or the polypeptide part of a conjugate of the invention, optionally in glycosylated form, can be produced by any suitable method known in the art. Such methods include constructing a nucleotide sequence encoding the polypeptide and expressing the sequence in a suitable transformed or transfected host. Preferably, the host cell is a gammacarboxylating host cell such as a mammalian cell. However, polypeptides of the invention can be produced, albeit less efficiently, by chemical synthesis or a combination of chemical synthesis or a combination of chemical synthesis and recombinant DNA technology.

[0196] A nucleotide sequence encoding a polypeptide or the polypeptide part of a conjugate of the invention can be constructed by isolating or synthesizing a nucleotide sequence encoding the parent FVII, such as hFVII with the amino acid sequence shown in SEQ ID NO:1 and then changing the nucleotide sequence so as to effect introduction (i.e., insertion or substitution) or removal (i.e., deletion or substitution) of the relevant amino acid residue(s).

[0197] The nucleotide sequence is conveniently modified by site-directed mutagenesis in accordance with conventional methods. Alternatively, the nucleotide sequence is prepared by chemical synthesis, e.g., by using an oligonucleotide synthesizer, wherein oligonucleotides are designed based on the amino acid sequence of the desired polypeptide, and preferably selecting those codons that are favored in the host cell in which the recombinant polypeptide will be produced. For example, several small oligonucleotides coding for portions of the desired polypeptide can be synthesized and assembled by PCR, ligation or ligation chain reaction (LCR) (Barany, PNAS 88:189-193, 1991). The individual oligonucleotides typically contain 5' or 3' overhangs for complementary assembly.

[0198] Alternative nucleotide sequence modification methods are available for producing polypeptide variants for high throughput screening, for instance methods which involve homologous cross-over such as disclosed in U.S. Pat. No. 5,093,257, and methods which involve gene shuffling, i.e., recombination between two or more homologous nucleotide sequences resulting in new nucleotide sequences having a number of nucleotide alterations when compared to the starting nucleotide sequences. Gene shuffling (also known as DNA shuffling) involves one or more cycles of random fragmentation and reassembly of the nucleotide sequences, followed by screening to select nucleotide sequences encoding polypeptides with desired properties. In order for homology-based nucleic acid shuffling to take place, the relevant parts of the nucleotide sequences are preferably at least 50% identical, such as at least 60% identical, more preferably at least 70% identical, such as at least 80% identical. The recombination can be performed in vitro or in vivo.

[0199] Examples of suitable in vitro gene shuffling methods are disclosed by Stemmer et al. (1994), Proc. Natl. Acad. Sci. USA; vol. 91, pp. 10747-10751; Stemmer (1994), Nature, vol. 370, pp. 389-391; Smith (1994), Nature vol. 370, pp. 324-325; Zhao et al., Nat. Biotechnol. 1998, March; 16(3): 258-61; Zhao H. and Arnold, F B, Nucleic Acids

Research, 1997, Vol. 25. No. 6 pp. 1307-1308; Shao et al., Nucleic Acids Research 1998, Jan. 15; 26(2): pp. 681-83; and WO 95/17413.

[0200] An example of a suitable in vivo shuffling method is disclosed in WO 97/07205. Other techniques for mutagenesis of nucleic acid sequences by in vitro or in vivo recombination are disclosed e.g., in WO 97/20078 and U.S. Pat. No. 5,837,458. Examples of specific shuffling techniques include "family shuffling,""synthetic shuffling" and "in silico shuffling".

[0201] Family shuffling involves subjecting a family of homologous genes from different species to one or more cycles of shuffling and subsequent screening or selection. Family shuffling techniques are disclosed e.g., by Crameri et al. (1998), Nature, vol. 391, pp. 288-291; Christians et al. (1999), Nature Biotechnology, vol. 17, pp. 259-264; Chang et al. (1999), Nature Biotechnology, vol. 17, pp. 793-797; and Ness et al. (1999), Nature Biotechnology, vol. 17, 893-896.

[0202] Synthetic shuffling involves providing libraries of overlapping synthetic oligonucleotides based e.g., on a sequence alignment of homologous genes of interest. The synthetically generated oligonucleotides are recombined, and the resulting recombinant nucleic acid sequences are screened and if desired used for further shuffling cycles. Synthetic shuffling techniques are disclosed in WO 00/42561.

[0203] In silico shuffling refers to a DNA shuffling procedure, which is performed or modelled using a computer system, thereby partly or entirely avoiding the need for physically manipulating nucleic acids. Techniques for in silico shuffling are disclosed in WO 00/42560.

[0204] Once assembled (by synthesis, site-directed mutagenesis or another method), the nucleotide sequence encoding the polypeptide is inserted into a recombinant vector and operably linked to control sequences necessary for expression of the FVII in the desired transformed host cell.

[0205] It should of course be understood that not all vectors and expression control sequences function equally well to express the nucleotide sequence encoding a polypeptide described herein. Neither will all hosts function equally well with the same expression system. However, one of skill in the art can make a selection among these vectors, expression control sequences and hosts without undue experimentation. For example, in selecting a vector, the host must be considered because the vector must replicate in it or be able to integrate into the chromosome. The vector's copy number, the ability to control that copy number, and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered. In selecting an expression control sequence, a variety of factors should also be considered. These include, for example, the relative strength of the sequence, its controllability, and its compatibility with the nucleotide sequence encoding the polypeptide, particularly as regards potential secondary structures. Hosts should be selected by consideration of their compatibility with the chosen vector, the toxicity of the product coded for by the nucleotide sequence, their secretion characteristics, their ability to fold the polypeptide correctly, their fermentation or culture requirements, and the ease of purification of the products coded for by the nucleotide sequence.

[0206] The recombinant vector can be an autonomously replicating vector, i.e., a vector, which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g., a plasmid. Alternatively, the vector is one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

[0207] The vector is preferably an expression vector, in which the nucleotide sequence encoding the polypeptide of the invention is operably linked to additional segments required for transcription of the nucleotide sequence. The vector is typically derived from plasmid or viral DNA. A number of suitable expression vectors for expression in the host cells mentioned herein are commercially available or described in the literature. Useful expression vectors for eukaryotic hosts, include, for example, vectors comprising expression control sequences from SV40, bovine papilloma virus, adenovirus and cytomegalovirus. Specific vectors are, e.g., pcDNA3.1(+)\Hyg (Invitrogen, Carlsbad, Calif., USA) and pCI-neo (Stratagene, La Jola, Calif., USA). Useful expression vectors for yeast cells include the 2μ plasmid and derivatives thereof, the POT1 vector (U.S. Pat. No. 4,931, 373), the pJSO37 vector described in Okkels, Ann. New York Acad. Sci. 782, 202-207, 1996, and pPICZ A, B or C (Invitrogen). Useful vectors for insect cells include pVL941, pBG311 (Cate et al., "Isolation of the Bovine and Human Genes for Mullerian Inhibiting Substance And Expression of the Human Gene In Animal Cells," Cell, 45, pp. 685-98 (1986), pBluebac 4.5 and pMelbac (both available from Invitrogen). Useful expression vectors for bacterial hosts include known bacterial plasmids, such as plasmids from *E. coli*, including pBR322, pET3a and pET12a (both from Novagen Inc., WI, USA), wider host range plasmids, such as RP4, phage DNAs, e.g., the numerous derivatives of phage lambda, e.g., NM989, and other DNA phages, such as M13 and filamentous single stranded DNA phages.

[0208] Other vectors for use in this invention include those that allow the nucleotide sequence encoding the polypeptide to be amplified in copy number. Such amplifiable vectors are well known in the art. They include, for example, vectors able to be amplified by DHFR amplification (see, e.g., Kaufman, U.S. Pat. No. 4,470,461, Kaufman and Sharp, "Construction of A Modular Dihydrafolate Reductase cDNA Gene: Analysis Of Signals Utilized For Efficient Expression," Mol. Cell. Biol., 2, pp. 1304-19 (1982)) and glutamine synthetase ("GS") amplification (see, e.g., U.S. Pat. No. 5,122,464 and EP 338,841).

[0209] The recombinant vector can further comprise a DNA sequence enabling the vector to replicate in the host cell in question. An example of such a sequence (when the host cell is a mammalian cell) is the SV40 origin of replication. When the host cell is a yeast cell, suitable sequences enabling the vector to replicate are the yeast plasmid 2μ replication genes REP 1-3 and origin of replication.

[0210] The vector can also comprise a selectable marker, e.g., a gene the product of which complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or the *Schizosaccharomyces pombe* TPI gene (described by P. R. Russell, Gene 40, 1985, pp. 125-130), or one which confers resistance to a drug, e.g., ampicillin, kanamycin, tetracyclin, chloramphenicol, neomycin, hygro-

mycin or methotrexate. For *Saccharomyces cerevisiae*, selectable markers include ura3 and leu2. For filamentous fungi, selectable markers include amdS, pyrG, arcB, niaD and sC.

[0211] The term "control sequences" is defined herein to include all components, which are necessary or advantageous for the expression of the polypeptide of the invention. Each control sequence can be native or foreign to the nucleic acid sequence encoding the polypeptide. Such control sequences include, but are not limited to, a leader sequence, polyadenylation sequence, propeptide sequence, promoter, enhancer or upstream activating sequence, signal peptide sequence, and transcription terminator. At a minimum, the control sequences include a promoter.

[0212] A wide variety of expression control sequences can be used in the present invention. Such useful expression control sequences include the expression control sequences associated with structural genes of the foregoing expression vectors as well as any sequence known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof.

[0213] Examples of suitable control sequences for directing transcription in mammalian cells include the early and late promoters of SV40 and adenovirus, e.g., the adenovirus 2 major late promoter, the MT-1 (metallothionein gene) promoter, the human cytomegalovirus immediate-early gene promoter (CMV), the human elongation factor 1α (EF-1α) promoter, the *Drosophila* minimal heat shock protein 70 promoter, the Rous Sarcoma Virus (RSV) promoter, the human ubiquitin C (UbC) promoter, the human growth hormone terminator, SV40 or adenovirus E1b region polyadenylation signals and the Kozak consensus sequence (Kozak, M. *J Mol Biol* 1987 Aug. 20; 196(4):947-50).

[0214] In order to improve expression in mammalian cells a synthetic intron can be inserted in the 5' untranslated region of the nucleotide sequence encoding the polypeptide. An example of a synthetic intron is the synthetic intron from the plasmid pCI-Neo (available from Promega Corporation, WI, USA).

[0215] Examples of suitable control sequences for directing transcription in insect cells include the polyhedrin promoter, the P10 promoter, the Autographa californica polyhedrosis virus basic protein promoter, the baculovirus immediate early gene 1 promoter and the baculovirus 39K delayed-early gene promoter, and the SV40 polyadenylation sequence. Examples of suitable control sequences for use in yeast host cells include the promoters of the yeast α-mating system, the yeast triose phosphate isomerase (TPI) promoter, promoters from yeast glycolytic genes or alcohol dehydrogenase genes, the ADH2-4c promoter, and the inducible GAL promoter. Examples of suitable control sequences for use in filamentous fungal host cells include the ADH3 promoter and terminator, a promoter derived from the genes encoding *Aspergillus oryzae* TAKA amylase triose phosphate isomerase or alkaline protease, an *A. niger* α-amylase, *A. niger* or *A. nidulans* glucoamylase, *A. nidulans* acetamidase, *Rhizomucor miehei* aspartic proteinase or lipase, the TPI1 terminator and the ADH3 terminator. Examples of suitable control sequences for use in bacterial host cells include promoters of the lac system, the trp system, the TAC or TRC system, and the major promoter regions of phage lambda.

[0216] The presence or absence of a signal peptide will, e.g., depend on the expression host cell used for the production of the polypeptide to be expressed (whether it is an intracellular or extracellular polypeptide) and whether it is desirable to obtain secretion. For use in filamentous fungi, the signal peptide can conveniently be derived from a gene encoding an *Aspergillus* sp. amylase or glucoamylase, a gene encoding a *Rhizomucor miehei* lipase or protease or a *Humicola lanuginosa lipase*. The signal peptide is preferably derived from a gene encoding *A. oryzae* TAKA amylase, *A. niger* neutral α-amylase, *A. niger* acid-stable amylase, or *A. niger glucoamylase*. For use in insect cells, the signal peptide can conveniently be derived from an insect gene (cf. WO 90/05783), such as the Lepidopteran *manduca sexta* adipokinetic hormone precursor, (cf. U.S. Pat. No. 5,023,328), the honeybee meiittin (Invitrogen), ecdysteroid UDPglucosyltransferase (egt) (Murphy et al., Protein Expression and Purification 4, 349-357 (1993) or human pancreatic lipase (hpl) (Methods in Enzymology 284, pp. 262-272, 1997). A preferred signal peptide for use in mammalian cells is that of hFVII or the murine Ig kappa light chain signal peptide (Coloma, M (1992) J. Imm. Methods 152:89-104). For use in yeast cells suitable signal peptides have been found to be the α-factor signal peptide from *S. cereviciae* (cf. U.S. Pat. No. 4,870,008), a modified carboxypeptidase signal peptide (cf. L. A. Valls et al., Cell 48, 1987, pp. 887-897), the yeast BAR1 signal peptide (cf. WO 87/02670), the yeast aspartic protease 3 (YAP3) signal peptide (cf. M. Egel-Mitani et al., Yeast 6, 1990, pp. 127-137), and the synthetic leader sequence TA57 (WO98/32867). For use in *E. coli* cells a suitable signal peptide have been found to be the signal peptide ompA (EP581821).

[0217] The nucleotide sequence of the invention encoding a FVII polypeptide, whether prepared by site-directed mutagenesis, synthesis, PCR or other methods, can optionally include a nucleotide sequence that encode a signal peptide. The signal peptide is present when the polypeptide is to be secreted from the cells in which it is expressed. Such signal peptide, if present, should be one recognized by the cell chosen for expression of the polypeptide. The signal peptide can be homologous (e.g., be that normally associated with hFVII) or heterologous (i.e., originating from another source than hFVII) to the polypeptide or can be homologous or heterologous to the host cell, i.e., be a signal peptide normally expressed from the host cell or one which is not normally expressed from the host cell. Accordingly, the signal peptide can be prokaryotic, e.g., derived from a bacterium such as *E. coli*, or eukaryotic, e.g., derived from a mammalian, or insect or yeast cell.

[0218] Any suitable host can be used to produce the polypeptide or polypeptide part of the conjugate of the invention, including bacteria, fungi (including yeasts), plant, insect, mammal, or other appropriate animal cells or cell lines, as well as transgenic animals or plants. Examples of bacterial host cells include grampositive bacteria such as strains of *Bacillus*, e.g., *B. brevis* or *B. subtilis, Pseudomonas* or *Streptomyces*, or gramnegative bacteria, such as strains of *E. coli*. The introduction of a vector into a bacterial host cell can, for instance, be effected by protoplast transformation (see, e.g., Chang and Cohen, 1979, *Molecular General Genetics* 168: 111-115), using competent cells (see, e.g., Young and Spizizin, 1961, *Journal of Bacteriology* 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, *Journal of Molecular Biology* 56: 209-221), electroporation (see,

e.g., Shigekawa and Dower, 1988, *Biotechniques* 6: 742-751), or conjugation (see, e.g., Koehler and Thorne, 1987, *Journal of Bacteriology* 169: 5771-5278). Examples of suitable filamentous fungal host cells include strains of *Aspergillus*, e.g., *A. oryzae, A. niger*, or *A. nidulans, Fusarium* or *Trichoderma*. Fungal cells can be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known per se. Suitable procedures for transformation of *Aspergillus* host cells are described in EP 238 023 and U.S. Pat. No. 5,679,543. Suitable methods for transforming *Fusarium* species are described by Malardier et al., 1989, *Gene* 78: 147-156 and WO 96/00787. Examples of suitable yeast host cells include strains of *Saccharomyces*, e.g., *S. cerevisiae, Schizosaccharomyces, Klyveromyces, Pichia*, such as *P. pastoris* or *P. methanolica, Hansen ula*, such as *H. Polymorpha* or *Yarrowia*. Yeast can be transformed using the procedures described by Becker and Guarente, In Abelson, J. N. and Simon, M. I., editors, *Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology*, Volume 194, pp 182-187, Academic Press, Inc., New York; Ito et al., I983, *Journal of Bacteriology* 153: 163; Hinnen et al., 1978, *Proceedings of the National Academy of Sciences USA* 75: 1920: and as disclosed by Clontech Laboratories, Inc, Palo Alto, Calif., USA (in the product protocol for the Yeastmaker™ Yeast Transformation System Kit). Examples of suitable insect host cells include a *Lepidoptora* cell line, such as *Spodoptera frugiperda* (Sf9 or Sf21) or Trichoplusioa ni cells (High Five) (U.S. Pat. No. 5,077,214). Transformation of insect cells and production of heterologous polypeptides therein can be performed as described by Invitrogen. Examples of suitable mammalian host cells include Chinese hamster ovary (CHO) cell lines, (e.g., CHO-K1; ATCC CCL-61), Green Monkey cell lines (COS) (e.g., COS 1 (ATCC CRL-1650), COS 7 (ATCC CRL-1651)); mouse cells (e.g., NS/O), Baby Hamster Kidney (BHK) cell lines (e.g., ATCC CRL-1632 or ATCC CCL-10), and human cells (e.g., HEK 293 (ATCC CRL-1573)), as well as plant cells in tissue culture. Additional suitable cell lines are known in the art and available from public depositories such as the American Type Culture Collection, Rockville, Md. Also, the mammalian cell, such as a CHO cell, can be modified to express sialyltransferase, e.g., 1,6-sialyltransferase, e.g., as described in U.S. Pat. No. 5,047,335, in order to provide improved glycosylation of the FVII or FVIIa polypeptide.

[0219] In order to increase secretion it can be of particular interest to produce the polypeptide of the invention together with an endoprotease, in particular a PACE (Paired basic amino acid converting enzyme) (e.g., as described in U.S. Pat. No. 5,986,079), such as a Kex2 endoprotease (e.g., as described in WO 00/28065).

[0220] Methods for introducing exogeneous DNA into mammalian host cells include calcium phosphate-mediated transfection, electroporation, DEAE-dextran mediated transfection, liposome-mediated transfection, viral vectors and the transfection method described by Life Technologies Ltd, Paisley, UK using Lipofectamin 2000. These methods are well known in the art and e.g., described by Ausbel et al. (eds.), 1996, Current Protocols in Molecular Biology, John Wiley & Sons, New York, USA. The cultivation of mammalian cells are conducted according to established methods, e.g., as disclosed in (Animal Cell Biotechnology, Methods and Protocols, Edited by Nigel Jenkins, 1999, Human Press Inc, Totowa, N.J., USA and Harrison M A and Rae I F, General Techniques of Cell Culture, Cambridge University Press 1997).

[0221] In the production methods of the present invention, the cells are cultivated in a nutrient medium suitable for production of the polypeptide using methods known in the art. For example, the cell can be cultivated by shake flask cultivation, small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermenters performed in a suitable medium and under conditions allowing the polypeptide to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or can be prepared according to published compositions (e.g., in catalogues of the American Type Culture Collection). If the polypeptide is secreted into the nutrient medium, the polypeptide can be recovered directly from the medium. If the polypeptide is not secreted, it can be recovered from cell lysates.

[0222] The resulting polypeptide can be recovered by methods known in the art. For example, the polypeptide can be recovered from the nutrient medium by conventional procedures including, but not limited to, centrifugation, filtration, extraction, spray drying, evaporation, or precipitation.

[0223] The polypeptides can be purified by a variety of procedures known in the art including, but not limited to, chromatography (e.g., ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (e.g., preparative isoelectric focusing), differential solubility (e.g., ammonium sulfate precipitation), SDS-PAGE, or extraction (see, e.g., *Protein Purification*, J.-C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989).

[0224] Single chain FVII can be purified and activated to two-chain FVIIa by a number of methods as described in the literature (Broze and Majerus, 1980, J. Biol. Chem. 255:1242-47 and Hedner and Kisiel, 1983, J. Clin. Invest. 71:1836-41). Another method whereby single chain FVII can be purified is by incorporation of Zn ions during purification as described in U.S. Pat. No. 5,700,914.

[0225] In a preferred embodiment, the polypeptide is purified as a single chain FVII, which further is PEGylated. The PEGylated FVII single chain polypeptide is activated by either use of an immobilized enzyme (e.g., factors IIa, IXa, Xa and XIIa) or by autoactivation using a positively charged ion exchange matrix or the like.

[0226] It is advantageous to first purify FVII in its single chain form, then PEGylate (if desired) and last activate by one of the methods described above or by autoactivation as described by Pedersen et al, 1989, Biochemistry 28: 9331-36. The advantage of carrying out PEGylation before activation is that PEGylation of the new aminoterminal formed by cleavage of R152-I153 is avoided. PEGylation of this new amino terminal would render the molecule inactive since the formation of a hydrogen bond between D242 and the amino terminal of I153 is necessary for activity.

[0227] Pharmaceutical Composition of the Invention and its Use

[0228] In a further aspect, the present invention relates to a composition, in particular to a pharmaceutical composition, comprising a polypeptide or conjugate (including an inactive conjugate as described further above) of the invention and a pharmaceutically acceptable carrier or excipient.

[0229] The conjugate, the polypeptide or the pharmaceutical composition according to the invention can be used as a medicament.

[0230] Preferably, the polypeptide or the (active) conjugate can be used for the manufacture of a medicament for the treatment or prophylaxis of a FVIIa/TF-related disease or disorder in a mammal. For example, the polypeptide or the (active) conjugate can be used for the manufacture of a medicament for the treatment or prophylaxis of diseases wherein increased clot formation is desirable, such as treatment of patients having diseases resulting in inadequate blood coagulation in response to damage to blood vessels. In particular, the polypeptide or the (active) conjugate can be used for the manufacture of a medicament for the treatment of hemophiliacs, hemophiliacs with inhibitors to FVIII and FIX, patients with thrombocytopenia, patients with thrombocytopathies, such as Glanzmann's thrombastenia platelet release defects and storage pool defects, patients with von Willebrand's disease, patients with liver diseases, or otherwise healthy people with severe bleeding problems, e.g., due to trauma or major surgery, who have developed inhibitors to FVIIa, bleeding disorders such as hemophiliacs and other typically associated with severe tissue damages.

[0231] Analogously, the inactive conjugate of the invention can be used for the manufacture of a medicament for the treatment or prophylaxis of a FVIIa/TF-related disease or disorder in a mammal. For example, the inactive conjugate of the invention can be used for the manufacture of a medicament for the treatment or prophylaxis of diseases where decreased clot formation is desirable, such as prophylaxis or treatment of patients being in hypercoagulable states, such as patients with sepsis, deep-vein thrombosis, patients in risk of myocardial infections or thrombotic stroke, pulmonary embolism, patients with acute coronary syndromes (myocardial infarction and unstable angina pectoris), patients undergoing coronary cardiac, prevention of cardiac events and restonosis for patients receiving angioplasty, patients with peripheral vascular diseases. The inactive conjugate of the invention can also be used for the manufacture of a medicament for the treatment of respiratory diseases, tumor growth and metastasis.

[0232] In another aspect, the polypeptide, the (active) conjugate or the pharmaceutical composition comprising the (active) conjugate of the invention can be used in a method for treating a mammal having a FVIIa/TF-related disease or disorder (such as one or more of the diseases or disorders mentioned above), comprising administering to a mammal in need thereof an effective amount of such a polypeptide, conjugate or composition.

[0233] Analogously, the inactive conjugate or the pharmaceutical composition comprising the inactive conjugate of the invention can be used in a method for treating a mammal having a FVIIa/TF-related disease or disorder (such as one or more of the diseases or disorders mentioned above), comprising administering to a mammal in need thereof an effective amount of such an inactivated conjugate or composition.

[0234] The polypeptides or conjugates of the invention is administered to patients in a therapeutically effective dose, normally one approximately paralleling that employed in therapy with rFVII such as NovoSeven®, or at higher dosage. By "therapeutically effective dose" herein is meant a dose that is sufficient to produce the desired effects in relation to the condition for which it is administered. The exact dose will depend on the circumstances, and will be ascertainable by one skilled in the art using known techniques. Normally, the dose should be capable of preventing or lessening the severity or spread of the condition or indication being treated. It will be apparent to those of skill in the art that an effective amount of a polypeptide, conjugate or composition of the invention depends, inter alia, upon the disease, the dose, the administration schedule, whether the polypeptide or conjugate or composition is administered alone or in conjunction with other therapeutic agents, the plasma half-life of the compositions, and the general health of the patient. Preferably, the polypeptide, conjugate, or composition of the invention is administered in an effective dose, in particular a dose which is sufficient to normalize the coagulation disorder.

[0235] The polypeptide or conjugate of the invention is preferably administered in a composition including a pharmaceutically acceptable carrier or excipient. "Pharmaceutically acceptable" means a carrier or excipient that does not cause any untoward effects in patients to whom it is administered. Such pharmaceutically acceptable carriers and excipients are well known in the art (see, for example, Remington's Pharmaceutical Sciences, 18th edition, A. R. Gennaro, Ed., Mack Publishing Company [1990]; Pharmaceutical Formulation Development of Peptides and Proteins, S. Frokjaer and L. Hovgaard, Eds., Taylor & Francis [2000]; and Handbook of Pharmaceutical Excipients, 3rd edition, A. Kibbe, Ed., Pharmaceutical Press [2000]).

[0236] The polypeptide or conjugate of the invention can be formulated into pharmaceutical compositions by well-known methods. Suitable formulations are described by Remington's Pharmaceutical Sciences by E. W. Martin (Mark Publ. Co., 16th Ed., 1980).

[0237] The polypeptide or conjugate of the invention can be used "as is" and/or in a salt form thereof. Suitable salts include, but are not limited to, salts with alkali metals or alkaline earth metals, such as sodium, potassium, calcium and magnesium, as well as e.g., zinc salts. These salts or complexes can be present as a crystalline and/or amorphous structure.

[0238] The pharmaceutical composition of the invention can be administered alone or in conjunction with other therapeutic agents. These agents can be incorporated as part of the same pharmaceutical composition or can be administered separately from the polypeptide or conjugate of the invention, either concurrently or in accordance with another treatment schedule. In addition, the polypeptide, conjugate or pharmaceutical composition of the invention can be used as an adjuvant to other therapies.

[0239] A "patient" for the purposes of the present invention includes both humans and other mammals. Thus the methods are applicable to both human therapy and veterinary applications.

[0240] The pharmaceutical composition of the polypeptide or conjugate of the invention can be formulated in a variety of forms, e.g., as a liquid, gel, lyophilized, or as a compressed solid. The preferred form will depend upon the particular indication being treated and will be apparent to one skilled in the art.

[0241] In particular, the pharmaceutical composition of the polypeptide or conjugate of the invention can be formulated in lyophilised or stable soluble form. The polypeptide or the conjugate can be lyophilised by a variety of procedures known in the art. A polypeptide or the conjugate can be a stable soluble form by the removal or shielding of proteolytic degradation sites. The advantage of obtaining a stable soluble preparation lies in easier handling for the patient and, in the case of emergencies, quicker action, which potentially can become life saving. The preferred form will depend upon the particular indication being treated and will be apparent to one of skill in the art.

[0242] The administration of the formulations of the present invention can be performed in a variety of ways, including, but not limited to, orally, subcutaneously, intravenously, intracerebrally, intranasally, transdermally, intraperitoneally, intramuscularly, intrapulmonary, vaginally, rectally, intraocularly, or in any other acceptable manner. The formulations can be administered continuously by infusion, although bolus injection is acceptable, using techniques well known in the art, such as pumps or implantation. In some instances the formulations can be directly applied as a solution or spray.

Parenterals

[0243] A preferred example of a pharmaceutical composition is a solution designed for parenteral administration. Although in many cases pharmaceutical solution formulations are provided in liquid form, appropriate for immediate use, such parenteral formulations can also be provided in frozen or in lyophilized form. In the former case, the composition must be thawed prior to use. The latter form is often used to enhance the stability of the active compound contained in the composition under a wider variety of storage conditions, as it is recognized by those skilled in the art that lyophilized preparations are generally more stable than their liquid counterparts. Such lyophilized preparations are reconstituted prior to use by the addition of one or more suitable pharmaceutically acceptable diluents such as sterile water for injection or sterile physiological saline solution.

[0244] In case of parenterals, they are prepared for storage as lyophilized formulations or aqueous solutions by mixing, as appropriate, the polypeptide having the desired degree of purity with one or more pharmaceutically acceptable carriers, excipients or stabilizers typically employed in the art (all of which are termed "excipients"), for example buffering agents, stabilizing agents, preservatives, isotonifiers, nonionic surfactants or detergents, antioxidants and/or other miscellaneous additives.

[0245] Buffering agents help to maintain the pH in the range which approximates physiological conditions. They are typically present at a concentration ranging from about 2 mM to about 50 mM Suitable buffering agents for use with the present invention include both organic and inorganic acids and salts thereof such as citrate buffers (e.g., monosodium citrate-disodium citrate mixture, citric acid-trisodium citrate mixture, citric acid-monosodium citrate mixture, etc.), succinate buffers (e.g., succinic acid-monosodium succinate mixture, succinic acid-sodium hydroxide mixture, succinic acid-disodium succinate mixture, etc.), tartrate buffers (e.g., tartaric acid-sodium tartrate mixture, tartaric acid-potassium tartrate mixture, tartaric acid-sodium hydroxide mixture, etc.), fumarate buffers (e.g., fumaric acid-monosodium fumarate mixture, fumaric acid-disodium fumarate mixture, monosodium fumarate-disodium fumarate mixture, etc.), gluconate buffers (e.g., gluconic acid-sodium glyconate mixture, gluconic acid-sodium hydroxide mixture, gluconic acid-potassium glyuconate mixture, etc.), oxalate buffer (e.g., oxalic acid-sodium oxalate mixture, oxalic acid-sodium hydroxide mixture, oxalic acid-potassium oxalate mixture, etc.), lactate buffers (e.g., lactic acid-sodium lactate mixture, lactic acid-sodium hydroxide mixture, lactic acid-potassium lactate mixture, etc.) and acetate buffers (e.g., acetic acid-sodium acetate mixture, acetic acid-sodium hydroxide mixture, etc.). Additional possibilities are phosphate buffers, histidine buffers and trimethylamine salts such as Tris.

[0246] Stabilizers refer to a broad category of excipients which can range in function from a bulking agent to an additive which solubilizes the therapeutic agent or helps to prevent denaturation or adherence to the container wall. Typical stabilizers can be polyhydric sugar alcohols (enumerated above); amino acids such as arginine, lysine, glycine, glutamine, asparagine, histidine, alanine, ornithine, L-leucine, 2-phenylalanine, glutamic acid, threonine, etc., organic sugars or sugar alcohols, such as lactose, trehalose, stachyose, mannitol, sorbitol, xylitol, ribitol, myoinisitol, galactitol, glycerol and the like, including cyclitols such as inositol; polyethylene glycol; amino acid polymers; sulfur-containing reducing agents, such as urea, glutathione, thioctic acid, sodium thioglycolate, thioglycerol, α-monothioglycerol and sodium thiosulfate; low molecular weight polypeptides (i.e., <10 residues); proteins such as human serum albumin, bovine serum albumin, gelatin or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; monosaccharides such as xylose, mannose, fructose and glucose; disaccharides such as lactose, maltose and sucrose; trisaccharides such as raffinose, and polysaccharides such as dextran. Stabilizers are typically present in the range of from 0.1 to 10,000 parts by weight based on the active protein weight.

[0247] Preservatives are added to retard microbial growth, and are typically added in amounts of about 0.2%-1% (w/v). Suitable preservatives for use with the present invention include phenol, benzyl alcohol, meta-cresol, methyl paraben, propyl paraben, octadecyldimethylbenzyl ammonium chloride, benzalkonium halides (e.g., benzalkonium chloride, bromide or iodide), hexamethonium chloride, alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol and 3-pentanol.

[0248] Isotonicifiers are added to ensure isotonicity of liquid compositions and include polyhydric sugar alcohols, preferably trihydric or higher sugar alcohols, such as glycerin, erythritol, arabitol, xylitol, sorbitol and mannitol. Polyhydric alcohols can be present in an amount between 0.1% and 25% by weight, typically 1% to 5%, taking into account the relative amounts of the other ingredients.

[0249] Non-ionic surfactants or detergents (also known as "wetting agents") can be present to help solubilizing the therapeutic agent as well as to protect the therapeutic polypeptide against agitation-induced aggregation, which also permits the formulation to be exposed to shear surface stress without causing denaturation of the polypeptide. Suit-

able non-ionic surfactants include polysorbates (20, 80, etc.), polyoxamers (184, 188 etc.), Pluronic® polyols, polyoxyethylene sorbitan monoethers (Tween®-20, Tween®-80, etc.).

[0250] Additional miscellaneous excipients include bulking agents or fillers (e.g., starch), chelating agents (e.g., EDTA), antioxidants (e.g., ascorbic acid, methionine, vitamin E) and cosolvents.

[0251] The active ingredient can also be entrapped in microcapsules prepared, for example, by coascervation techniques or by interfacial polymerization, for example hydroxymethylcellulose, gelatin or poly-(methylmethacylate) microcapsules, in colloidal drug delivery systems (for example liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, supra.

[0252] Parenteral formulations to be used for in vivo administration must be sterile. This is readily accomplished, for example, by filtration through sterile filtration membranes.

[0253] Sustained Release Preparations

[0254] Examples of sustained-release preparations include semi-permeable matrices of solid hydrophobic polymers containing the polypeptide or conjugate, the matrices having a suitable form such as a film or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate) or poly(vinylalcohol)), polylactides, copolymers of L-glutamic acid and ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the ProLease® technology or Lupron Depot® (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(−)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for long periods such as up to or over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated polypeptides remain in the body for a long time, they can denature or aggregate as a result of exposure to moisture at 37° C., resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S—S bond formation through thio-disulfide interchange, stabilization can be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

[0255] The invention is further described in the following non-limiting examples.

Materials and Methods

[0256] Methods Used to Determine the Amino Acids to be Modified

Accessible Surface Area (ASA)

[0257] The computer program Access (B. Lee and F. M. Richards, J. Mol. Biol. 55: 379-400 (1971)) version 2 (© 1983 Yale University) are used to compute the accessible surface area (ASA) of the individual atoms in the structure. This method typically uses a probe-size of 1.4 Å and defines the Accessible Surface Area (ASA) as the area formed by the center of the probe. Prior to this calculation all water molecules and all hydrogen atoms should be removed from the coordinate set, as should other atoms not directly related to the protein.

Fractional ASA of Side Chain

[0258] The fractional ASA of the side chain atoms is computed by division of the sum of the ASA of the atoms in the side chain with a value representing the ASA of the side chain atoms of that residue type in an extended Ala-x-Ala tripeptide (See Hubbard, Campbell & Thornton (1991) J. Mol. Biol. 220, 507-530). For this example the CA atom is regarded as a part of the side chain of Glycine residues but not for the remaining residues. The following table is used as standard 100% ASA for the side chain:

Ala	69.23 Å^2
Arg	200.35 Å^2
Asn	106.25 Å^2
Asp	102.06 Å^2
Cys	96.69 Å^2
Gln	140.58 Å^2
Glu	134.61 Å^2
Gly	32.28 Å^2
His	147.00 Å^2
Ile	137.91 Å^2
Leu	140.76 Å^2
Lys	162.50 Å^2
Met	156.08 Å^2
Phe	163.90 Å^2
Pro	119.65 Å^2
Ser	78.16 Å^2
Thr	101.67 Å^2
Trp	210.89 Å^2
Tyr	176.61 Å^2
Val	114.14 Å^2

[0259] Residues not detected in the structure are defined as having 100% exposure as they are thought to reside in flexible regions. The gamma-carboxy glutamic acids at positions 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35 are all defined as being 100% exposed.

Determining Distances Between Atoms

[0260] The distance between atoms is most easily determined using molecular graphics software e.g., InsightII® v. 98.0, MSI INC.

Catalytic Site Region

[0261] The catalytic site region is defined as any residues having at least one atom within 10 Å of any atom in the catalytic triad (residues H193, D242, S344).

Determination of Tissue Factor Binding Site

[0262] The receptor-binding site is defined as comprising of all residues having their accessible surface area changed upon receptor binding. This is determined by at least two ASA calculations; one on the isolated ligand(s) in the ligand(s)/receptor(s) complex and one on the complete ligand(s)/receptor(s) complex.

[0263] Methods for Testing FVII and FVIIa Properties

Measurement of Functional In Vivo Half-Life

[0264] Measurement of in vivo biological half-life can be carried out in a number of ways as described in the literature.

An example of an assay for the measurement of in vivo half-life of rFVIIa or variants thereof is described in FDA reference number 96-0597. Briefly, FVII clotting activities are measured in plasma drawn prior to and during a 24-hour period after administration of the conjugate, polypeptide or composition. The median apparent volume of distribution at steady state is measured and the median clearance determined.

[0265] Measurement of Reduced Sensitivity to Proteolytic Degradation

[0266] A composition containing the conjugate (100-750 μg/ml, preferably 600 μg/ml), 1.5 mg Ca^{2+}/ml (as calcium chloride), mannitol (30 mg/ml), polysorbate 80 (0.1 mg/ml), sodium chloride (3 mg/ml) and glycyl-glycine buffer (1.3 mg/ml, pH 5.5) is prepared.

[0267] A similar composition containing wild-type rFVIIa is prepared.

[0268] The initial clotting activity or, alternatively, the initial amidolytic activity is then determined as described in the section entitled "*Method for measuring the clotting activity*" or as described in the section entitled "*Method of measuring low levels of catalytic activity*" or "*Method of measuring the catalytic activity.*"

[0269] The compositions are then incubated at 37° C. until the composition containing the wild-type rFVII has lost at least 25%, preferably at least 50%, of its initial clotting or amidolytic activity.

[0270] The clotting or amidolytic activity of the composition containing the conjugate of the invention is then measured.

[0271] The reduced sensitivity to proteolytic degradation of the conjugate of the invention as compared to wild-type rFVIIa is then expressed in percentage.

[0272] Alternative Methods for Measuring Reduced Sensitivity to Proteolytic Degradation

[0273] Proteolytic degradation can be measured using the assay described in U.S. Pat. No. 5,580,560, Example 5, where proteolysis is autoproteolysis.

[0274] Furthermore, reduced proteolysis can be tested in an in vivo model using radiolabelled samples and comparing proteolysis of wild type and conjugates by withdrawing blood samples and subjecting these to SDS-PAGE and autoradiography.

[0275] Irrespectively of the assay used for determining proteolytic degradation, "reduced proteolytic degradation" is intended to mean a measurable reduction in cleavage compared to that obtained by non-conjugated wild type FVIIa as measured by gel scanning of Coomassie stained SDS-PAGE gels, HPLC or as measured by conserved catalytic activity in comparison to wild type using the chromogenic assay described below.

Determination of the Molecular Weight of rFVII and Conjugates Thereof

[0276] The molecular weight of conjugated or unconjugated rFVII or conjugates thereof is determined by either SDS-PAGE, gel filtration, Western Blots, matrix assisted laser desorption mass spectrometry or equilibrium centrifugation, e.g., SDS-PAGE according to Laemmli, U.K., Nature Vol 227 (1970), pp. 680-85.

Method of Measuring Low Levels of Catalytic Activity

[0277] The amidolytic activity in dilute samples of FVII/FVIIa and fermentation liquid/conditioned medium can be determined using COASET® FVII (Chromogenix, Art. No 82 19 00). The amidolytic activity is determined in accordance with the manufacturer's instructions. Briefly, FX is present in surplus and converted to FXa by FVIIa at 37° C. The generated FXa then hydrolyses the chromogenic substrate S2765 (N-α-Cbo-D-Arg-Gly-Arg-pNA) resulting in liberation of the chromophoric molecule, para-nitro-anillin (pNA) absorbing light at wavelength 405 nm. The reaction is stopped by addition of acetic acid. The amount of FVII/FVIIa in the sample is determined by comparison to a standard curve prepared from FVIIa (ranging from 125 pg/ml to 1 ng/ml in assay buffer).

Method of Measuring the Catalytic Activity

[0278] The ability of the conjugates to cleave small peptide substrates can be measured using the chromogenic substrate S-2288 (D-Ile-Pro-Arg-p-nitroanilide). Recombinant FVIIa is diluted in 0.1 M Tris, 0.1 M NaCl, 5 mM $CaCl_2$, pH 8.3 containing 0.1% BSA. The reaction is started by addition of the S-2288 substrate to 1 mM and the absorption at 405 nm is measured after incubation for 30 min. at 37° C.

Method of Measuring the Clotting Activity

[0279] FVIIa activity is measured using a standard one-stage clotting assay essentially as described in WO92/15686. Briefly, the sample to be tested is diluted in 50 mM Tris (pH 7.5), 0.1% BSA and 100 μl is incubated with 100 μl of FVII deficient plasma and 200 μl of thromboplastin C containing 10 mM Ca^{++}. Clotting times are measured and compared to a standard curve using a pool of citrated normal human plasma in serial dilution.

Method of Measuring the Anticoagulant Activity

[0280] The anticoagulant activity of an inactive FVII or FVIIa conjugate can be measured using the one-stage clotting assay described above (Method of measuring the clotting activity) where the inactive conjugate competes with wild-type FVII for a limited amount of relipidated tissue factor. The assay is performed essentially as described in WO 92/15686, example III, which is hereby incorporated as reference. The ability of the inactive conjugate to prolong the clotting time of wild-type FVII is recorded and taken as a measure of anticoagulant activity.

EXAMPLES

Example 1

[0281] The X-ray structure of hFVIIa in complex with soluble tissue factor by Banner et. al., J Mol Biol, 1996; 285:2089 is used for this example. It is noted that the numbering of residues in the reference does not follow the sequence. Here we have used the sequential numbering according to SEQ ID NO:1. The gamma-carboxy glutamic acids at positions 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35 are all here named GLU (three letter abbreviation) or E (one letter abbreviation). Residues 143-152 are not present in the structure.

[0282] Surface Exposure

[0283] Performing fractional ASA calculations on FVII fragments alone combined with the definition of accessibilities of non standard and/or missing residues described in the methods resulted in the following residues having more than 25% of their side chain exposed to the surface: A1, N2, A3, F4, L5, E6, E7, L8, R9, P10, S12, L13, E14, E16, K18, E19, E20, Q21, S23, F24, E25, E26, R28, E29, F31, K32, D33, A34, E35, R36, K38, L39, W41, I42, S43, S45, G47, D48, Q49, A51, S52, S53, Q56, G58, S60, K62, D63, Q64, L65, Q66, S67, I69, F71, L73, P74, A75, E77, G78, R79', E82, T83, H84, K85, D86, D87, Q88, L89, I90, V92, N93, E94, G97, E99, S103, D104, H105, T106, G107, T108, K109, S111, R113, E116, G117, S119, L120, L121, A122, D123, G124, V125, S126, T128, P129, T130, V131, E132, I140, L141, E142, K143, R144, N145, A146, S147, K148, P149, Q150, G151, R152, G155, K157, V158, P160, K161, E163, L171, N173, G174, A175, N184, T185, I186, H193, K197, K199, N200, R202, N203, I205, S214, E215, H216, D217, G218, D219, S222, R224, S232, T233, V235, P236, G237, T238, T239, N240, H249, Q250, P251, V253, T255, D256, E265, R266, T267, E270, R271, F275, V276, R277, F278, L280, L287, L288, D289, R290, G291, A292, T293, L295, E296, N301, M306, T307, Q308, D309, L311, Q312, Q313, R315, K316, V317, G318, D319, S320, P321, N322, T324, E325, Y326, Y332, S333, D334, S336, K337, K341, G342, H351, R353, G354, Q366, G367, T370, V371, G372, R379, E385, Q388, K389, R392, S393, E394, P395, R396, P397, G398, V399, L400, L401, R402, P404 and P406.

[0284] The following residues had more than 50% of their side chain exposed to the surface: A1, A3, F4, L5, E6, E7, L8, R9, P10, E14, E16, K18, E19, E20, Q21, S23, E25, E26, E29, K32, A34, E35, R36, K38, L39, I42, S43, G47, D48, A51, S52, S53, Q56, G58, S60, K62, L65, Q66, S67, I69, F71, L73, P74, A75, E77, G78, R79, E82, H84, K85, D86, D87, Q88, L89, I90, V92, N93, E94, G97, T106, G107, T108, K109, S111, E116, S119, L121, A122, D123, G124, V131, E132, L141, E142, K143, R144, N145, A146, S147, K148, P149, Q150, G151, R152, G155, K157, P160, N173, G174, A175, K197, K199, N200, R202, S214, E215, H216, G218, R224, V235, P236, G237, T238, H249, Q250, V253, D256, T267, F275, R277, F278, L288, D289, R290, G291, A292, T293, L295, N301, M306, Q308, D309, L311, Q312, Q313, R315, K316, G318, D319, N322, E325, D334, K341, G354, G367, V371, E385, K389, R392, E394, R396, P397, G398, R402, P404 and P406.

[0285] Tissue Factor Binding Site

[0286] Performing ASA calculations the following residues in human FVII change their ASA in the complex. These residues were defined as constituting the receptor binding site: L13, K18, F31, E35, R36, L39, F40, I42, S43, S60, K62, D63, Q64, L65, I69, C70, F71, C72, L73, P74, F76, E77, G78, R79, E82, K85, Q88, I90, V92, N93, E94, R271, A274, F275, V276, R277, F278, R304, L305, M306, T307, Q308, D309, Q312, Q313, E325 and R379.

[0287] Active Site Region

[0288] The active site region is defined as any residue having at least one atom within a distance of 10 Å from any atom in the catalytic triad (residues H193, D242, S344): I153, Q167, V168, L169, L170, L171, Q176, L177, C178, G179, G180, T181, V188, V189, S190, A191, A192, H193, C194, F195, D196, K197, I198, W201, V228, I229, I230, P231, S232, T233, Y234, V235, P236, G237, T238, T239, N240, H241, D242, I243, A244, L245, L246, V281, S282, G283, W284, G285, Q286, T293, T324, E325, Y326, M327, F328, D338, S339, C340, K341, G342, D343, S344, G345, G346, P347, H348, L358, T359, G360, I361, V362, S363, W364, G365, C368, V376, Y377, T378, R379, V380, Q382, Y383, W386, L387, L400 and F405.

The Ridge of the Active Site Binding Cleft

[0289] The ridge of the active site binding cleft region was defined by visual inspection of the FVIIa structure 1FAK-.pdb as: N173, A175, K199, N200, N203, D289, R290, G291, A292, P321 and T370.

Example 2

Design of an Expression Cassette for Expression of Human Blood Coagulation Factor VII in Mammalian Cells

[0290] The DNA sequence shown in SEQ ID NO:2, encompassing the short form of the full length cDNA encoding human blood coagulation factor VII with its native short signal peptide (Hagen et al., 1986. PNAS 83:2412), was synthesized in order to facilitate high expression in mammalian cells. First the ATG start codon context was modified according to the Kozak consensus sequence (Kozak, M. *J Mol Biol* 1987 Aug. 20; 196(4):947-50), so that there is a perfect match to the consensus sequence upstream of the ATG start codon. Secondly the open reading frame of the native human blood coagulation factor cDNA was modified by making a bias in the codon usage towards the codons frequently used in highly expressed human genes. Further, two translational stop codons was inserted at the end of the open reading frame in order to facilitate efficient translational stop. The fully synthetic and expression optimized human FVII gene was assembled from 70-mer DNA oligonucleotides and finally amplified using end primers inserting BamHI and HindIII sites at the 5' and 3' ends respectively using standard PCR techniques, which resulted in the following sequence (SEQ ID NO:4):

```
ggatcccgccaccatggtcagccaggccctccgcctcctgtgcctgctcc

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gaaaagcggaacgctagcaaaccccagggccggatcgtcggcgggaaggt

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gctaccgctctcgagctgatggtgctcaacgtcccccggctgatgaccca

ggactgcctgcagcagtcccgcaaagtgggggactcccccaatatcacgg

agtatatgttttgcgctggctatagcgatggctccaaggatagctgcaag

ggggactccggcgggccccatgccacgcactatcgcgggacctggtacct

caccgggatcgtcagctggggccagggctgcgccacggtggggcactttg

gcgtctacacgcgcgtcagccagtacattgagtggctgcagaagctcatg

cggagcgaaccccggcccggggtgctcctgcgggccccttttcccttgata

aaagctt

[0291] A vector for the cloning of the generated PCR product encompassing the expression cassette for native human blood coagulation factor VII was prepared by cloning the intron from pCINeo (Promega). The synthetic intron from pCI-Neo was amplified using standard PCR conditions as described above and the primers:

CBProFpr174:
5'- AGCTGGCTAGCCACTGGGCAGGTAAGTATCA -3'
and

CBProFpr175:
5'- TGGCGGGATCCTTAAGAGCTGTAATTGAACT -3'

[0292] resulting in a 332 bp PCR fragment. The fragment was cut with NheI and BamHI before cloning into pcDNA3.1/HygR (obtained from In Vitro Gen) resulting in PF#34.

[0293] The expression cassette for native human blood coagulation factor VII was cloned between the BamHI and HindIII sites of PF#34, resulting in plasmid PF#226.

Example 3

Construction of Expression Cassettes Encoding Variant Forts of Human Blood Coagulation Factor VII that are Having an Additional In Vivo Glycosylation Site.

[0294] Sequence overhang extension (SOE) PCR was used for generating constructs having variant human blood coagulation factor open reading frames with substituted codons. In the SOE-PCR both the N-terminal part and the C-terminal part of the FVII open reading frame was first amplified in individual primary PCRs.

[0295] For example, in order to change the codons for R315 and V317 to the codons for N315 and T317 the following primers were used pair vice for the primary PCR's:

CBProFpr216: 5'-CTTAAGGATCCCGCCACCATGGTCAGCCAG-3'
and

CBProFpr229: 5'-GGAGTCCCCGGTTTTGTTGGACTGCTGC-3',
and

CBProFpr221: 5'-ACTTAAGCTTTTATCAAGGGA-3'
and

CBProFpr228: 5'-GCAGCAGTCCAACAAAACCGGGGACTCC-3'.

[0296] The primary PCR products were then combined and the terminal primers (CBProFpr216 and CBProFpr221) added resulting in the secondary full-length product encoding the desired R315N+V317T FVII variant. The secondary PCR product was trimmed with BamHI and HindIII before cloning into the vector PF#34 between the BamHI and HindIII sites resulting in PF#249.

[0297] Furthermore, in cases where the introduced mutation(s) were sufficiently close to a unique restriction endonuclease site in the expression plasmid variant genes were constructed using construction procedure encompassing a single PCR step and a subsequent cloning. For instance, the substitution K143N+N145T was introduced by use of the PCR primers:

CBProFpr226: 5'-CATTCTAGAAAACCGGACCGCTAGCAAACC-3'
and

CBProFpr221: 5'-ACTTAAGCTTTTATCAAGGGA-3'

in a single PCR reaction.

[0298] The PCR product was subsequently cloned using the restriction endo-nuclease sites XbaI and HindIII.

[0299] Using the above strategy, the following glycosylation conjugates were prepared and their amidolytic activities were tested as described in the section entitled *Method of measuring low levels of catalytic activity*. Furthermore, some of the conjugates were subjected to the one-stage clotting assay described in the section entitled *Method of measuring the clotting activity*. The obtained results are compiled in the following table.

Glycosylation Conjugate	Amidolytic activity	Clotting activity
T106N	+	+
K143N + N145T	+	nd
V253N	+	nd
R290N + A292T	–	nd
G291N	–	nd
R315N + V317T	+	+
K143N + N145T + R315N + V317T	+	nd

+: Activity measurable;
–: Activity not measurable;
nd: not determined

Example 4

Improvement of Glycosylation Site Utility by Introduction of Another Proximal (N-Terminally Located) Glycosylation Site.

[0300] In order to prevent autolysis of wild type human FVII a glycosylation site was introduced at position 315 by

making the substitutions: R315N and V317T as described above, resulting in PF#249.

[0301] Upon transfection of CHO K1 cells, using Lipofactamine 2000, low transient expression levels were obtained. Assaying the twenty-four hour transient supernatant by the amidiolytic assay, COASET® FVII, indicated that the variant was active. After selection using 400 μg/ml Hygromycin B a pool of stable clones was obtained. This pool expresses the R315N+V317T variant at approximately 0.2 μg/ml allowing for Western blotting analysis for determination of the degree of usage of the introduced glycosylation site. A twenty-four hour supernatant from the stable pool was assayed by Western blotting and revealed partial usage of the introduced glycosylation site at position 315, approximately one-half of the fully processed secreted protein is glycosylated. However, if the native glycosylation site at position 145 is moved to position 143, by making the substitutions K143N+N145T, the introduced glycosylation site at position 315 is completely glycosylated as judged by Western blotting.

Example 5

Expression of FVII in HEK 293 Cells

[0302] The cell line HEK 293 (ATCC # CRL-1573) was seeded at 20% confluence in T-25 flasks using DMEM, high glucose 10% heat inactivated FCS (Gibco/BRL Cat # 10091), 5 μg/ml phylloquinone and allowed to grow until confluent. The confluent mono cell layer was transfected with 1, 2, 5, 10 and 20 μg of the plasmid p226 described above using the Lipofectamine 2000 transfection agent (Life technologies) according to the manufactures instructions. After 24 hours post transfection a medium sample was drawn. The FVII concentration in the 24 hour transient expression experiments was on average 0.15 μg/ml.

[0303] Subsequently, selection medium containing 100 μg/ml Hygromycin B was administered to the cells. After three weeks of selection, with renewal of the medium every 3rd or 4th day, the Hygromycin resistant cells were confluent in the flasks transfected with 1 μg of plasmid DNA and 2 μg of plasmid DNA. The cells from each of the five flasks were harvested and pooled. The resulting stable pool of transfectants expressing native human blood coagulation factor VII was frozen in liquid nitrogen according to standard procedures.

Example 6

Generation of HEK293 Cells Stably Expressing FVII.

[0304] A vial of HEK293 PF#226 transfectant pool was thawed and the cells seeded in a 75 cm^2 tissue flask containing 15 ml of DMEM high glucose, 10% FCS, phylloquinone (5 μg/ml), 100 U/l penicillin, 100 μg/l streptomycin, which was used for all subsequent experiments, and grown for 24 hours. The cells were harvested, diluted and plated in 96 well micro titer plates at a cell density of 1/2 cell/well. After 12 days colonies of about 20-100 cells were present in the wells and those wells containing only one colony were labeled. After a further two days of growth an additional 100 μl medium was added to all wells. Two days later the media in all wells containing only one colony were changed. The first colonies were transferred to 25 cm^2 tissue flasks culturing after 3 days and depending on the level of confluency the colonies were transferred to 25 cm^2 tissue flasks culturing the next 11 days. When confluent in T-25 tissue flasks the medium was changed and clones allowed to secrete FVII into the growth medium for 24 hours, after which the supernatants were harvested and assayed for the presence of factor VII using the COASET FVII amidolytic assay. One clone, C18, was found to express 29 μg/ml FVII.

Example 7

[0305] Expression of FVII Glycosylation Variants with No Amidolytic Activity Capable of Inhibiting the Function of FVIIa

[0306] The expression plasmids for expression of the active site ridge mutants R290N+A292T and G291N were constructed essentially as described in example 3.

[0307] Using the amidiolytic assay COASET® FVII (see above) the ability of the two FVII glycosylation variants, R290N+A292T and G291N, to inhibit the activity of rFVIIa was evaluated. The plasmids PF#250 encoding R290N+A292T and the plasmid PF#294 encoding G291N were transfected into near confluent serum grown HEK293 cells using Lipofectamin 2000. The transfected cells were incubated at 37° C. with 5% CO_2 for three hours post transfection before the medium was changed to serum free medium (DMEM, ITS-A, ExCyte, Phylloquinone, P/S). Forty hours post transfection the conditioned medium was harvested and cleared by centrifugation for analysis.

[0308] A standard curve was made from rFVIIa: 0.0125 ng, 0.025 ng, 0.05 ng, 0.075 ng and 0.1 ng. Fifty μl aliquots of undiluted, 2-fold diluted, 5 fold diluted, 10-fold diluted and 50-fold diluted condition medium from either of the two inactive glycosylation variants R290N+A292T and G291N or conditioned medium from a mock transfection were spiked with 0.025 ng rFVIIa. When assayed in the COASET FVII assay 0.025 ng of FVIIa corresponded to a signal of OD_{405}=0.35 (first run) and OD_{405}=0.26 (second run).

[0309] The obtained results are compiled in the following table:

Glycosylation Conjugate	Dilution	OD_{405}	
Standard		0.35	0.26
Mock	50	0.38	0.19
	25	0.31	0.16
	10	0.21	0.15
	5	0.22	0.14
	1	0.08	0.07
R290N + A292T	50	0.23	—
	25	0.12	—
	10	0.07	—
	5	0.04	—
	1	0.04	—
G291N	50	—	0.16
	25	—	0.08
	10	—	0.06
	5	—	0.05
	1	—	0.04

[0310] As it appears from the above data the glycosylation conjugates G291N and R290N+A292T inhibit the function rFVIIa.

Example 8

Purification of FVII and Subsequent Activation

[0311] Purification of wild-type Factor VII and conjugates thereof was performed at 4° C. The supernatant from cells expressing the conjugate (or wild-type FVII) was sterile filtered (0.22 μm) and diluted 2 fold in cold milliQ water. EDTA was added to 5 mM, pH adjusted to 8.6 and conductivity was below 10 mS/cm. The sample was applied onto a Q-sepharose Fast Flow resin (Pharmacia) equilibrated at 4° C. in 10 mM Tris pH 8.6. After application of the sample the column washed in 10 mM Tris (pH 8.6), 150 mM NaCl, until the absorption at 280 nm reached baseline levels. Then the column was equilibrated in 10 mM Tris (pH 8.6), 100 mM NaCl. Bound conjugate (or wild-type FVII) was eluted with 10 mM Tris (pH 8.6), 100 mM NaCl, 5 mM $CaCl_2$. Fractions enriched in conjugate (or wild-type FVII) was pooled and concentrated by dialysis or by using Vivaspin concentration units (Vivascience).

[0312] Auto-activation of the conjugate (or wild-type FVII) was obtained by concentration and incubation of the eluted protein in 10 mM Tris (pH 7.8-8.6), 100 mM NaCl, 5 mM $CaCl_2$.

[0313] Alternatively, the conjugate (or wild-type FVII) was activated at 37° C. by Factor Xa coupled to CNBr-activated sepharose in 10 mM Tris (pH 7.4-8.0) 100 mM NaCl, 5 mM $CaCl_2$.

[0314] The conjugate (or wild-type FVIIa) was buffer-exchanged into a solution containing 10 mM $CaCl_2$, 50 mM NaCl, 3% mannitol, 0.05% Tween80, buffered at pH 5.6 and sterilfiltered before storage at −80° C.

Example 9

N-Terminal Pegylation of FVII

[0315] Factor VII was conjugated with methoxy polyethylene glycol (mPEG) having a molecular weight of about 5 kDa using M-PEG-CHO (M-ALD-5000, obtained from Shearwater) in a buffer containing 10 mM sodium citrate, 20 mM $CaCl_2$, 100 mM NaCl, pH 5.5. M-PEG-CHO was present in 50-100-fold molar excess, and the protein concentration was 0.2-0.5 mg/ml. The reaction was carried out in 300-1500 μl batches at room temperature for 1 hour with agitation, and $NaBH_3CN$ was added to 500-1000 fold excess and incubation continued over night with agitation at room temperature.

[0316] PEGylated FVII was buffer exchanged to buffer A (10 mM Tris pH 7.6) and applied at 4° C. on a mono Q column (Pharmacia) equilibrated in buffer A. Bound protein was eluted in a gradient from 0-100% B (10 mM Tris (pH 7.6), 500 mM NaCl) over 40 column volumes.

Example 10

Pharmakokinetic Studies in Rats

[0317] Both wild-type FVII and the conjugates of the invention are formulated in 1.3 mg/ml glycyl-glycin buffer pH 5.5 containing 1.5 mg/ml $CaCl_2$, 30 mg/ml mannitol, 0.1 mg/ml polysorbat 80 and 3 mg/ml NaCl. For determination of in vivo half-life each of the preparations are administered to Sprague-Dawley rats as one intra venous bolus injection. The injections are given slowly over about 10 seconds to reduce potential risk of heart failure due to high C^{2+} concentration. Blood samples are drawn from each of the nine anaesthetized rats at suitable intervals, e.g., 1 minute, 15 minutes, 30 minutes, 45 minutes and 1 hour after injection. The blood samples are collected in 1 ml tubes containing 50 μl Citrate-phosphate-dextrose solution with adenine (Sigma #C4431) in order to prevent coagulation. Immediately after collection the samples are stored at about 0° C. until centrifugation and subsequent collection of the citrate plasma supernatants for assay. Samples are assayed by the one-stage clotting assay as described in the section *Method of measuring the clotting activity* and the half-lives are then calculated.

[0318] While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be clear to one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention. For example, all the techniques, methods, compositions, apparatus and systems described above can be used in various combinations. All publications, patents, patent applications, or other documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication, patent, patent application, or other document were individually indicated to be incorporated by reference for all purposes.

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Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln
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His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu
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Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn
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Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser
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Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr
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Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys
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Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile
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 50                  55                  60                  65

cag agc tat atc tgc ttc tgc ctg cct gcc ttt gag ggg cgc aat tgc      357
Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn Cys
                 70                  75                  80

gaa acc cat aag gat gac cag ctg att tgc gtc aac gaa aac ggg ggc      405
Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly Gly
             85                  90                  95

tgc gag cag tac tgc agc gat cac acg ggc acg aag cgg agc tgc cgc      453
Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys Arg
        100                 105                 110

tgc cac gaa ggc tat agc ctc ctg gct gac ggg gtg tcc tgc acg ccc      501
Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr Pro
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Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg Asn
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gct agc aaa ccc cag ggc cgg atc gtc ggc ggg aag gtc tgc cct aag      597
Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro Lys
                150                 155                 160

ggg gag tgc ccc tgg cag gtc ctg ctc ctg gtc aac ggg gcc cag ctg      645
Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln Leu
            165                 170                 175

tgc ggc ggg acc ctc atc aat acc att tgg gtc gtg tcc gcc gct cac      693
Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala His
        180                 185                 190

tgc ttc gat aag att aag aat tgg cgg aac ctc atc gct gtg ctc ggc      741
Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu Gly
    195                 200                 205

gaa cac gat ctg tcc gag cat gac ggg gac gaa cag tcc cgc cgg gtg      789
Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg Val
210                 215                 220                 225

gct cag gtc atc att ccc tcc acc tat gtg cct ggc acg acc aat cac      837
Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn His
                230                 235                 240

gat atc gct ctg ctc cgc ctc cac cag ccc gtc gtg ctc acc gat cac      885
Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp His
            245                 250                 255

gtc gtg cct ctg tgc ctg cct gag cgg acc ttt agc gaa cgc acg ctg      933
Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr Leu
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gct ttc gtc cgc ttt agc ctc gtg tcc ggc tgg ggc cag ctg ctc gac      981
Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu Asp
    275                 280                 285

cgg ggc gct acc gct ctc gag ctg atg gtg ctc aac gtc ccc cgg ctg     1029
Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg Leu
290                 295                 300                 305

atg acc cag gac tgc ctg cag cag tcc cgc aaa gtg ggg gac tcc ccc     1077
Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser Pro
                310                 315                 320

aat atc acg gag tat atg ttt tgc gct ggc tat agc gat ggc tcc aag     1125
Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser Lys
            325                 330                 335

gat agc tgc aag ggg gac tcc ggc ggg ccc cat gcc acg cac tat cgc     1173
Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr Arg
        340                 345                 350

ggg acc tgg tac ctc acc ggg atc gtc agc tgg ggc cag ggc tgc gcc     1221
Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys Ala
    355                 360                 365

acg gtg ggg cac ttt ggc gtc tac acg cgc gtc agc cag tac att gag     1269
Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile Glu
370                 375                 380                 385

tgg ctg cag aag ctc atg cgg agc gaa ccc cgg ccc ggg gtg ctc ctg     1317
Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu Leu
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cgg gcc cct ttc cct tgataa                                         1338
Arg Ala Pro Phe Pro
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             20                  25                  30

Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp
         35                  40                  45

Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln
     50                  55                  60

Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn
 65                  70                  75                  80

Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly
                 85                  90                  95

Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys
            100                 105                 110

Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr
        115                 120                 125

Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg
    130                 135                 140

Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro
145                 150                 155                 160

Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln
                165                 170                 175

Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala
            180                 185                 190

His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu
        195                 200                 205

Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg
    210                 215                 220

Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn
225                 230                 235                 240

His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp
                245                 250                 255

His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr
            260                 265                 270

Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu
        275                 280                 285

Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg
    290                 295                 300

Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser
305                 310                 315                 320

Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser
                325                 330                 335

Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr
            340                 345                 350

Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys
        355                 360                 365

Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile
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Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu
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385 390 395 400

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gcgccgggcc aatgcctttc tggaagagct ccgccctggc tccctggaac gcgaatgcaa 180

agaggaacag tgcagctttg aggaagcccg ggagattttc aaagacgctg agcggaccaa 240

actgttttgg attagctata gcgatggcga tcagtgcgcc tccagccctt gccagaacgg 300

gggctcctgc aaagaccagc tgcagagcta tatctgcttc tgcctgcctg cctttgaggg 360

gcgcaattgc gaaacccata aggatgacca gctgatttgc gtcaacgaaa acgggggctg 420

cgagcagtac tgcagcgatc acacgggcac gaagcggagc tgccgctgcc acgaaggcta 480

tagcctcctg gctgacgggg tgtcctgcac gcccacggtg gaataccctt gcgggaagat 540

tcccattcta gaaaagcgga acgctagcaa accccagggc cggatcgtcg gcgggaaggt 600

ctgccctaag ggggagtgcc cctggcaggt cctgctcctg gtcaacgggg cccagctgtg 660

cggcgggacc ctcatcaata ccatttgggt cgtgtccgcc gctcactgct tcgataagat 720

taagaattgg cggaacctca tcgctgtgct cggcgaacac gatctgtccg agcatgacgg 780

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gaccaatcac gatatcgctc tgctccgcct ccaccagccc gtcgtgctca ccgatcacgt 900

cgtgcctctg tgcctgcctg agcggacctt tagcgaacgc acgctggctt tcgtccgctt 960

tagcctcgtg tccggctggg gccagctgct cgaccggggc gctaccgctc tcgagctgat 1020

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caccgggatc gtcagctggg gccagggctg cgccacggtg gggcactttg gcgtctacac 1260

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  1               5
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1.-**67**. (canceled)

68. A nucleic acid comprising a nucleotide sequence which encodes a polypeptide comprising an amino acid sequence which (a) differs from the hFVII or hFVIIa sequence SEQ ID NO:1 in 1-15 amino acid residues and (b) comprises an introduced in vivo N-glycosylation site relative to SEQ ID NO:1, wherein the introduced in vivo N-glycosylation site comprises the substitution V253N.

69. The nucleic acid of claim 68, wherein the encoded polypeptide further comprises at least one additional introduced in vivo N-glycosylation site.

70. The nucleic acid of claim 69, wherein the at least one additional introduced in vivo N-glycosylation site comprises the substitution I205S/T.

71. The nucleic acid of claim 69, wherein the at least one additional introduced in vivo N-glycosylation site comprises the substitutions R315N+V317S/T.

72. The nucleic acid of claim 69, wherein the encoded polypeptide comprises two additional introduced in vivo N-glycosylation sites comprising the substitutions I205S/T and T267N.

73. The nucleic acid of claim 69, wherein the additional introduced in vivo N-glycosylation site is introduced in a position relative to SEQ ID NO:1 occupied by an amino acid residue with more than 25% of its side chain exposed to the solvent.

74. The nucleic acid of claim 69, wherein the additional introduced in vivo N-glycosylation site comprises an amino acid substitution in a position selected from the group consisting of 28-48, 139-147, 286-294, 311-319, 338-345 and 388-406 relative to SEQ ID NO:1.

75. An expression vector comprising the nucleic acid of claim 68.

76. A host cell comprising the nucleic acid of claim 68.

77. The host cell of claim 76, wherein the host cell is a eukaryotic host cell.

78. A method of producing a conjugate, comprising

providing a culture comprising a eukaryotic host cell capable of in vivo N-glycosylation, said host cell comprising a nucleic acid comprising a nucleotide sequence which encodes a polypeptide comprising an amino acid sequence which (a) differs from the hFVII or hFVIIa sequence SEQ ID NO:1 in 1-15 amino acid residues and (b) comprises an introduced in vivo N-glycosylation site relative to SEQ ID NO:1, wherein the introduced in vivo N-glycosylation site comprises the substitution V253N, and wherein the nucleic acid is operably linked to a control sequence necessary for expression of the polypeptide in the host cell; and

culturing the culture under conditions which permit expression and N-glycosylation of the polypeptide.

* * * * *

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ORIGINAL ARTICLE

Interspecies exchange mutagenesis of the first epidermal growth factor-like domain of human factor VII

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Summary. The first epidermal growth factor-like (EGF1) domain of human factor VII (FVII) is essential for binding to tissue factor (TF). We hypothesized that the previously observed increased coagulant activity of rabbit plasma (i.e. FVII) with human TF might be explained by the five non-conserved amino acids in the rabbit vs. the human FVII EGF1 domain. Accordingly, we 'rabbitized' the human FVII EGF1 domain either by exchanging the entire EGF1 domain creating human FVII(rabEGF1) or by the single amino acid substitutions S53N, K62E, P74A, A75D and T83K. After transient expression in HEK293 cells, the recombinant FVII (rFVII) mutant proteins were analyzed for biological activity and binding affinity to human TF by competitive enzyme-linked immunosorbent assay (ELISA). Biological activity of the unpurified rFVII mutant proteins was either depressed or statistically unchanged vs. rFVII(WT). However, three of six rFVII mutant proteins had increased affinity for human TF in the rank order rFVII(rabEGF1) (3.3-fold) > rFVII(K62E) (2.9-fold) > rFVII(A75D) (1.7-fold). The mutant protein rFVII(K62E) was then permanently expressed and purified. Fully activated, purified rFVIIa(K62E) had a twofold greater clotting activity and 2.8-fold greater direct FVIIa amidolytic activity when compared with rFVIIa(WT). Quantitation of the affinity of TF binding by surface plasmon resonance indicated that the K_D of purified rFVII(K62E) for human soluble TF (sTF) was 1.5 nM compared with 7.5 nM for rFVII(WT), i.e. fivefold greater affinity. We conclude that substitution of selected amino acid residues of the FVII EGF1 domain facilitated the creation of human rFVII chimeric proteins with both enhanced biological activity and increased affinity for TF.

Keywords: coagulation, EGF, FVII, rabbit, tissue factor.

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Introduction

Factor VII (FVII), a 50-kDa glycoprotein of human plasma [1] is essential for the initiation of the clotting cascade in man [2,3]. Recent evidence has supported a role for activated FVII (FVIIa) in tissue factor (TF)-mediated signal transduction [4,5], tumor angiogenesis and metastasis [6,7] and the inflammatory response during disseminated intravascular coagulation [8]. In the quest for new anticoagulant and anti-inflammatory drugs, a number of strategies have been explored in attempts to inhibit the FVII–TF interaction. This is a formidible biological task as both zymogen FVII and FVIIa bind with high affinity to human sTF with a K_D of 7.5 and 5.1 nM, respectively [9]. New, potentially important anticoagulants include humanized monoclonal antibodies to TF [10] and variants of human sTF [11]. A different approach resulted in the description of inhibitory peptides to exosites in the heavy chain of human FVII [12,13]. To date, studies of both natural [14–16] and site-directed mutants of FVII [17] have generally described FVII mutant proteins with decreased affinity for TF with one exception being the M156Q mutation [18]. The impetus for this work was an early observation of Janson *et al*. [19] who described a fourfold increased clotting activity of rabbit-plasma FVII vs. human-plasma FVII, on incubation with human TF. As 43% of the contact area of FVII with TF lies within the FVII epidermal growth factor-like (EGF1) domain [20], we postulated that the discrepancy in FVII clotting activities noted might reside in the five amino acid residue differences between the rabbit [21] and human FVII EGF1 domains. Accordingly, we synthesized human FVII chimeras in which either the entire EGF1 domain was substituted with the rabbit EGF1 domain or each of the five rabbit amino acids was individually substituted for its human counterpart. This resulted in the synthesis of human rFVII mutant proteins with enhanced affinity for human TF and increased enzymatic activity. A preliminary report of this work was presented at the XIX Congress of the International Society on Thrombosis and Haemostasis [22].

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Experimental procedures

Reagents

Human FVII cDNA was subcloned in the *Eco*RI–*Hin*dIII site of the expression vector pCMV5 as described [15]. Cloning vector pUC19 DNA was obtained from New England Biolabs (Beverly, MA, USA). Plasmid DNA was amplified in *Escherichia coli* XL-1 Blue (Stratagene, La Jolla, CA, USA). Oligonucleotide synthesis and automated DNA sequence analysis were performed in the molecular biology facility MOBIX (McMaster University). Dulbecco's modified Eagles medium (DMEM)-Ham's F12 media, bromochloroindolyl phosphate (BCIP), nitroblue tetrazolium (NBT) and para-nitrophenyl phosphate (PNPP) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Lipofectin reagent was from InVitrogen Corp. (San Diego, CA, USA). The tissue culture medium supplement bovine albumin–insulin–transferrin (BIT9500) was from Stem Cell Technologies (Vancouver, Canada). Vitamin K_1 was purchased from EMD Biosciences (La Jolla, CA, USA). Purified plasma-derived human factor FVII (pdFVII) and pdFVIIa was from Enzyme Research Labs (South Bend, IN, USA). Purified, recombinant wild-type human factor VII [rFVII(WT)] was kindly donated by Dr R. Kelley, Genentech, Inc. (South San Francisco, CA, USA). Purified rFVIIa(WT) was also purchased from Novo Nordisk Inc. (Bagsvaerd, Denmark) or purified in our laboratory from rFVII(WT)HEK293 cell-line culture medium as required. The chromogenic peptidyl substrates Pefachrome FVIIa (CH_3SO_2-D-CHA-But-Arg-pNA.AcOH) and S-2222 were purchased from Pentapharm Ltd. (Basel, Switzerland) and Chromogenix (Westchester, OH, USA), respectively. Human thromboplastin (Thromborel S) was a product of Dade Behring (Marburg, Germany). Streptavidin conjugated to alkaline phosphatase was from Jackson Immune Research Laboratories (West Grove, PA, USA). The total-protein bicinchoninic acid (BCA) and Bradford Coomassie blue assay reagents were from Pierce Scientific Co. (Rockford, IL, USA).

Mutagenesis of the FVII EGF1 domain

Oligonucleotide site-directed mutagenesis (Clontech, Palo Alto, CA, USA) was performed on the FVII EGF1 domain in the vector pUC19 as previously described [15]. The mutagenic primers employed were S53(5′-AGT-3′) → N (5′-GTGTGCCTCA**AAC**CCATGCCAGAATG-3′), K62 (5′-AAG-3′) → E (5′-GGGCTCCTGC**GAG**GACCAGCTC-3′), P74(5′-CCT-3′) → A (5′-GCTTCTGCCTC**GCT**GCCTTC GAG-3′), A75(5′-GCC-3′) → D (5′-CTGCCTCCCT**GAC**T TCGAGGGC-3′), and T83(5′-ACG-3′) → K (5′-GCCGGAA GTGTGAG**AAA**CACAAGGATGACC-3′). Human rFVII with a rabbit EGF1 domain (Fig. 1), i.e. rFVII(rabEGF1) was created by site-directed mutagenesis using the unique restriction sites *Bst*EII and *Nsi*I at the 5′ and 3′ ends of the human FVII EGF1 domain, respectively. The *Bst*EII site was generated using the primer 5′-CTTACAGTGAT**GGTGACC**

```
        53        62          74/75      83
        ↓         ↓           ↓↓         ↓
DGDQCASSPCQNGGSCKDQLQSYICFCLPAFEGRNCET      Human
::::::: :::::::: ::.:::::::::  :::::::
DGDQCASNPCQNGGSCEDQIQSYICFCLADFEGRNCEK      Rabbit
```

Fig. 1. Alignment of the EGF1 domains of human and rabbit FVII. The N-terminal amino acid residues 46–83 of FVII were aligned using the software program GENEPRO. The five non-conserved amino acid residues at positions 53, 62, 74, 75 and 83 are highlighted in bold.

AGTGTGCCTC-3′. This base substitution did not change the amino acid sequence of human FVII. The *Nsi*I site was generated using the primer 5′-CGGAACTGTGAG**ATGCAT** AAGGATGACCAGC-3′. Creation of the new *Nsi*I restriction site also altered the amino acid sequence of human FVII changing residue T83 → M. After excision of the human FVII EGF1 domain DNA by *Bst*EII–*Nsi*I restriction endonuclease digestion and subcloning of the rabbit FVII EGF1 domain DNA in its place, the codon at position 83 was corrected from M83 → K by site-directed mutagenesis using the primer 5′-GGTCGCAACTGTGAG**AAACAC**AAGGATGACCA GC-3′. Rabbit FVII EGF1 domain DNA was prepared using rabbit FVII template cDNA [21] by a standard polymerase chain reaction utilizing the forward primer 5′-TACAATGAT**GGTGACC**AGTGTGCCTCC-3′ and the reverse primer 5′-TCTT**ATGCAT**CTCACAGTTGCGACCC TCG-3′. The fidelity of all FVII mutant DNAs were confirmed by automated DNA sequence analysis.

Mammalian cell culture and transient expression of rFVII mutant proteins

Wild-type and mutant FVII cDNAs in the vector pCMV5 were transfected into HEK293 cells using Lipofectin reagent as previously described [23]. HEK293 cells were routinely maintained in DMEM-F12 medium supplemented with 10% fetal calf serum, 100 U mL^{-1} penicillin–streptomycin and 100 ng mL^{-1} vitamin K_1. HEK293 cell-conditioned media (3 mL medium, approximately 1×10^6 cells/35 mm well, 6×35 mm wells per FVII cDNA) containing the serum-free supplement BIT9500 were collected for analysis 72 h post-transfection and concentrated sixfold using an Amicon centrifugal filtration device, 10 kDa molecular weight cut-off (Millipore Corporation, Billerica, MA, USA) prior to quantitation by FVII-specific ELISA.

Permanent expression of rFVII mutant proteins

A HEK293 cell line permanently expressing rFVII(K62E) was established essentially as described [21]. Briefly, the rFVII(K62E) mutant cDNA was subcloned into the *Eco*RI–*Hin*dIII site of the expression vector pCMV5 and co-transfected into HEK293 cells with the selection plasmid pSV2neo. After 2–3 weeks post-transfection, G418-resistant clones were assayed for synthesis of human rFVII by ELISA. Optimal FVII-synthesizing cell clones were expanded into NUNC triple-flask cell factories and the supernatant medium

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was collected weekly. Purification of rFVII from HEK293 cell-conditioned medium was greatly facilitated by the use of phenol red-free DMEM-F12 supplemented with BIT9500. Confluent HEK293 cells remained adherent to the plastic substratum and continued to synthesize rFVII normally for 3–4 weeks in the above medium.

Purification of rFVII mutant proteins

Both rFVII(WT) and rFVII(K62E) mutant protein were purified using a modification of the Q-Sepharose pseudoaffinity chromatography technique [24]. Briefly, HEK293 cell serum-free conditioned medium was collected and benzamidine and Na_2 ethylenediaminetetraacetic acid (EDTA) were added to final concentrations of 10 and 5 mM, respectively. The medium was stored frozen at −40 °C. One liter of HEK293 cell-conditioned medium was concentrated to 250 mL using a Millipore Masterflex pump and prep-scale tangential flow filter (TFF) cartridge (30 kDa molecular weight cut-off). The 250-mL concentrate was dialyzed overnight against 20 mM Tris, pH 8.0, 50 mM NaCl, 0.05% azide, 1 mM benzamidine, 1 mM EDTA at 4 °C. The dialyzed sample was readjusted to 10 mM benzamidine and 5 mM EDTA. Conductivity of the dialyzed sample was routinely < 10 μmhos. If not, distilled water was added. Chromatography was performed using a Q-Sepharose fast flow column (1.5×25 cm, bed volume 50 mL; Amersham Biosciences, Baie d'Urfe, Canada) equilibrated with 3 column volumes of 20 mM Tris pH 8.0, 50 mM NaCl, 10 mM benzamidine, 5 mM EDTA. All subsequent chromatographic steps were conducted at 4 °C. The dialyzed, concentrated medium was applied to the column at a flow rate of 2 mL min^{-1}. The column was then washed with 5 column volumes of equilibration buffer followed by 5 column volumes of equilibration buffer without EDTA. rFVII was eluted from the column with 250 mL of equilibration buffer without EDTA containing 10 mM $CaCl_2$. The majority of rFVII was eluted in the first 150 mL. Eluted rFVII was concentrated to 5–10 mL using an Amicon-stirred ultrafiltration cell with a YM10 Diaflo 10-kDa molecular weight cut-off ultrafiltration membrane (Millipore Corporation). The purity of the starting rFVII concentrate and the eluted protein were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining for total protein as well as Western blot analysis using biotinylated, polyclonal sheep anti-human rFVII IgG (described below). Western blotting was performed using streptavidin–alkaline phosphatase and the insoluble chromogenic substrates BCIP–NBT essentially as described [21]. A second passage and calcium elution from Q-sepharose was sometimes needed to achieve $> 95\%$ pure rFVII/rFVIIa.

Quantitation of FVII antigen and total protein

Total rFVII antigen levels were determined by solid-phase 'trap' ELISA in 96-well microtiter plates. Briefly, a single sheep was hyperimmunized with 300–400 μg of purified human rFVII and total sheep IgG purified as previously described [21]. Routinely, 500 ng of the above polyclonal sheep anti-human rFVII IgG was coated per microtiter well using carbonate antigen coating buffer (0.15 M Na_2CO_3, 0.35 M $NaHCO_3$, pH 9.6) overnight at 4 °C. All proteins and unknown samples were diluted in phosphate-buffered saline (PBS)-Tween buffer (25 mM Na phosphate, pH 8.0, 0.15 M NaCl, 0.05% Tween 20) containing 0.1% bovine serum albumin as a carrier protein; 100 ng per well of biotinylated polyclonal sheep anti-human rFVII IgG was employed as the detecting antibody. Biotinylated antibody binding to human rFVII was quantitated using streptavidin–alkaline phosphatase and the enzyme substrate PNPP (1 mg mL^{-1} in diethanolamine buffer, 5 mM $MgCl_2$, pH 9.8). Either purified plasma-derived human FVII or purified human rFVII were used to generate a standard curve. After subtraction of background (routinely < 0.1 OD unit) data were plotted as A_{405nm} vs. the rFVII antigen concentration. The assay was linear in the range 1–25 ng mL^{-1} rFVII antigen. Serial dilutions of normal human pooled plasma (20–30 individuals per pool) confirmed the specificity and sensitivity of the assay for FVII antigen. Quantitation of the FVII antigen in unknown samples was estimated using a single sample dilution having an A_{405nm} between 0.2 and 1.0 OD, determined in triplicate. Total protein concentrations were determined either by BCA assay or the Bradford Coomassie blue reagent.

Coagulant and amidolytic activity of rFVII mutant proteins

Coagulant activity of the various FVII samples was measured in quadruplicate by prothrombin time (PT) assay using FVII-depleted human plasma prepared in our laboratory and human thromboplastin reagent (Thromborel S) essentially as previously described [23]. Purified rFVII was quantitatively converted to rFVIIa as previously described in detail [25]. Amidolytic activity of unpurified rFVIIa in transient expression experiments was determined indirectly using the FXa chromogenic peptide substrate S-2222 as previously described [27]. Amidolytic activity of purified rFVIIa was determined directly using the FVIIa chromogenic peptide substrate Pefachrome FVIIa in a reaction mixture containing 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM $CaCl_2$, 50 ng FVIIa, human thromboplastin and 400 μM Pefachrome FVIIa essentially as described by the manufacturer. After addition of substrate the reaction rate was determined at 15′ intervals over a period of 3 h using an automated microtiter plate reader (EL808, Bio-Tek Instruments, Winooski, VT, USA). The maximum change in absorbance (ΔA_{405nm}) per hour per microgram FVIIa for each sample was calculated as the mean ± SEM from triplicate determinations.

Determination of the relative affinity of rFVII mutant proteins for TF by competitive ELISA

The binding of biotinylated, plasma-derived FVII to relipidated, full-length rTF and the quantitation of the relative affinity of

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rFVII mutant proteins for rTF by inhibition of biotinylated FVII binding (IC_{50}) has been described in detail elsewhere [25].

Determination of the absolute affinity of rFVII mutant protein for sTF by surface plasmon resonance

Binding affinities were determined by surface plasmon resonance using a BIAcore 3000 instrument (Biacore Inc., Piscataway, NJ, USA). Briefly, a non-neutralizing anti-TF monoclonal antibody 5G6 [9] was immobilized on Pioneer B1 sensor chips at high density by random coupling through amino groups as recommended by the manufacturer (Biacore Inc.). Flow cells were subsequently reacted with recombinant sTF. Dilutions of wild-type or mutant FVII molecules were then injected into the BIAcore flow cell and binding kinetics were determined as previously described [25,26]. Data from reference cells containing the same amount of anti-TF antibody but no sTF were subtracted to correct for non-specific binding.

Statistical analysis

Linear regression analysis, *t*-tests, analysis of variance and standard error of the mean were performed using the InStat 3.05 software package (GraphPad Software, San Diego, CA, USA) for Windows 98. All experiments were performed two or more times and a representative experiment was subjected to statistical analysis.

Results

DNA mutagenesis, transient expression and characterization of unpurified human rFVII EGF1 mutant proteins

Alignment of the 38 amino acids of the human and rabbit FVII EGF1 domains (Fig. 1) illustrates that there are five non-conserved amino acid residues. Using site-directed DNA mutagenesis we 'rabbitized' the human FVII molecule at each of these amino acid residues individually to create rFVII(S53N), rFVII(K62E), rFVII(P74A), rFVII(A75D) and rFVII(T83K). The collective effect of all five amino acid changes was examined by restriction endonuclease excision of the human FVII EGF1 domain DNA and substitution of the polymerase chain reaction (PCR)-generated rabbit FVII EGF1 DNA to create rFVII(rabEGF1). Three days after liposome-mediated pCMV5(rFVII) DNA transfection into HEK293 cells, transient expression of rFVII(WT) and mutant rFVII molecules was quantitated by FVII-specific ELISA. Generally, rFVII mutant proteins and rFVII(WT) were expressed at levels between 70 and 130 ng rFVII mL^{-1} culture medium with the exception of rFVII(A75D) and rFVII(rabEGF1) which were secreted at levels below 25 ng FVII mL^{-1}. Specific activities of the rFVII mutant proteins with single amino acid changes for either the peptidyl substrate S-2222 (indirect amidolytic assay) or a macromolecular substrate (PT assay) were either depressed or not statistically different from wild-type rFVII (data not shown).

Table 1 Binding of transiently expressed rFVII mutant proteins to TF

FVII mutant	Relative affinity for TF (IC_{50})	Relative increase in affinity for TF
WT	40 ± 10	1.0
S53N	106 ± 40	0.4
K62E	14 ± 4*	2.9
P74A	44 ± 20	0.9
A75D	24 ± 10	1.7
T83K	38 ± 4	1.1
rabEGF1	12 ± 6*	3.3

The data show the normalized competitive ELISA IC_{50} values (pM FVII) for inhibition of biotinylated plasma FVII binding to relipidated, full-length human TF. Data are the mean ± SEM, $n \geq 3$.
*A statistically significant difference when compared with the mean of FVII(WT), $P \leq 0.05$, using the Tukey–Kramer multiple comparisons *t*-test.

rFVII(rabEGF1) exhibited an approximate twofold increase in coagulant activity which was not of statistical significance (data not shown). However, multiple determinations of the affinity of each of the mutant rFVII proteins for immobilized human TF by competitive ELISA (Table 1) indicated that transiently expressed rFVII(K62E) and rFVII(rabEGF1) bound 2.9- and 3.3-fold more tightly than did rFVII(WT). Other rFVII EGF1 mutant proteins were either unchanged or decreased in their affinity for TF. In some experiments, the mutant protein rFVII(A75D) appeared to have enhanced affinity for TF but the data did not achieve statistical significance.

Functional analysis of purified rFVII(K62E)

rFVII(K62E) was permanently expressed in HEK293 cells and purified to homogeneity. rFVII(K62E) was >95% pure as judged by SDS-PAGE with Coomassie blue staining and Western blot analysis (data not shown). Purification of rFVII(K62E) resulted in 10–20% activation of rFVII(K62E) to rFVIIa(K62E). rFVII(WT) from Genentech was 50% activated to rFVIIa whereas pdFVII was purely in the zymogen form (data not shown). The relative affinity of purified rFVII(K62E) for full-length relipidated human TF calculated by competitive ELISA (Fig. 2) was substantially greater than either human plasma FVII (IC_{50} is 19-fold lower) or human rFVII(WT) (IC_{50} is 18-fold lower). On average, using three different preparations, rFVII(K62E) exhibited 5-fold tighter binding than rFVII(WT) in competitive ELISA experiments. These results were confirmed independently by surface plasmon resonance experimentation on two of the above FVII preparations. In this representative experiment (Table 2), testing the identical FVII preparations used in Fig. 2, the k_a of rFVII(K62E) for sTF was 5.6-fold higher than that of rFVII(WT) resulting in a K_D of rFVII(K62E) for human sTF of 1.5 nM, i.e. 5-fold lower than rFVII(WT) and more than 10-fold lower than plasma FVII. In addition to its enhanced affinity for TF, rFVIIa(K62E) exhibited a 2.0- and 5.5-fold increase in

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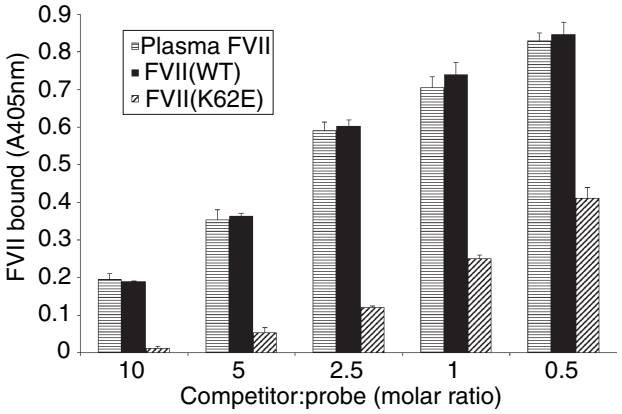


Fig. 2. Relative affinity of purified rFVII(K62E) mutant protein for TF. rFVII(WT) was from Genentech Inc. The data illustrate the inhibition of binding of biotinylated, plasma-derived zymogen FVII to full-length, relipidated human TF via competitive ELISA. Data are the mean ± SEM of triplicate determinations.

Table 2 Absolute affinity of purified FVII for human sTF determined by surface plasmon resonance

FVII sample	$k_a \times 10^5$ (M^{-1} s^{-1})	$k_d \times 10^{-3}$ (s^{-1})	K_D (nM)
rFVII(WT)	3.9 ± 0.5	2.9 ± 0.1	7.5 ± 1.1
pdFVII	1.4 ± 0.1	3.7 ± 0.1	26.0 ± 0.9
rFVII(K62E)	22.1 ± 3.5	3.2 ± 0.03	1.5 ± 0.2**

Purified rFVII(WT) was from Genentech Inc. Data are mean ± SEM. **A statistically significant difference between rFVII(K62E) and rFVII(WT), $P \leq 0.01$ using a two-tailed *t*-test, Welch-corrected. For rFVII(WT), $n = 6$; pdFVII and rFVII(K62E), $n = 3$.

Table 3 Comparative enzyme activity of purified rFVIIa

FVII sample	Clotting activity (U mg^{-1})	Indirect amidolytic assay (ΔA_{405nm} min^{-1} μg^{-1})	Direct amidolytic assay (ΔA_{405nm} h^{-1} μg^{-1})
rFVIIa (WT)	20740 ± 4680	35.3 ± 2.2	2.09 ± 0.02
pdFVIIa	7540 ± 785	36.5 ± 2.1	2.88 ± 0.06
rFVIIa (K62E)	41635 ± 385**	39.3 ± 2.4	5.89 ± 0.62**

Both rFVIIa(WT) and rFVIIa(K62E) were purified in our laboratory. Data are mean ± SEM. $n \geq 3$.
**A statistically significant difference between rFVIIa(K62E) and rFVIIa(WT) in column 1 and plasma FVIIa in column 3, $P \leq 0.01$, using the Tukey–Kramer multiple comparisons *t*-test.

coagulant activity (Table 3) when compared with rFVIIa(WT) and pdFVIIa, respectively. It is of interest to note that the amidolytic activity of rFVIIa(K62E) for the FVIIa-specific substrate Pefachrome VIIa was increased 2.8-fold vs. rFVIIa(WT), whereas there was no statistically significant difference in activity of the FVIIa preparations in the classical indirect FXa-dependent amidolytic assay. Both preparations of rFVIIa(WT) and rFVIIa(K62E) compared in Table 3 were purified in our laboratory and 100% activated to the rFVIIa form as judged by SDS-PAGE followed by Coomassie blue staining and densitometry.

Discussion

The first epidermal growth factor-like domain of human coagulation FVII is essential for high-affinity binding to its cell-surface receptor TF [14,20,27,28]. Initially, replacement of the entire human FVII EGF1 domain with the homologous rabbit FVII EGF1 region resulted in the formation of a chimeric human rFVII(rabEGF1) molecule which exhibited greater than a threefold increase in affinity for human TF (Table 1) and an approximate twofold increase in specific clotting activity with human TF relative to pdFVII (data not shown). Subsequently, we examined the effects of the five individual amino acid differences between the human and rabbit FVII EGF1 domains (Fig. 1). Transient expression of the rFVII mutants in HEK293 cells revealed that only rFVII(K62E) demonstrated a statistically significant increase in affinity for human TF (Table 1). The purified rFVII(K62E) mutant protein exhibited a fivefold increased affinity for human TF (Fig. 2, Table 2). The biological activity of rFVIIa(K62E) was elevated twofold in coagulant activity and 2.8-fold in direct amidolytic activity (Table 3) relative to human rFVIIa(WT). These data confirm and extend previous observations [19,29,30] all of which described significant differences in the affinity and/or activity of FVII for homologous vs. non-homologous TF.

We previously described two naturally occurring mutations of the FVII EGF1 domain which affect binding to TF. Both mutants rFVII(N57D) [15] and rFVII(R79Q) [14,31] exhibited a five- to ten-fold decrease in TF binding but the mechanisms of the two defects differed. Amino acid residue R79 of FVII was shown to form both hydrophobic and hydrogen bonds with TF [20], whereas the mutation FVII(N57D) did not directly alter FVII contact with TF but caused a misfolding of the FVII EGF1 domain [15]. rFVII(R79Q) mutant protein bound normally to the EGF1 conformation-sensitive monoclonal antibody 231-7 but rFVII(N57D) mutant protein did not [15]. Notably, the increased affinity of rFVII(K62E) protein for TF is associated with enhanced binding of rFVII(K62E) protein to monoclonal antibody 231-7 (data not shown), suggesting that the K → E substitution at position 62 has resulted in a conformational change in the rFVII(K62E) EGF1 domain. Although K62 is far removed from the FVII–TF interface [20], the K → E substitution potentially affects its neighboring amino acid residues D63, Q64, I69, C70 and F71 all of which either directly contact TF and/or act as ligands for the single Ca^{++} bound within the FVII EGF1 domain. Recently, reverse-charge mutations of surface-exposed amino acid residues have been correlated with faster association and tighter binding protein complexes in other biological systems [32]. Thus the increased affinity of rFVII(K62E) for TF is likely because of either electrostatic or conformational changes (or a combination of the two) in the EGF1 domain of FVII.

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Naturally occurring mutants of the EGF1 domain have not been reported for either FVII or FIX at the K62/K63 amino acid residue, respectively. Furthermore, the C–K–D tripeptide sequence is absolutely conserved in the FIX EGF1 and FX EGF1 domains across species but highly variable in protein C and FVII. The FVII EGF1 amino acid residue 62 is variously K (man), D (chicken), E (rabbit and bovine), Q (mouse and rat) and T (zebrafish) [21,33,34]. We are currently analyzing the effect of each of these interspecies EGF1 domain amino acid substitutions, i.e. K62 $\rightarrow$ X on the biological activity of FVII.

A number of studies provide evidence for reciprocal allosteric interaction(s) between the EGF1 and protease domains of FVII [35–38]. Perturbations of the FVII EGF1 domain including monoclonal antibody 231-7 binding [34] and site-directed mutagenesis affecting the high-affinity Ca++ binding site [36] have been shown to either enhance or decrease rFVIIa catalytic activity. We therefore suggest that the K62 $\rightarrow$ E amino acid substitution enhances rFVIIa activity via an allosteric effect on the protease domain. Our results are an interesting contrast to the recent data of Persson [39] and Harvey *et al*. [40], who have demonstrated that replacement of selected amino acid residues in the protease domain and Gla domain of rFVII, respectively, can substantially increase the enzymatic activity of rFVIIa without a concomittent change in the affinity of these rFVII mutants for TF.

Recombinant FVIIa is currently approved for use in hemophilia A patients with acquired inhibitors to FVIII [41]. In addition, chemically inactivated rFVIIa, termed rFVIIai, which exhibits increased affinity for TF *in vitro* [42], has been shown to be an effective inhibitor of thrombosis *in vivo* in a baboon model [8] and to enhance apoptosis of tumor cells *in vitro* [43–45]. The clinical use of human rFVIIa/rFVIIai could be greatly facilitated by the advent of mutants of rFVIIa with either increased enzymatic activity (in hemophilia) or increased affinity for TF (a better competitive inhibitor) in patients with thrombosis and cancer.

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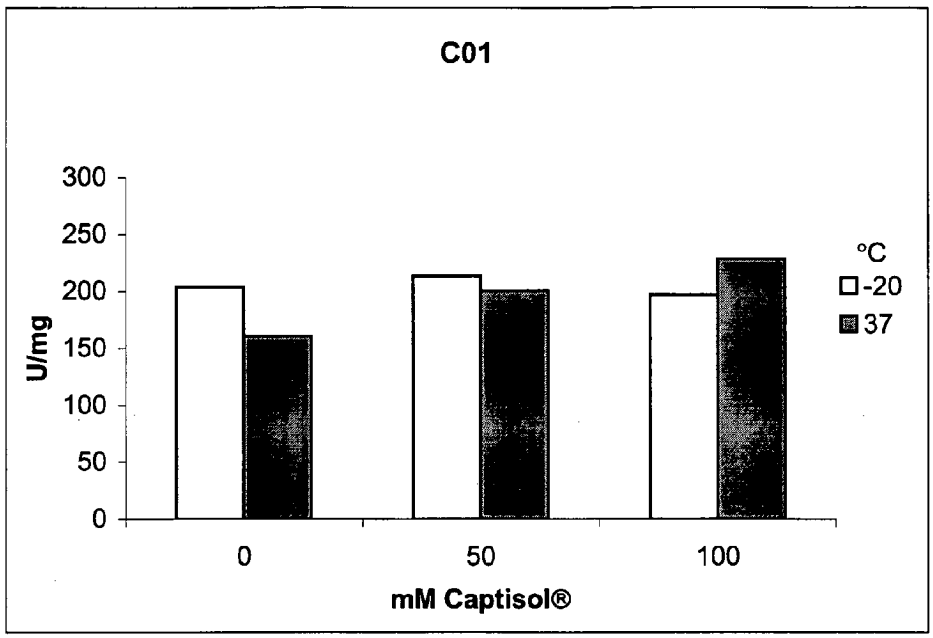
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(54) Title: FORMULATIONS OF VITAMIN K-DEPENDENT POLYPEPTIDES AND SULFOALKYL ETHER CYCLOEXTRINS



(57) Abstract: Stabilized formulations, in particular liquid formulations such as an aqueous solution, comprising a vitamin K-dependent polypeptide such as protein C or factor VII/VIIa and a sulfoalkyl ether cyclodextrin derivative, e.g. a salt of a beta cyclodextrin sulfobutyl ether.

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FORMULATIONS OF VITAMIN K-DEPENDENT POLYPEPTIDES AND SULFOALKYL ETHER CYCLOEXTRINS

FIELD OF THE INVENTION

The present invention relates to formulations of vitamin K-dependent polypeptides, in particular stabilized formulations comprising a vitamin K-dependent polypeptide such as protein C or factor VII and a sulfoalkyl ether cyclodextrin derivative.

BACKGROUND OF THE INVENTION

Blood coagulation is a process consisting of a complex interaction of various blood components, or factors, which eventually give rise to a fibrin clot. Generally, blood components participating in the coagulation "cascade" are proenzymes or zymogens, i.e. enzymatically inactive proteins that are converted into an active form by action of an activator. Regulation of blood coagulation is accomplished enzymatically by proteolytic inactivation of the procoagulation factors Va and VIIIa achieved by activated protein C (Esmon, *J Biol Chem* 1989; 264; 4743-4746).

Vitamin K-dependent proteins contain 9 to 13 gamma-carboxyglutamic acid (Gla) residues in their amino terminal 45 residues. The Gla residues are produced by enzymes in the liver that utilize vitamin K to carboxylate the side chains of glutamic acid residues in protein precursors.

Vitamin K-dependent proteins are involved in a number of biological processes, of which the most well-described is blood coagulation (reviewed in Furie et al. *Cell* (1998) 53:505-518). Vitamin K-dependent proteins include protein Z, protein S, prothrombin, factor X (FX) , factor IX (FIX) , protein C, factor VII (FVII) and Gas6. The latter protein functions in cell growth regulation (Matsubara et al. *Dev. Biol.* (1996) 180:499-510) The Gla residues are needed for proper calcium binding and membrane interaction by these proteins. Although the Gla-containing regions of these proteins show a high degree of sequence homology, they have at least a 1000-fold range in membrane affinity (McDonald et al. *Biochemistry* (1997) 36:5120-5137. Vitamin K-dependent proteins are reviewed e.g. by Nelsestuen et al., *Vitam. Horm.* (2000) 58:355-89.

Protein C is a serine protease that circulates in the plasma as a zymogen with a half-life of approximately 7 hours, and plasma levels are typically in the range of 3-5 μg/l. It is produced *in vivo* in the liver as a single chain precursor polypeptide of 461 amino acids. The protein C precursor comprises a 42 amino acid residue signal and propeptide sequence that includes a conserved 18 amino acid propeptide sequence found in all vitamin K-dependent

proteins (Stanley et al., *Biochemistry* (1999) 38:15681-7). This precursor polypeptide undergoes multiple post-translational modifications, including a) cleavage of the signal sequence and the propeptide sequence; b) cleavage of lysine and arginine residues (positions 156 and 157) to make a two-chain inactive zymogen (a 155 amino acid light chain attached via a disulfide bridge to a 262 amino acid heavy chain); c) vitamin K-dependent carboxylation of nine glutamic acid residues of the light chain resulting in nine gamma-carboxyglutamic acid residues in the N-terminal region of the light chain; and d) carbohydrate attachment at four sites (one in the light chain and three in the heavy chain). Finally, the two-chain zymogen may be activated by removal of a dodecapeptide (the activation peptide) at the N-terminus of the heavy chain (positions 158-169), producing the activated protein C (APC).

Protein C is activated by limited proteolysis by thrombin in complex with thrombomodulin on the lumenal surface of the endothelial cell. As explained above, activation liberates a 12 amino acid activation peptide from the N-terminal of the heavy chain. The APC has a half-life of approximately 15 minutes in plasma.

In the presence of its cofactor, protein S, APC proteolytically inactivates factors Va and VIIIa, thereby reducing thrombin generation (Esmon, *Thromb Haemost* 1993; 70; 29-35). Protein S circulates reversibly bound to another plasma protein, C4b-binding protein. Only free protein S serves as a cofactor for APC. Since C4b-binding protein is an acute phase reactant, the plasma levels of this protein vary greatly in many diseases and thus influence the anticoagulant activity of the protein C system.

The gene encoding human protein C maps to chromosome 2q13-q14 (Patracchini et al., *Hum Genet* 1989; 81; 191-192), spans over 11 kb, and comprises a coding region (exons II to IX) and a 5' untranslatable region encompassing exon I. The protein domains encoded by exons II to IX show considerable homology with other vitamin K-dependent coagulation proteins such as factor IX and X. Exon II codes for a signal peptide, while exon III codes for a propeptide and a 38 amino acid sequence containing 9 Glu residues. The propeptide contains a binding site for the carboxylase that transforms the Glu residues into dicarboxylic acid (Gla) able to bind calcium ions, a step required for phospholipid binding (Cheung et al., *Arch Biochem Biophys* 1989; 274; 574-581). Exons IV, V and VI encode a short connection sequence and two EGF-like domains, respectively. Exon VII encodes both a domain encompassing the 12 amino acid activation peptide and the dipeptide 156-157 which, when cleaved off, yields the mature two-chain form of the protein. Exons VIII and IX encode the serine protease domain.

The complete amino acid sequence of human protein C has been reported by Foster et al., *PNAS USA* 1986; 82; 4673-4677 and includes a signal peptide, a propeptide, a light chain, a heavy chain and an activation peptide. The sequence is available from the Swiss-Prot protein sequence database under entry name PRTC_HUMAN and primary accession number P04070.

APC is inhibited in plasma by the protein C inhibitor as well as by alpha-1-antitrypsin and alpha-2-macroglobulin.

The experimental three-dimensional structure of human APC (in a Gla-domainless form) has been determined to 2.8 Å resolution and reported by Mather et al., *EMBO J* 1996; 15; 6822-6831. The structure included a covalently bound inhibitor (D-Phe-Pro-Arg chloromethylketone, PPACK).

APC is used for the treatment of genetic and acquired protein C deficiency and has been suggested for use as an anticoagulant in patients with some forms of Lupus, following stroke or myocardial infarction, after venous thrombosis, disseminated intravascular coagulation (DIC), septic shock, emboli such as pulmonary emboli, transplantation, such as bone marrow transplantation, burns, major surgery/trauma and adult respiratory stress syndrome (ARDS).

Recombinant APC is produced by Eli Lilly and Co. and marketed under the name Xigris®. Xigris® is sold as a lyophilized powder to be reconstituted with sterile water immediately prior to administration.

Since APC is used for treatment of acute conditions such as septic shock, it would be advantageous to be able to store APC as a stable, ready-to-use formulation, e.g. an aqueous solution, that could be rapidly administered without the need to first reconstitute a dry powder in water. However, the lack of stability of APC in solution has thus far prevented the development of such stable liquid formulations.

APC is known to degrade at increasing temperature as well as elevated pH. A major degradation pathway is the cleavage of the K308-E309 peptide bond in the heavy chain, leading to two fragments of approximately equal size, with N-terminals starting at L170 and E309, respectively. The latter 111 amino acid fragment, constituting the C-terminal end of the heavy chain from E309 to P419, is referred to as the "EAK fragment" in EP 0662513 A1, which discloses methods for minimizing degradation of protein C by maintaining the molecule at a lowered pH, in a denaturing agent or at an extreme salt concentration, in particular by maintaining APC at a pH between 6.3 and 7.0. Although APC was found to have maxi-

mum amidolytic activity at pH 7.4, EP 0662513 A1 teaches reducing autodegradation by avoiding pH conditions at which APC has maximal activity, i.e. by lowering pH to below 7.0.

US 6,162,629 describes another APC degradation pathway involving autodegradation on either side of H10 near the N-terminal of the light chain, resulting in removal of the first nine or ten residues of the light chain, including Gla residues at positions 6 and 7 that are involved in anticoagulation. It is described that at low pH of less than 6.3 this autodegradation pathway at the N-terminal of the light chain predominates over autodegradation between residues 308-309 of the heavy chain, but that the N-terminal autodegradation can be reduced by use of a pH of 5.5-6.3, preferably about 6.0, in combination with an ionic strength of greater than 150 mM. Although the use of low pH and a high ionic strength reportedly reduced the autodegradation of APC, it is worth noting that processing of APC under these conditions is described as in the context of ultimately isolating the protein in lyophilized form.

Thus, in spite of the clinical advantages that would be provided by a stable, liquid formulation of activated protein C, such formulations have yet to be developed.

FVII is synthesized in the liver and secreted into the blood as a single-chain glycoprotein with a molecular weight of 53 kDa (Broze & Majerus, *J. Biol. Chem.* 1980; 255:1242-1247). The FVII zymogen is converted into an activated form (FVIIa) by proteolytic cleavage at a single site, R152-I153, resulting in two chains linked by a single disulfide bridge. FVIIa in complex with tissue factor (TF) is able to convert both FIX and FX into their activated forms, followed by reactions leading to rapid thrombin production and fibrin formation (Østerud & Rapaport, *PNAS USA* 1977; 74:5260-5264).

FVII undergoes post-translational modifications, including vitamin K-dependent carboxylation resulting in ten γ-carboxyglutamic acid residues in the N-terminal region of the molecule. Thus, residues number 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35 shown in SEQ ID NO:2 are γ-carboxyglutamic acids residues in the Gla domain important for FVII activity. Other post-translational modifications include sugar moiety attachment at two naturally occurring N-glycosylation sites at positions 145 and 322, respectively, and at two naturally occurring O-glycosylation sites at positions 52 and 60, respectively.

The gene coding for human FVII (hFVII) has been mapped to chromosome 13 at q34-qter 9 (de Grouchy et al., *Hum Genet* 1984; 66:230-233). It contains nine exons and spans 12.8 Kb (O'Hara et al., *PNAS USA* 1987; 84:5158-5162). The gene organisation and protein structure of FVII are similar to those of other vitamin K-dependent procoagulant proteins, with exons 1a and 1b encoding for signal sequence; exon 2 the propeptide and Gla do-

main; exon 3 a short hydrophobic region; exons 4 and 5 the epidermal growth factor-like domains; and exons 6 through 8 the serine protease catalytic domain (Yoshitake et al., *Biochemistry* 1985; 24: 3736-3750).

Reports exist on experimental three-dimensional structures of human FVIIa (hFVIIa) (Pike et al., *PNAS USA*, 1999; 96:8925-30 and Kemball-Cook et al., *J. Struct. Biol.*, 1999; 127:213-223), of hFVIIa in complex with soluble tissue factor using X-ray crystallographic methods (Banner et al., *Nature*, 1996; 380:41 and Zhang et al., *J. Mol. Biol.*, 1999; 285: 2089), and of smaller fragments of hFVII (Muranyi et al., *Biochemistry*, 1998; 37:10605 and Kao et al., *Biochemistry*, 1999; 38:7097).

Some protein-engineered variants of FVII have been reported (Dickinson & Ruf, J *Biol. Chem.*, 1997;272:19875-19879, Kemball-Cook et al., *J. Biol. Chem.*, 1998; 273:8516-8521, Bharadwaj et al., *J. Biol. Chem.*, 1996; 271:30685-30691, Ruf et al., *Biochemistry*, 1999; 38:1957-1966).

Reports exist on expression of FVII in BHK or other mammalian cells (WO 92/15686, WO 91/11514 and WO 88/10295) and co-expression of FVII and kex2 endoprotease in eukaryotic cells (WO 00/28065).

An inactive form of FVII in which arginine 152 and/or isoleucine 153 is/are modified has been reported in WO 91/11514. These amino acids are located at the activation site. WO 96/12800 describes inactivation of FVIIa by a serine proteinase inhibitor. Inactivation by carbamylation of FVIIa at the α-amino acid group I153 has been described by Petersen et al., *Eur. J. Biochem.*, 1999;261:124-129. The inactivated form is capable of competing with hFVII or hFVIIa for binding to TF and inhibiting clotting activity. The inactivated form of FVIIa is suggested to be used for treatment of patients in a hypercoagulable state, such as patients with sepsis, or at risk of myocardial infarction or thrombotic stroke.

WO 98/32466 suggests that FVII, among many other proteins, may be PEGylated (i.e. bound to polyethylene glycol) but does not contain any further information in this respect. WO 01/58935 discloses a new strategy for developing FVII or FVIIa molecules having *inter alia* an increased half-life by means of directed conjugation.

Commercial preparations of recombinant human FVIIa (rhFVIIa) are sold under the trade name NovoSeven®. NovoSeven® is indicated for the treatment of bleeding episodes in hemophilia A or B patients and is currently the only rhFVIIa product available for effective and reliable treatment of bleeding episodes. NovoSeven® is a freeze-dried FVIIa product which is reconstituted before use; it contains a relatively low FVIIa concentration. A vial (1.2 mg) of NovoSeven® contains 1.2 mg rhFVIIa, 5.84 mg NaCl, 2.94 mg $CaCl_2,2H_2O$, 2.64 mg

glycylglycine, 0.14 mg polysorbate 80 and 60.0 mg mannitol; it is reconstituted to pH 5.5 by 2.0 ml water for injection. When reconstituted, the protein is stable for use for 24 hours. Accordingly, no liquid ready-for-use or concentrated FVII products are currently commercially available.

A stable soluble preparation of FVIIa would provide the advantage of easier handling for patients, medical care personnel, etc. Further, in case of emergencies such as severe bleeding caused e.g. by acute trauma or surgery, a ready-to-use soluble formulation of FVIIa would be able to be administered quicker than a dry formulation that must first be reconstituted with water, which could potentially be life saving and/or prevent complications caused by loss of blood.

Attempts to prevent proteolytic degradation by site-directed mutagenesis at major proteolytic sites are disclosed in WO 88/10295, and attempts to prepare stabilized liquid compositions of FVIIa are disclosed in WO 03/055511 and WO 03/055512. Nevertheless, in spite of the clinical advantages that would be provided by a stable, liquid formulation of FVIIa, such formulations have yet to be developed.

BRIEF DISCLOSURE OF THE INVENTION

The object of the present invention is to provide a stabilized formulation for vitamin K-dependent polypeptides, preferably a liquid formulation having a sufficient stability to allow storage in liquid form for a desired length of time, without the need for e.g. freezing or freeze-drying, while maintaining a sufficient level of protein activity. This would e.g. make it possible to rapidly administer the vitamin K-dependent polypeptide to a patient without first having to reconstitute a freeze-dried powder in water, thus saving valuable time in acute clinical situations.

Surprisingly, it has been found that adding a sulfoalkyl ether cyclodextrin derivative to a liquid formulation of a vitamin K-dependent polypeptide in its activated form results in improved stability of the polypeptide. In a first aspect, the invention thus relates to a composition comprising a vitamin K-dependent polypeptide and a sulfoalkyl ether cyclodextrin derivative.

In another aspect, the invention relates to a method for increasing the stability of a vitamin K-dependent polypeptide in a pharmaceutical composition, the method comprising incorporating into said composition a sulfoalkyl ether cyclodextrin derivative and optionally at least one buffering agent.

In an additional aspect, the invention relates to a method for preparing the above composition, comprising mixing a vitamin K-dependent polypeptide with a sulfoalkyl ether cyclodextrin derivative.

In further aspects, the invention relates to a method for treating a condition treatable by administration of a vitamin K-dependent polypeptide comprising administering to a patient in need thereof a therapeutically effective amount of a composition of the invention; and use of a composition of the invention for the manufacture of a medicament for treatment of a condition treatable by administration of a vitamin K-dependent polypeptide.

In a particular aspect, the invention relates to a composition comprising an activated protein C polypeptide and a sulfoalkyl ether cyclodextrin derivative; a method for increasing the stability of an activated protein C polypeptide in a pharmaceutical composition, the method comprising incorporating into said composition a sulfoalkyl ether cyclodextrin derivative and optionally at least one buffering agent; a method of treating a condition treatable by administration of activated protein C, e.g. sepsis or septic shock, comprising administering to a patient in need thereof a therapeutically effective amount of a composition of the invention comprising APC; and use of a composition of the invention for the manufacture of a medicament for treatment of a condition treatable by administration of activated protein C.

In another particular aspect, the invention relates to a composition comprising a factor VIIa polypeptide and a sulfoalkyl ether cyclodextrin derivative; a method for increasing the stability of a factor VIIa polypeptide in a pharmaceutical composition, the method comprising incorporating into said composition a sulfoalkyl ether cyclodextrin derivative and optionally at least one buffering agent; a method of treating a condition treatable by administration of FVIIa, e.g. patients with clotting factor deficiencies (such as hemophilia A or B or deficiency of clotting factors XI or VII) or trauma patients, comprising administering to a patient in need thereof a therapeutically effective amount of a composition of the invention comprising FVIIa; and use of a composition of the invention for the manufacture of a medicament for treatment of a condition treatable by administration of FVIIa.

DRAWING DESCRIPTION

Figures 1-4 show the amidolytic activity of APC samples treated with 0, 50 or 100 mM Captisol®. Samples stored at –20°C (white) or 37° (shaded) were analyzed for their ability to cleave a synthetic substrate. Figure 1 shows the activity of Xigris®, Figure 2 shows the activity of recombinant APC having the wild-type sequence ("WT"), Figure 3 shows the activity of the APC variant "C01", and Figure 4 shows the activity of APC variant "C02".

DETAILED DISCLOSURE OF THE INVENTION

Definitions

In the context of the present application the following definitions apply:

The term "stabilized" is intended to mean that the composition has increased storage stability as compared to a composition which does not comprise the sulfoalkyl ether cyclodextrin derivative. For instance, the increased storage stability may be observed in a liquid formulation stored as such or stored in a frozen state and thawed prior to use, in a dried form, e.g. lyophilized, spray-dried or air-dried form, for later reconstitution into a liquid form prior to use, in a solid form, e.g. intended for pulmonary or nasal delivery, and/or in any other form, e.g. made for a special drug delivery system (such as microspheres or the like). The increased storage stability may e.g. be measured in terms of increased activity as compared to a reference composition when subjected to the same storage conditions and/or in terms of reduced aggregate formation. The increased storage stability is thus intended to comprise physical stability, e.g. reduced aggregate formation, and/or chemical stability, e.g. reduced degradation.

The term "activity" is intended to mean an *in vitro* and/or *in vivo* activity as determined by any suitable assay known to be relevant for the vitamin K-dependent polypeptide in question. For APC, an example of a suitable activity assay is the analysis for enzyme activity (amidolytic activity) described below. Suitable activity assays for FVIIa are also described below.

The term "aggregate formation" is intended to mean a physical interaction between vitamin K-dependent polypeptides that results in formation of oligomers. Aggregate formation is undesirable since in most cases this leads to reduced or even lost activity and/or increased immunogenicity.

The term "protein C precursor" refers to the DNA-encoded form of protein C that includes an 18-residue propeptide, the light chain (residues 1-155), the Lys-Arg dipeptide (residues 156-157) and the heavy chain (residues 158-419), including the activation peptide (residues 158-169). The protein C precursor may also include a signal peptide, e.g. the native signal peptide of human protein C, or alternatively an altered version of the human protein C signal peptide or a heterologous signal peptide selected according the particular expression system used.

The term "one-chain zymogen protein C" refers to the one-chain inactive form of protein C, which includes the light chain (residues 1-155), the Lys-Arg dipeptide (residues

156-157), and the heavy chain (residues 158-419), including the activation peptide (residues 158-169), as shown in SEQ ID NO:1.

The term "two-chain zymogen protein C" refers to the two-chain inactive form of protein C, which includes the light chain (residues 1-155) and the heavy chain (residues 158-419), including the activation peptide (residues 158-169) (but without the Lys-Arg dipeptide between the light chain and the heavy chain), shown in SEQ ID NO:1. The term "zymogen protein C" is intended to refer to both the one-chain form and the two-chain form of the zymogen protein C.

The terms "activated protein C", "activated human protein C", "APC" or "human APC" are used about the activated zymogen and include the light chain (residues 1-155) and the heavy chain without the activation peptide (i.e. residues 170-419) of SEQ ID NO:1. The amino acid sequence of activated protein C may be referred to as "the APC part" of the amino acid sequence of SEQ ID NO:1.

The term "protein C" encompasses all of the above-mentioned forms of protein C, i.e. the "protein C precursor" form, the "zymogen protein C" form (the one-chain form as well as the two-chain form) and "activated protein C", as well as variants thereof.

The "Gla domain" of protein C comprises amino acid residues 1-45 of SEQ ID NO:1.

The "EGF domains" of protein C comprise amino acid residues 55-134 of SEQ ID NO:1.

The "active site region" of protein C is defined as including those amino acid residues that are described as belonging to the active site in WO 02/32461, namely: L170, I171, D172, G173, Q184, V185, V186, L187, L188, D189, S190, K191, K192, K193, L194, A195, C196, G197, A198, T208, A209, A210, H211, C212, M213, D214, E215, S216, K217, K218, L219, L220, L228, I240, V243, V245, N248, Y249, S250, K251, S252, T253, T254, D255, N256, D257, I258, A259, L261, T295, L296, V297, T298, G299, W300, G301, Y302, H303, S304, S305, R306, E307, K308, E309, A310, K311, R312, N313, R314, T315, F316, I321, I323, P324, V326, C331, V334, M335, S336, N337, M338, V339, M343, L344, C345, A346, G347, I348, L349, D351, R352, Q353, D354, A355, C356, E357, G358, D359, S360, G361, G362, P363, M364, G376, L377, V378, S379, W380, G381, E382, G383, C384, G385, L386, L387, H388, N389, Y390, G391, V392, Y393 and T394.

The term "FVII" or "FVII polypeptide" refers to a FVII molecule provided in single chain form and includes human wild-type FVII as well as variants thereof. The term "FVIIa" or "FVIIa polypeptide" refers to a FVIIa molecule provided in its activated two-chain form

and includes human wild-type FVIIa as well as variants thereof. When the amino acid sequence of SEQ ID NO:2 is used to describe the amino acid sequence of FVIIa it will be understood that the peptide bond between R152 and I153 of the single-chain form has been cleaved, and that one of the chains comprises amino acid residues 1-152, the other chain amino acid residues 153-406.

The terms "rFVII" and "rFVIIa" refer to FVII and FVIIa polypeptides produced by recombinant techniques.

The terms "hFVII" and "hFVIIa" refer to human wild-type FVII and FVIIa, respectively, where hFVII has the amino acid sequence shown in SEQ ID NO:2

The terms "rhFVII" and "rhFVIIa" refer to human wild-type FVII and FVIIa, respectively, produced by recombinant means. An example of rhFVIIa is NovoSeven®.

The term "vitamin K-dependent polypeptide" is intended to cover any polypeptide which requires vitamin K as a cofactor for carboxylation of glutamic acid residues, including precursor, activated or variant forms thereof. The formation of Gla residues in vitamin K-dependent polypeptides is necessary to allow the protein to chelate calcium ions and hence exert its biological activity. Specific examples of vitamin K-dependent polypeptides include protein C, FVII, FIX, protein S, protein Z, prothrombin, FX and Gas6 (including activated and/or variant forms thereof).

The term "amino acid residue" is intended to include any natural or synthetic amino acid residue, and is primarily intended to indicate an amino acid residue contained in the group consisting of the 20 naturally occurring amino acids, i.e. selected from the group consisting of alanine (Ala or A), cysteine (Cys or C), aspartic acid (Asp or D), glutamic acid (Glu or E), phenylalanine (Phe or F), glycine (Gly or G), histidine (His or H), isoleucine (Ile or I), lysine (Lys or K), leucine (Leu or L), methionine (Met or M), asparagine (Asn or N), proline (Pro or P), glutamine (Gln or Q), arginine (Arg or R), serine (Ser or S), threonine (Thr or T), valine (Val or V), tryptophan (Trp or W) and tyrosine (Tyr or Y) residues.

The terminology used for identifying amino acid positions/substitutions is illustrated as follows with reference to the sequence of human protein C (SEQ ID NO:1) as an example: A39 in a given amino acid sequence indicates that position number 39 is occupied by an alanine residue. A39S indicates that the alanine residue of position 39 is substituted with a serine residue. Alternative substitutions are indicated with a "/", e.g., A39S/T means that the alanine residue of position 39 is substituted with either a serine residue or a threonine residue. Multiple substitutions are indicated with a "+", e.g., A39S+K251N means that the alanine residue of position 39 is substituted with a serine residue and that the lysine residue in posi-

tion 251 is substituted with an asparagine residue. The insertion of an additional amino acid residue is indicated in the following way: Insertion of a serine residue after A39 is indicated by A39AS. An insertion may alternatively be indicated by an asterisk, e.g. addition of an arginine residue after position 156 may be indicated by *156R. Deletion of an amino acid residue is indicated by an asterisk. For example, deletion of the alanine residue in position 39 is indicated by A39*.

The term "non-polypeptide moiety" refers to a non-polypeptide molecule that is capable of conjugating to an attachment group of the vitamin K-dependent polypeptide. Examples of such non-polypeptide moieties include polymer molecules, sugar moieties, lipophilic compounds and organic derivatizing agents. The non-polypeptide moiety can be directly covalently joined to the attachment group or it can be indirectly covalently joined to the attachment group through an intervening moiety, such as a bridge, spacer or linker moiety or moieties. Preferred examples of non-polypeptide moieties are a polymer molecule, in particular a linear or branched polyethylene glycol (PEG) or other polyalkylene glycol, and a sugar moiety, in particular an N- or O-linked oligosaccharide generally attached by *in vivo* glycosylation, typically an N-linked oligosaccharide.

The term "differs" or "differs from" refers to the fact that the vitamin K-dependent polypeptides in the compositions of the invention may comprise various substitutions, insertions, additions or deletions in relation to the native (typically human wild-type) polypeptide. Such alterations, e.g. with the aim of introducing at least one site for conjugation to a non-polypeptide moiety, may e.g. be performed with the aim of improving or altering the effect of the polypeptide or increasing the half-life. For APC, desired alterations/improvements may include increasing the anti-inflammatory effect and/or lowering the anticoagulant activity of the variant. For FVII, desired alterations/improvements may include increasing the phospholipid membrane-binding properties and/or the clotting activity. Additional alterations may further include, for example, truncation of the N- and/or C-terminus by one or more amino acid residues, or addition of one or more extra residues at the N- and/or C-terminus, e.g. addition of a methionine residue at the N-terminus as well as "conservative amino acid substitutions", i.e. substitutions performed within groups of amino acids with similar characteristics, e.g. small amino acids, acidic amino acids, polar amino acids, basic amino acids, hydrophobic amino acids and aromatic amino acids.

Examples of conservative substitutions include amino acids within the respective groups listed in the table below.

1	Alanine (A)	Glycine (G)	Serine (S)	Threonine (T)
2	Aspartic acid (D)	Glutamic acid (E)		
3	Asparagine (N)	Glutamine (Q)		
4	Arginine (R)	Histidine (H)	Lysine (K)	
5	Isoleucine (I)	Leucine (L)	Methionine (M)	Valine (V)
6	Phenylalanine (F)	Tyrosine (Y)	Tryptophan (W)	

Vitamin K-dependent polypeptides that may be used in the composition of the invention thus include not only the human form of such vitamin K-dependent polypeptides, but also variants thereof.

The term "variant" is intended to cover a vitamin K-dependent polypeptide that differs in one or more amino acid residues from its parent polypeptide, normally in 1-15 amino acid residues (such as in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acid residues), e.g. in 1-10, 1-8, 1-6, 1-5, 1-4 or 1-3 amino acid residues, e.g. one or two amino acid residues. The parent polypeptide in the present context is in particular the wild-type human form of the vitamin K-dependent polypeptide, such as human protein C (SEQ ID NO:1) or human FVII (SEQ ID NO:2), or the activated form of a wild-type human vitamin K-dependent polypeptide, e.g. the APC part of SEQ ID NO:1.

The term "modified" or "modification" includes a substitution, an insertion or a deletion.

The term "introduce" is primarily intended to mean substitution of an existing amino acid residue, but may also mean insertion of an additional amino acid residue.

The term "remove" is primarily intended to mean substitution of the amino acid residue to be removed with another amino acid residue, but may also mean deletion (without substitution) of the amino acid residue to be removed.

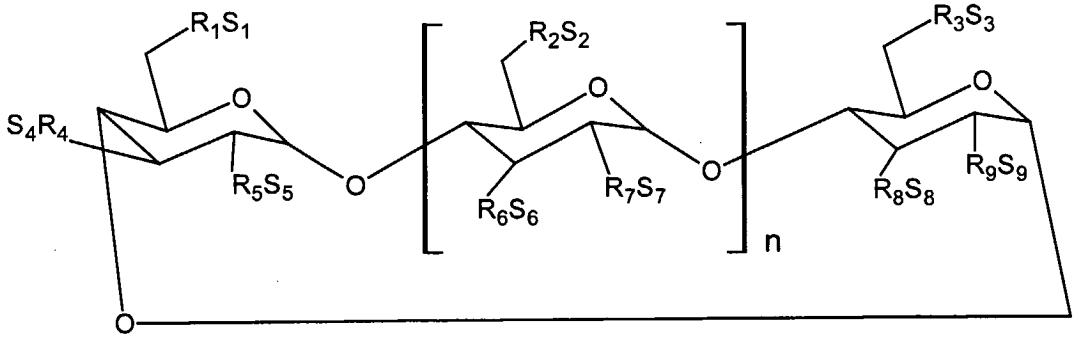
The term "nucleotide sequence" is intended to indicate a consecutive stretch of two or more nucleotide molecules. The nucleotide sequence may be of genomic, cDNA, RNA, semi-synthetic or synthetic origin, or any combination thereof.

In the present description and claims, any reference to "a" component, e.g. in the context of a non-polypeptide moiety, an amino acid residue, a substitution, a buffer, a cation, etc., is intended to refer to one or more of such components, unless stated otherwise or unless it is clear from the particular context that this is not the case. For example, the expression "a component selected from the group consisting of A, B and C" is intended to include all combinations of A, B and C, i.e. A, B, C, A+B, A+C, B+C or A+B+C.

The sulfoalkyl ether cyclodextrin derivative

Sulfoalkyl ether cyclodextrin derivatives suitable for use in the compositions of the invention include any of the derivatives described in WO 91/11172, US 5,134,127, US 5,376,645 and US 5,874,418, the contents of which are incorporated herein by reference.

In a preferred embodiment, the sulfoalkyl ether cyclodextrin derivative is a compound of the following formula (I):



wherein:

n is 4, 5 or 6,

R_1, R_2, R_3, R_4, R_5, R_6, R_7, R_8, and R_9 are each independently -O- or a -O-(C_2-C_6 alkyl)-SO_3- group, wherein at least one of R_1 and R_2 is an -O-(C_2-C_6 alkyl)-SO_3- group, and

S_1, S_2, S_3, S_4, S_5, S_6, S_7, S_8, and S_9 are each independently a pharmaceutically acceptable cation.

In one particular embodiment of the compound of formula (I), n is 4 ("alpha cyclodextrin"). In another embodiment of the compound of formula (I), n is 5 ("beta cyclodextrin"). In a further embodiment of the compound of formula (I), n is 6 ("gamma cyclodextrin"). Preferably, the compound is a derivative of beta cyclodextrin.

In a further embodiment of the compound of formula (I) at least one of R_1 and R_2 is -O-$(CH_2)_m$-SO_3-, and m is 2, 3, 4, 5 or 6.

In a further embodiment of the compound of formula (I) each of R_1 and R_2 are independently selected from -$OCH_2CH_2CH_2SO_3$- and -$OCH_2CH_2CH_2CH_2SO_3$-.

In a further embodiment of the compound of formula (I) at least one of R_4, R_6, and R_8 is independently -O-(C_2-C_6 alkyl)-SO_3-; and R_5, R_7, and R_9 are all -O-.

In a further embodiment of the compound of formula (I) S_1, S_2, S_3, S_4, S_5, S_6, S_7, S_8 and S_9 are each independently a pharmaceutically acceptable cation selected from H^+, an alkali metal (e.g. Li^+, Na^+, K^+), an alkaline earth metal (e.g., Ca^{+2}, Mg^{+2}), an ammonium ion

and an amine cation such as the cations of (C_1-C_6) alkylamines, piperidine, pyrazine, (C_1-C_6) alkanolamine and (C_4-C_8)cycloalkanolamine.

In a further embodiment of the compound of formula (I) S_1, S_2, S_3, S_4, S_5, S_6, S_7, S_8 and S_9 are independently selected from H^+, an alkali metal cation, an alkaline earth metal cation, a quaternary ammonium cation, a tertiary ammonium cation, and a secondary ammonium cation.

In a further embodiment at least one of R_4, R_6, and R_8 is independently -O-(C_2-C_6 alkyl)-SO_3-; and R_5, R_7, and R_9 are all -O-.

The "alkyl" group as used herein may be linear, cyclic or branched, and saturated or unsaturated (i.e., containing at least one double bond), including divalent alkylene groups and monovalent alkyl groups, respectively. The term "alkanol" likewise includes linear, cyclic and branched, as well as saturated and unsaturated alkyl components of the alkanol groups, in which the hydroxyl groups may be situated at any position on the alkyl moiety. The term "cycloalkanol" includes unsubstituted or substituted (e.g., by methyl or ethyl) cyclic alcohols.

The presently preferred sulfoalkyl ether cyclodextrin derivative is a salt of beta cyclodextrin sulfobutyl ether, e.g. based on any one or more of the pharmaceutically acceptable cations listed above. In particular, it may be in the form of an alkali metal or an alkaline earth metal salt, such as a lithium salt, a sodium salt, a potassium salt, a calcium salt or a magnesium salt, or an ammonium salt. An example of a preferred salt form is a sodium salt. A suitable beta cyclodextrin sulfobutyl ether is commercially available as Captisol® from CyDex Inc., Overland Park, Kansas, USA). Captisol®, also termed SBE7-β-CD, is in the form of a sodium salt having approximately seven sulfobutyl ether substitutions per molecule.

The sulfoalkyl ether cyclodextrin derivative will generally be present in compositions of the invention in a concentration of from about 1 mg/ml to about 200 mg/ml. Typically, the concentration of the sulfoalkyl ether cyclodextrin derivative will be at least about 10 mg/ml, such as at least about 20 mg/ml, at least about 30 mg/ml or at least about 40 mg/ml, more typically at least about 50 mg/ml, and up to about 190 mg/ml, such as up to about 180 mg/ml, up to about 170 mg/ml, up to about 160 mg/ml or up to about 150 mg/ml. The sulfoalkyl ether cyclodextrin derivative may thus be present in a concentration range of, for example, about 10-190 mg/ml, 20-180 mg/ml, 30-170 mg/ml or 40-160 mg/ml. A more typical concentration will be in the range of about 50-150 mg/ml.

Compositions of the invention comprising a vitamin K-dependent polypeptide and a sulfoalkyl ether cyclodextrin derivative will generally have a pH in the range of from about 5

to about 8, such as a range of from about 5 to about 7, about 6 to about 7, about 6 to about 8, or about 7 to about 8. Surprisingly, it has been found that use of a sulfobutyl ether cyclodextrin derivative (Captisol®) results in improved stability of liquid formulations of protein C at a pH of above 7. In one particular embodiment, the composition of the invention thus has a pH of above about 7.0, for example from about 7.1 to about 8.5, preferably from about 7.2 to about 8.0, such as from about 7.2 to about 7.8 or about 7.2 to about 7.6.

It will be apparent that any reference to "a" or "the" sulfoalkyl ether cyclodextrin derivative is intended to refer to at least one sulfoalkyl ether cyclodextrin derivative, i.e. the invention is intended to encompass compositions comprising more than one such derivative, even though the compositions of the invention will typically contain only a single sulfoalkyl ether cyclodextrin derivative.

Vitamin K-dependent polypeptides

As explained above, the vitamin K-dependent polypeptide is in particular selected from the group consisting of protein C, FVII, FIX, protein S, protein Z, prothrombin, FX and Gas6 (including activated and/or variant forms thereof).

In one aspect, the invention thus relates to a composition that comprises a sulfoalkyl ether cyclodextrin derivative and a vitamin K-dependent polypeptide selected from the group consisting of:

- human wild-type protein C, preferably in its activated form, or a variant thereof comprising 1-15 amino acid modifications relative to the amino acid sequence of human wild-type protein C,
- human wild-type FVII, preferably in its activated form, or a variant thereof comprising 1-15 amino acid modifications relative to the amino acid sequence of human wild-type FVII,
- human wild-type Factor IX (FIX), preferably in its activated form, or a variant thereof comprising 1-15 amino acid modifications relative to the amino acid sequence of human wild-type FIX,
- human wild-type protein S, preferably in its activated form, or a variant thereof comprising 1-15 amino acid modifications relative to the amino acid sequence of human wild-type protein S,
- human wild-type protein Z, preferably in its activated form, or a variant thereof comprising 1-15 amino acid modifications relative to the amino acid sequence of human wild-type protein Z,

- human wild-type prothrombin, preferably in its activated form, or a variant thereof comprising 1-15 amino acid modifications relative to the amino acid sequence of human wild-type prothrombin,
- human wild-type Factor X (FX), preferably in its activated form, or a variant thereof comprising 1-15 amino acid modifications relative to the amino acid sequence of human wild-type FX, and
- human wild-type Gas6, preferably in its activated form, or a variant thereof comprising 1-15 amino acid modifications relative to the amino acid sequence of human wild-type Gas6.

Examples of variants of the above-mentioned vitamin K-dependent polypeptides are given in US 6,017,882. Thus, the vitamin K-dependent polypeptides mentioned above may comprise at least one amino acid substitution in the Gla domain, such as at least substitution in a position selected from the group consisting of position 10, 11, 28, 32 and 33. Preferably, the amino acid substitution is at amino acid 12, 32 or 33. It will be understood that since FIX contains one additional amino acid residue in the Gla domain, the above-mentioned positions must be corrected accordingly, i.e. in case of FIX the at least one substitution is preferably selected from the group consisting of position 11, 12, 29, 33 and 34, in particular position 11, 33 or 34. These substitutions may be either conservative or non-conservative. Non-conservative amino acid substitutions replace an amino acid with an amino acid of a different class and may result in a substantial change in the hydrophobicity of the vitamin K-dependent polypeptide or in the bulk of a residue side chain. In addition, non-conservative substitutions may make a substantial change in the charge of the vitamin K-dependent polypeptide, such as reducing electropositive charges or introducing electronegative charges. Examples of non-conservative substitutions include substitution of a basic amino acid for a non-polar amino acid, or a polar amino acid for an acidic amino acid.

Protein C polypeptides

Activated protein C polypeptides suitable for use in compositions of the invention include human APC with the wild-type sequence as well as variants of hAPC having one or more mutations, e.g. having at least one substitution, insertion or deletion, typically at least one substitution. These additional mutations may e.g. be aimed at increasing the anti-inflammatory effect, increasing the half-life and/or lowering the anticoagulant activity of the resulting polypeptide. Typically, such mutations are aimed at introducing and/or at removing

at least one amino acid residue comprising an attachment group for a non-polypeptide moiety.

In one embodiment, the protein C polypeptide may thus include at least one introduced *in vivo* N-glycosylation site, where an "N-glycosylation site" has the sequence N-X-S/T/C'', wherein X is any amino acid residue except proline, N is asparagine and S/T/C is either serine, threonine or cysteine, preferably serine or threonine, and most preferably threonine. An introduced *in vivo* N-glycosylation site may be created by a substitution selected from the group consisting of D172N+K174S, D172N+K174T, D189N+K191S, D189N+K191T, S190N+K192S, S190N+K192T, K191N+K193S, K191N+K193T, K192N+L194S, K192N+L194T, K193N+A195S, K193N+A195T, D214N, D214N+S216T, E215N+K217S, E215N+K217T, S216N+K218S, S216N+K218T, K217N+L219S, K217N+L219T, K218N+L220S, K218N+L220T, L220N+R222S, L220N+R222T, V243N+V245S, V243N+V245T, V245N+P247S, V245N+P247T, S250N, S250N+S252T, K251N, K251N+T253S, S252N, S252N+T254S, T253N+D255S, T253N+D255T, T254N+N256S, T254N+N256T, D255N+D257S, D255N+D257T, L296N, L296N+T298S, Y302N, Y302N+S304T, H303N, H303N+S305T, S304N+R306S, S304N+R306T, S305N+E307S, S305N+E307T, T315N+V317S, T315N+V317T, F316N+L318S, F316N+L318T, V334N, V334N+S336T, S336N+M338S, S336N+M338T, V339S, V339T, M338N, M338N+S340T, I348N+G350S, I348N+G350T, L349N+D351S, L349N+D351T, D351N+Q353S, D351N+Q353T, R352N+D354S, R352N+D354T, E357N+D359S, E357N+D359T, G383N+G385S, G383N+G385T, L386N+H388S, L386N+H388T, L387N+N389S, L387N+N389T, H388N+Y390S and H388N+Y390T.

Preferred substitutions for introduction of an *in vivo* N-glycosylation site are selected from the group consisting of D189N+K191S, D189N+K191T, S190N+K192S, S190N+K192T, K191N+K193S, K191N+K193T, D214N, D214N+S216T, K217N+L219S, K217N+L219T, K251N, K251N+T253S, S252N, S252N+T254S, T253N+D255S, T253N+D255T, Y302N, Y302N+S304T, S336N+M338S, S336N+M338T, V339S, V339T, M338N, M338N+S340T, G383N+G385S, G383N+G385T, L386N+H388S and L386N+H388T; preferably from the group consisting of D189N+K191S, D189N+K191T, K191N+K193T, D214N, D214N+S216T, K251N, K251N+T253S, S252N, S252N+T254S, T253N+D255S, T253N+D255T, Y302N, Y302N+S304T, S336N+M338S, S336N+M338T, V339S, V339T, M338N, M338N+S340T, G383N+G385S, G383N+G385T, L386N+H388S and L386N+H388T; such as from the group consisting of D189N+K191T, K191N+K193T, D214N, K251N, S252N, T253N+D255T, Y302N, S336N+M338T, V339T, M338N,

G383N+G385T and L386N+H388T; in particular from the group consisting of D189N+K191T, D214N, D251N, and L386N+H388T. Particularly preferred substitutions include D214N and K251N. See WO 02/32461 for further information on variants of protein C with introduced glycosylation sites.

As is also described in WO 02/32461, at least some of the charged residues of protein C interact with each other. For example, K251 is believed to form a salt bridge to D214. Moreover, a cluster of negatively charged amino acid residues (D214, E215 and E357) is present in the protein. Therefore, another group of amino acid substitutions of interest include substitutions where a charged amino acid residue in the active site region and having at least 25% of its side chain exposed to the surface (as defined in WO 02/32461) is substituted with an amino acid residue having no charge, in particular an amino acid residue having no charge but a polar side chain (Gly, Ser, Thr, Cys, Tyr, Asn or Gln) or with an amino acid residue having an opposite charge.

Specific examples of substitutions to an amino acid residue with an opposite charge include D172K, D172R, D189K, D189R, K191D, K191E, K192D, K192E, K193D, K193E, D214K, D214R, E215K, E215R, K217D, K217E, K218D, K218E, K251D, K251E, D255K, D255R, R306D, R306E, E307K, E307R, K308D, K308E, E309K, E309R, R312D, R312E, D351K, D351R, R352D, R352E, E357K, E357R, E382K and E382R, such as D214K, D214R, E215K, E215R, K251D, K251E, E357K or E357R, e.g. D214K, D214R, K251D or K251E. Specific examples of substitutions to an amino acid residue having a polar side chain include D172G/S/T/C/Y/N/Q, D189G/S/T/C/Y/N/Q, K191G/S/T/C/Y/N/Q, K192G/S/T/C/Y/N/Q, K193G/S/T/C/Y/N/Q, D214G/S/T/C/Y/N/Q, E215G/S/T/C/Y/N/Q, K217G/S/T/C/Y/N/Q, K218G/S/T/C/Y/N/Q, K251G/S/T/C/Y/N/Q, D255G/S/T/C/Y/N/Q, R306G/S/T/C/Y/N/Q, E307G/S/T/C/Y/N/Q, K308G/S/T/C/Y/N/Q, E309G/S/T/C/Y/N/Q, R312G/S/T/C/Y/N/Q, D351G/S/T/C/Y/N/Q, R352G/S/T/C/Y/N/Q, E357G/S/T/C/Y/N/Q and E382G/S/T/C/Y/N/Q, such as D214G/S/T/C/Y/N/Q, E215G/S/T/C/Y/N/Q, K251G/S/T/C/Y/N/Q or E357G/S/T/C/Y/N/Q, e.g. D214Q, E215Q, K251Q or E357Q, in particular K251Q.

It will be understood that when the APC polypeptide variant comprises one or more *in vivo* glycosylation sites, the polypeptide must be expressed in a host cell capable of attaching sugar (oligosaccharide) moieties at the glycosylation site(s). Preferred host cells thus include, in particular, mammalian cells. Further information on suitable host cells is provided below.

In a further embodiment, at least one attachment group for a non-polypeptide moiety may be introduced in the EGF domains of protein C with the aim of producing variants that have a decreased anticoagulant activity as compared to human APC and with anti-inflammatory properties that have not been substantially changed as compared to human APC. Such variants are disclosed in US 2003/0018175 A1, which is hereby incorporated by reference.

More particularly, such variants comprise at least one sugar moiety covalently attached to a protein C polypeptide having an amino acid sequence which differs from that of a parent protein C polypeptide, in particular human protein C (SEQ ID NO:1), in that at least one *in vivo* glycosylation site has been introduced by a substitution selected from the group consisting of H66N, H66N+T68S, I73N, I73N+S75T, S75N, S75N+S77T, D79N+R81S, D79N+R81T, E92N, E92N+S94T, G104N, G104N+T106S, R117N, R117N+S119T, D128N+L130S and D128N+L130T, preferably from the group consisting of H66N, I73N+S75T, S75N+S77T, D79N+R81T, E92N, G104N, R117N+S119T and D128N+L130T. A particularly preferred substitution in the EGF domains is H66N.

In another embodiment, the protein C polypeptide may include at least one introduced amino acid residue comprising an attachment group for a polymer molecule, in particular an introduced cysteine residue. Preferably, such a cysteine residue is introduced in a position selected from the group consisting of D172, D189, S190, K191, K192, K193, D214, E215, S216, K217, K218, L220, V243, V245, S250, K251, S252, T253, T254, L296, Y302, H303, S304, S305, T315, F316, V334, S336, N337, M338, I348, L349, D351, R352, E357, G383, L386, L387 and H388; more preferably from the group consisting of D189, S190, K191, D214, K217, K251, S252, T253, Y302, S336, N337, M338, G383 and L386; such as from the group consisting of D189, K191, D214, K251, S252, T253, Y302, S336, N337, M338, G383 and L386; in particular from the group consisting of D189, D214, K251 and L386. The polymer molecule to be covalently attached to said introduced cysteine residue is in particular a linear or branched polyethylene glycol or other polyalkylene oxide.

Specific examples of activated PEG polymers particularly preferred for coupling to cysteine residues include the following linear PEGs: vinylsulfone-PEG (VS-PEG), preferably vinylsulfone-mPEG (VS-mPEG); maleimide-PEG (MAL-PEG), preferably maleimide-mPEG (MAL-mPEG) and orthopyridyl-disulfide-PEG (OPSS-PEG), preferably orthopyridyl-disulfide-mPEG (OPSS-mPEG). Such PEG or mPEG polymers will generally have a size of from about 1 kDa to about 40 kDa, such as from about 1 kDa to about 20 kDa, e.g. from

about 2 kDa to about 15 kDa, such as from about 3 kDa to about 10 kDa; for example about 5 kDa, about 6 kDa, about 10 kD, about 12 kDa or about 20 kDa.

For PEGylation to cysteine residues the protein C variant is usually treated with a reducing agent, such as dithiothreitol (DDT) prior to PEGylation. The reducing agent is subsequently removed by any conventional method, such as by desalting. Conjugation of PEG to a cysteine residue typically takes place in a suitable buffer at about pH 6-9 at temperatures of about 4°C to 25°C for periods up to about 16 hours.

It will be understood that the APC variant according to this aspect of the invention may contain combinations of more than one of the alterations described above. For example, in addition to having at least one introduced glycosylation site, the APC variant may comprise at least one introduced amino acid residue comprising an attachment site for a polymer polymer molecule such as PEG, and/or at least one introduced amino acid residue with an altered charge. The APC polypeptide variant may in this case e.g. contain a total of 1-6 attachment groups that are available for conjugation, such as 1, 2, 3, 4, 5, or 6 such attachment groups.

In a further embodiment, the protein C polypeptide may comprise at least one amino acid modification in the autolysis loop constituted by the amino acid residues in position 306-314 relative to SEQ ID NO:1 in order to achieve a reduced anticoagulant activity. This modification may e.g. include substitution of at least one of R306, E307, K308, E309, K311, R312 and R314 with an uncharged amino acid residue, e.g. A, V, L, I, F, W, P, G, S, T, Y, N or Q. By way of example, in one preferred embodiment, one, two, three, four or five of the residues in these positions may be substituted with an alanine residue.

Further information on protein C variants comprising advantageous substitutions of the type described above as well as conjugation of such variants to one or more non-polypeptide moieties is found in WO 02/32461, US 2003/0018175 A1 and PCT/DK03/00392, which are hereby incorporated herein by reference.

FVII polypeptides

FVIIa polypeptides suitable for use in compositions of the invention include human FVIIa (hFVIIa) with the wild-type sequence as well as variants of hFVIIa having one or more mutations, e.g. having at least one substitution, insertion or deletion, typically at least one substitution. These additional mutations may e.g. be aimed at increasing the half-life and/or increasing the clotting activity of the resulting polypeptide.

The term "clotting activity" is used to mean the activity measured in the "Whole Blood Assay" described herein. It will be understood that the activity measured in the "Whole Blood Assay" is the time needed to obtain clot formation. Thus, a lower clotting time corresponds to a higher clotting activity.

The term "increased clotting activity" is used to indicate that the clotting time of the polypeptide variant is statistically significantly decreased relative to that generated by rhFVIIa as determined under comparable conditions and when measured in the "Whole Blood Assay" described herein.

In one embodiment, the FVIIa polypeptide is a variant of hFVIIa which comprises at least one modification in the Gla domain. A number of modifications in the FVII Gla domain leading to an increased membrane binding affinity have been described in the art (see, for example, WO 99/20767 and WO 00/66753). Mutations of particular interest in the Gla domain are substitutions in one or more of P10, K32, D33, A34 and R36, as well as insertion of an amino acid residue between A3 and F4. Particularly preferred positions are P10 and K32. Preferably, the substitution in position 32 is K32E, the substitution in position 10 is P10Q, the substitution in position 33 is D33F, the substitution in position 34 is A34E, the substitution in position 36 is R36E, and the insertion between A3 and F4 is A3AY.

In another embodiment, the FVIIa polypeptide has been modified to result in a variant with an increased functional *in vivo* half-life, an increased plasma half-life and/or an increased Area Under the Curve when administered intravenously (AUC_{iv}), in particular as determined by intravenous administration to rats, and/or increased bioavailability and/or reduced sensitivity to proteolytic degradation. Such variants may allow lower doses and/or a longer duration between injections.

Interesting FVIIa variants include those wherein at least one amino acid residue comprising an attachment group for a non-polypeptide moiety has been introduced, and which have at least one non-polypeptide moiety covalently attached to at least one of said attachment groups. Numerous examples of relevant amino acid substitutions of this type are given in WO 01/58935, which is hereby incorporated by reference. See also WO 03/093465, incorporated by reference, which discloses FVII or FVIIa variants comprising an amino acid substitution in positions 10 and 32 and having one or more introduced N-glycosylation sites located outside the Gla domain.

Also, the polypeptide variant may be attached to a serine proteinase inhibitor to inhibit the catalytic site of the polypeptide or, alternatively, one of the active-site amino acid

residues may be mutated (such as S344A) to render the resulting molecule catalytically inactive.

The amino acid residue comprising an attachment group for a non-polypeptide moiety is selected on the basis of the nature of the non-polypeptide moiety of choice and, in most instances, on the basis of the method in which conjugation between the FVII polypeptide and the non-polypeptide moiety is to be achieved. For instance, when the non-polypeptide moiety is a polymer molecule such as a polyethylene glycol or polyalkylene oxide derived molecule, amino acid residues comprising an attachment group may be selected from the group consisting of lysine, cysteine, aspartic acid, glutamic acid, histidine, and tyrosine, preferably lysine, cysteine, aspartic acid and glutamic acid, more preferably lysine and cysteine, in particular cysteine.

When an attachment group for a non-polypeptide moiety is to be introduced into the FVII polypeptide, the position of the amino acid residue to be modified is preferably located at the surface of the FVII polypeptide, and more preferably occupied by an amino acid residue which has more than 25% of its side chain exposed to the surface preferably more than 50% of its side chain exposed to the surface. Such positions have been identified on the basis of an analysis of a 3D structure of the hFVII molecule as described in WO 01/58935.

Furthermore, the position is preferably selected from a part of the FVII polypeptide that is located outside the tissue factor binding site, the Gla domain, the active site region and/or the ridge of the active site binding cleft. These sites/regions are identified in Example 1 of WO 01/58935. In certain situations, however, e.g. in case an inactivated FVII polypeptide is desired, it may be advantageous to perform modifications in or close to the active site region and/or the ridge of the active site binding cleft. For example, it is contemplated that one or more attachment groups for the non-polypeptide moieties, such as attachment groups for *in vivo* N-glycosylation sites, may advantageously be introduced in the active site region or at the ridge of the active site binding cleft of the FVII polypeptide.

Functional *in vivo* half-life is *inter alia* dependent on the molecular weight of the protein, and the number of attachment groups needed for providing increased half-life thus depends on the molecular weight of the non-polypeptide moiety in question. In one embodiment, the FVIIa polypeptide has a molecular weight of at least 67 kDa, in particular at least 70 kDa, e.g., as measured by SDS-PAGE according to Laemmli, U.K., *Nature* Vol 227 (1970), p680-85. FVIIa itself has a molecular weight of about 53 kDa, and therefore an additional 10-20 kDa is required to obtain such an effect. This may, e.g., be provided by conjugating 2-4 10 kDa PEG molecules or otherwise as described in WO 01/58935.

The FVII polypeptide may contain 1-10 non-polypeptide moieties, typically 1-8 or 2-8 non-polypeptide moieties, preferably 1-5 or 2-5 non-polypeptide moieties, such as 1-4 or 1-3 non-polypeptide moieties, e.g. 1, 2 or 3 non-polypeptide moieties. Preferred non-polypeptide moieties include PEG, such as mPEG, or sugar moieties. It will also be apparent that a FVII polypeptide may contain, for example, at least one PEG moiety and at least one sugar moiety.

In one embodiment, the non-polypeptide moiety attached to a FVII variant may thus be a sugar moiety, i.e. the FVII polypeptide variant comprises at least one sugar moiety covalently attached to an introduced glycosylation site. Preferably said glycosylation site is an *in vivo* glycosylation site, in particular an *in vivo* N-glycosylation site, which has been introduced by substitution.

As used herein in the context of FVII, the term "naturally occurring glycosylation site" covers the glycosylation sites at positions N145, N322, S52 and S60. In a similar way, the term "naturally occurring *in vivo* O-glycosylation site" includes the positions S52 and S60, and the term "naturally occurring *in vivo* N-glycosylation site" includes positions N145 and N322. When the FVIIa polypeptide variant comprises one or more *in vivo* glycosylation sites, it will be apparent that the polypeptide must be expressed in a host cell capable of attaching sugar (oligosaccharide) moieties at the glycosylation site(s). Preferred host cells thus include, in particular, mammalian cells. Further information on suitable host cells is provided below.

Positions where glycosylation sites may be introduced in FVII are preferably selected from a part of the molecule that is located outside the TF binding site, the Gla domain, the active site region and the ridge of the active site cleft; see WO 01/58935. Specific examples of substitutions creating an *in vivo* N-glycosylation site include those selected from the group consisting of A51N, G58N, T106N, K109N, G124N, K143N+N145T, A175T, I205S, I205T, V253N, T267N, T267N+S269T, S314N+K316S, S314N+K316T, R315N+V317S, R315N+V317T, K316N+G318S, K316N+G318T, G318N and D334N. Preferred substitutions include those selected from the group consisting of A51N, G58N, T106N, K109N, G124N, K143N+N145T, A175T, I205T, V253N, T267N+S269T, S314N+K316T, R315N+V317T, K316N+G318T, G318N and D334N, more preferably those selected from the group consisting of T106N, A175T, I205T, V253N and T267N+S269T, in particular I205T or V253N.

In one embodiment, one *in vivo* N-glycosylation site has been introduced into FVII by substitution. In another embodiment, at least two *in vivo* N-glycosylation site sites have

been introduced by substitution. Specific examples of substitutions creating two *in vivo* N-glycosylation sites include those selected from the group consisting of A51N+G58N, A51N+T106N, A51N+K109N, A51N+G124N, A51N+K143N+N145T, A51N+A175T, A51N+I205T, A51N+V253N, A51N+T267N+S269T, A51N+S314N+K316T, A51N+R315N+V317T, A51N+K316N+G318T, A51N+G318N, A51N+D334N, G58N+T106N, G58N+K109N, G58N+G124N, G58N+K143N+N145T, G58N+A175T, G58N+I205T, G58N+V253N, G58N+T267N+S269T, G58N+S314N+K316T, G58N+R315N+V317T, G58N+K316N+G318T, G58N+G318N, G58N+D334N, T106N+K109N, T106N+G124N, T106N+K143N+N145T, T106N+A175T, T106N+I205T, T106N+V253N, T106N+T267N+S269T, T106N+S314N+K316T, T106N+R315N+V317T, T106N+K316N+G318T, T106N+G318N, T106N+D334N, K109N+G124N, K109N+K143N+N145T, K109N+A175T, K109N+I205T, K109N+V253N, K109N+T267N+S269T, K109N+S314N+K316T, K109N+R315N+V317T, K109N+K316N+G318T, K109N+G318N, K109N+D334N, G124N+K143N+N145T, G124N+A175T, G124N+I205T, G124N+V253N, G124N+T267N+S269T, G124N+S314N+K316T, G124N+R315N+V317T, G124N+K316N+G318T, G124N+G318N, G124N+D334N, K143N+N145T+A175T, K143N+N145T+I205T, K143N+N145T+V253N, K143N+N145T+T267N+S269T, K143N+N145T+S314N+K316T, K143N+N145T+R315N+V317T, K143N+N145T+K316N+G318T, K143N+N145T+G318N, K143N+N145T+D334N, A175T+I205T, A175T+V253N, A175T+T267N+S269T, A175T+S314N+K316T, A175T+R315N+V317T, A175T+K316N+G318T, A175T+G318N, A175T+D334N, I205T+V253N, I205T+T267N+S269T, I205T+S314N+K316T, I205T+R315N+V317T, I205T+K316N+G318T, I205T+G318N, I205T+D334N, V253N+T267N+S269T, V253N+S314N+K316T, V253N+R315N+V317T, V253N+K316N+G318T, V253N+G318N, V253N+D334N, T267N+S269T+S314N+K316T, T267N+S269T+R315N+V317T, T267N+S269T+K316N+G318T, T267N+S269T+G318N, T267N+S269T+D334N, S314N+K316T+R315N+V317T, S314N+K316T+G318N, S314N+K316T+D334N, R315N+V317T+K316N+G318T, R315N+V317T+G318N, R315N+V317T+D334N and G318N+D334N; preferably selected from the group consisting of T106N+A175T, T106N+I205T, T106N+V253N, T106N+T267N+S269T, A175T+I205T, A175T+V253N, A175T+T267N+S269T, I205T+V253N, I205T+T267N+S269T and V253N+T267N+S269T; more preferably selected from the group consisting of T106N+I205T, T106N+V253N and I205T+T267N+S269T, most preferably T106N+V253N.

In a still further embodiment of this aspect of the invention at least three *in vivo* N-glycosylation site sites have been introduced by substitution. Specific examples of substitutions creating three *in vivo* N-glycosylation sites include I205T+V253N+T267N+S269T and T106N+I205T+V253N.

As indicated above, the FVIIa variant according to this aspect of the invention may contain, in addition to at least one sugar moiety, at least one additional non-polypeptide moiety, in particular a polymer molecule such as PEG, i.e. one or more substitutions aimed at introducing at least one *in vivo* glycosylation site may be combined with one or more substitutions aimed at introducing at least one site for attachment of e.g. a PEG moiety. The FVII or FVIIa polypeptide variant may e.g. contain a total of 1-6 attachment groups that are available for conjugation, such as 1, 2, 3, 4, 5, or 6 such attachment groups.

As mentioned above, when the non-polypeptide moiety is a polymer molecule such as PEG, the attachment group in a preferred embodiment is cysteine. FVII/FVIIa contains 22 cysteine residues located outside the Gla domain, and disulfide bridges are established between the following cysteine residues: C50 and C61, C55 and C70, C72 and C81, C91 and C102, C98 and C112, C114 and C127, C135 and C262, C159 and C164, C178 and C194, C310 and C329, and between C340 and C368.

Thus, in an interesting embodiment at least one cysteine residue has been introduced, preferably by substitution, in the FVIIa polypeptide. Typically 1-10 cysteine residues have been introduced, preferably 1-8, 1-6, 1-4 or 1-3. In particular 1, 2 or 3 cysteine residues have been introduced by way of substitution.

In one embodiment, a cysteine residue may be introduced near or at the C-terminus. For example, a cysteine residue may be introduced, either by substitution or insertion, in position 400-406. Specific examples of substitutions include: L400C, L401C, R402C, A403C, P404C, F405C and P406C, in particular P406C. Specific examples of insertions include L400LC, L401LC, R402RC, A403AC, P404PC, F405FC and P406PC, in particular P406PC.

The polymer molecule to be attached to a cysteine residue is preferably a linear or branched polyethylene glycol or another polyalkylene oxide, in particular vinylsulfone-PEG (VS-PEG), preferably vinylsulfone-mPEG (VS-mPEG); maleimide-PEG (MAL-PEG), preferably maleimide-mPEG (MAL-mPEG); or orthopyridyl-disulfide-PEG (OPSS-PEG), preferably orthopyridyl-disulfide-mPEG (OPSS-mPEG).

When the FVIIa polypeptide variant comprises only one conjugatable cysteine residue, this residue is preferably conjugated to a non-polypeptide moiety with a molecular weight of from about 5 kDa to about 20 kDa, e.g. from about 10 kDa to about 20 kDa, such

as a molecular weight of about 5 kDa, about 10 kDa, about 12 kDa, about 15 kDa or about 20 kDa, either directly conjugated or indirectly through a low molecular weight polymer (as disclosed in WO 99/55377). When the FVIIa polypeptide variant comprises two or more conjugatable cysteine residues, normally each of the non-polypeptide moieties has a molecular weight of from about 5 to about 10 kDa, such as about 5 kDa or about 10 kDa.

In a further embodiment of the present invention, the FVIIa variant may, optionally in addition to the modifications described in the sections above, also contain mutations which are known to increase the intrinsic activity of the polypeptide, such as those described in WO 02/22776. Examples of such substitutions are those selected from the group consisting of V158D, E296D, M298Q, L305V and K337A. More preferably, said substitutions are selected from the group consisting of V158D+E296D+M298Q+L305V+K337A, V158D+E296D+M298Q+K337A, V158D+E296D+M298Q+L305V, V158D+E296D+M298Q, M298Q, L305V+K337A, L305V and K337A.

Moreover, the FVII variant may, optionally in addition to the modifications mentioned above, contain modifications which increase the tissue factor (TF) binding affinity. Examples of such modifications include at least one substitution selected from the group consisting of L39E, L39Q, L39H, I42R, S43H, S43Q, K62E, K62R, L65Q, L65S, F71D, F71Y, F71E, F71Q, F71N, E82Q, E82N, E82K, F275H and combinations thereof, in particular L65Q, F71Y, K62E, S43Q and combinations thereof.

In a further embodiment, the FVIIa variant may, optionally in addition to the modifications described in the sections above, also contain mutations which cause a decreased inhibition by TFPI. One such example is the substitution K341Q (disclosed by Neuenschwander et al., *Biochemistry* 1995; 34:8701-8707). Other examples include D196K, D196N, G237L, G237GAA and combinations thereof.

Methods for preparing polypeptides

Vitamin K-dependent polypeptides, optionally in glycosylated form, may be produced by any suitable method known in the art. Such methods include constructing a nucleotide sequence encoding the polypeptide and expressing the sequence in a suitable transformed or transfected host. Preferably, the host cell is a gamma-carboxylating host cell, in particular a mammalian cell.

A nucleotide sequence encoding a polypeptide precursor may be constructed by isolating or synthesizing a nucleotide sequence encoding the parent vitamin K-dependent polypeptide precursor, and then changing the nucleotide sequence so as to effect introduction (i.e.

insertion or substitution) or removal (i.e. deletion or substitution) of the relevant amino acid residue(s).

The nucleotide sequence may conveniently be modified by site-directed mutagenesis in accordance with conventional methods. Alternatively, the nucleotide sequence may be prepared by chemical synthesis, e.g. by using an oligonucleotide synthesizer, wherein oligonucleotides are designed based on the amino acid sequence of the desired polypeptide, and preferably selecting those codons that are favored in the host cell in which the recombinant polypeptide will be produced.

Persons skilled in the art will be capable of selecting suitable vectors, expression control sequences and hosts for expressing the polypeptide. The nucleotide sequence encoding a vitamin K-dependent polypeptide precursor, whether prepared by site-directed mutagenesis, synthesis, PCR or other methods, will generally include a nucleotide sequence that encodes a suitable signal peptide to allow secretion from the host cells.

Suitable host cells that may be used to produce the polypeptide precursor include, in particular, mammalian cells. Examples of suitable mammalian host cells include Chinese hamster ovary (CHO) cell lines, (e.g. CHO-K1; ATCC CCL-61), Green Monkey cell lines (COS) (e.g. COS 1 (ATCC CRL-1650), COS 7 (ATCC CRL-1651)); mouse cells (e.g. NS/O), Baby Hamster Kidney (BHK) cell lines (e.g. ATCC CRL-1632 or ATCC CCL-10), and human cells (e.g. HEK 293 (ATCC CRL-1573)).

The resulting polypeptide may be recovered from the nutrient medium by methods known in the art, e.g. using conventional procedures such as centrifugation, filtration, ultrafiltration, extraction and/or precipitation, and may be purified by a variety of procedures known in the art including, but not limited to, chromatography (e.g., ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (e.g., preparative isoelectric focusing), differential solubility (e.g., ammonium sulfate precipitation) or extraction (see, e.g., *Protein Purification* (2nd Edition), Janson and Ryden, editors, Wiley, New York, 1998).

For further information on preparing vitamin K-dependent polypeptides, see e.g. WO 00/66753.

Information on methods for preparing protein C and variants thereof may be found e.g. in WO 02/32461. Activation of the zymogen form of protein C to result in activated protein C may be performed in a manner known in the art, e.g. by using thrombin or thrombin and thrombomodulin to cleave the activation peptide, or alternatively by use of a snake venom activator. Another approach for producing activated protein C is to alter or replace the

native activation peptide. For example, EP 0319312 A2 describes replacement of the activation peptide of protein C with a non-native proteolytic cleavage site that leads to direct expression of activated protein C in recombinant host cells. A similar approach is disclosed in WO 03/035861, which describes chimeric protein C in which the native activation peptide has been replaced with a thrombin-cleavable sequence of fibrinopeptide A or a portion thereof in order to obtain enhanced activation by thrombin in the absence of thrombomodulin.

For further information on preparing FVII and FVIIa polypeptides, see e.g. WO 01/58935 and WO 03/093465.

Pharmaceutical compositions and use

Vitamin K-dependent polypeptides produced according to the present invention may be formulated as known in the art in a pharmaceutical composition comprising a polypeptide and at least one pharmaceutically acceptable carrier or excipient. In the present context, the term "pharmaceutically acceptable" means that the carrier or excipient, at the dosages and concentrations employed, will not cause any unwanted or harmful effects in the patients to which they are administered. Such pharmaceutically acceptable carriers and excipients are well known in the art (see Remington's Pharmaceutical Sciences, 19th edition, A. R. Gennaro, Ed., Mack Publishing Company, 1995; Pharmaceutical Formulation Development of Peptides and Proteins, S. Frokjaer and L. Hovgaard, Eds., Taylor & Francis, 2000; and Handbook of Pharmaceutical Excipients, 3rd edition, A. Kibbe, Ed., Pharmaceutical Press, 2000).

Compositions of the invention comprising APC may in particular be used for the manufacture of a medicament for treating or preventing a disease selected from the group consisting of stroke; myocardial infarction; after venous thrombosis; disseminated intravascular coagulation (DIC); sepsis; septic shock; emboli, such as pulmonary emboli; transplantation, such as bone marrow transplantation; burns; major surgery/trauma or adult respiratory stress syndrome (ARDS), in particular for the treatment of sepsis, including septic shock. The invention thus includes a method for treating or preventing such diseases or conditions by administering to a patient in need thereof an effective amount of a composition of the invention comprising activated protein C or a variant thereof and an sulfoalkyl ether cyclodextrin derivative.

Compositions of the invention comprising FVIIa may in particular be used for the manufacture of a medicament for treatment of a condition treatable by administration of

FVIIa, in particular for treating or preventing clotting factor deficiencies (such as hemophilia A or B or deficiency of clotting factors XI or VII) or trauma. The invention thus includes a method for treating or preventing such diseases or conditions by administering to a patient in need thereof an effective amount of a composition of the invention comprising FVIIa or a variant thereof and an sulfoalkyl ether cyclodextrin derivative.

A "patient" for the purposes of the present invention includes both humans and other mammals, i.e. the methods are applicable to both human therapy and veterinary applications.

The vitamin K-dependent polypeptides will be administered to patients in an effective dose. By "effective dose" herein is meant a dose that is sufficient to produce the desired effects in relation to the condition for which it is administered. The exact dose will depend on the disorder to be treated, and will be ascertainable by one skilled in the art using known techniques.

The polypeptides can be used "as is" and/or in a salt form thereof. Suitable salts include, but are not limited to, salts with alkali metals or alkaline earth metals, such as sodium, potassium, calcium and magnesium, as well as e.g. zinc salts. These salts or complexes may by present as a crystalline and/or amorphous structure.

The pharmaceutical composition of the invention may be administered alone or in conjunction with other therapeutic agents. These agents may be incorporated as part of the same pharmaceutical composition or may be administered separately, either concurrently or in accordance with another treatment schedule. In addition, the pharmaceutical composition of the invention may be used as an adjuvant to other therapies.

The composition of the invention is preferably in the form of an aqueous solution or suspension, typically an aqueous solution.

The administration of the formulations of the present invention can be performed in a variety of ways, including, but not limited to, orally, subcutaneously, intravenously, intracerebrally, intranasally, transdermally, intraperitoneally, intramuscularly, intrapulmonary, vaginally, rectally, intraocularly, or in any other acceptable manner. The liquid formulations of the invention will typically be administered continuously by infusion, although bolus injection may also be used in certain cases.

In the general discussion below regarding pharmaceutical compositions, it will be understood that compositions according to the invention will in all cases contain a sulfoalkyl ether cyclodextrin derivative as described above.

Parenteral compositions

An example of a pharmaceutical composition is a solution designed for parenteral administration. Although in many cases pharmaceutical solution formulations are provided in liquid form, appropriate for immediate use, such parenteral formulations may also be provided in frozen or in lyophilized form.

In case of parenterals, they are prepared for storage as lyophilized formulations or aqueous solutions by mixing, as appropriate, the polypeptide having the desired degree of purity with one or more pharmaceutically acceptable carriers, excipients or stabilizers typically employed in the art (all of which are termed "excipients"), for example buffering agents, stabilizing agents, preservatives, isotonifiers, non-ionic detergents, antioxidants and/or other miscellaneous additives.

Buffering agents help to maintain the pH in the range which approximates physiological conditions; see also the discussion of pH above. They are typically present at a concentration ranging from about 2 mM to about 50 mM. Suitable buffering agents for use with the present invention include both organic and inorganic acids and salts thereof such as citrate buffers (e.g., monosodium citrate-disodium citrate mixture, citric acid-trisodium citrate mixture, citric acid-monosodium citrate mixture, etc.), succinate buffers (e.g., succinic acid-monosodium succinate mixture, succinic acid-sodium hydroxide mixture, succinic acid-disodium succinate mixture, etc.), tartrate buffers (e.g., tartaric acid-sodium tartrate mixture, tartaric acid-potassium tartrate mixture, tartaric acid-sodium hydroxide mixture, etc.), fumarate buffers (e.g., fumaric acid-monosodium fumarate mixture, fumaric acid-disodium fumarate mixture, monosodium fumarate-disodium fumarate mixture, etc.), gluconate buffers (e.g., gluconic acid-sodium glyconate mixture, gluconic acid-sodium hydroxide mixture, gluconic acid-potassium glyuconate mixture, etc.), oxalate buffer (e.g., oxalic acid-sodium oxalate mixture, oxalic acid-sodium hydroxide mixture, oxalic acid-potassium oxalate mixture, etc.), lactate buffers (e.g., lactic acid-sodium lactate mixture, lactic acid-sodium hydroxide mixture, lactic acid-potassium lactate mixture, etc.) and acetate buffers (e.g., acetic acid-sodium acetate mixture, acetic acid-sodium hydroxide mixture, etc.). Additional possibilities are phosphate buffers, histidine buffers and trimethylamine salts such as Tris.

Preservatives are added to retard microbial growth, and are typically added in amounts of e.g. about 0.1%-2% (w/v). Suitable preservatives for use with the present invention include phenol, benzyl alcohol, meta-cresol, methyl paraben, propyl paraben, octadecyldimethylbenzyl ammonium chloride, benzalkonium halides (e.g. benzalkonium chloride,

bromide or iodide), hexamethonium chloride, alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol and 3-pentanol.

Isotonicifiers are added to ensure isotonicity of liquid compositions and include polyhydric sugar alcohols, preferably trihydric or higher sugar alcohols, such as glycerin, erythritol, arabitol, xylitol, sorbitol and mannitol. Polyhydric alcohols can be present in an amount between 0.1% and 25% by weight, typically 1% to 5%, taking into account the relative amounts of the other ingredients.

Stabilizers refer to a broad category of excipients which can range in function from a bulking agent to an additive which solubilizes the therapeutic agent or helps to prevent denaturation or adherence to the container wall. Typical stabilizers can be polyhydric sugar alcohols (enumerated above); amino acids such as arginine, lysine, glycine, glutamine, asparagine, histidine, alanine, omithine, L-leucine, 2-phenylalanine, glutamic acid, threonine, etc., organic sugars or sugar alcohols, such as lactose, trehalose, stachyose, mannitol, sorbitol, xylitol, ribitol, myoinisitol, galactitol, glycerol and the like, including cyclitols such as inositol; polyethylene glycol; amino acid polymers; sulfur-containing reducing agents, such as urea, glutathione, thioctic acid, sodium thioglycolate, thioglycerol, α-monothioglycerol and sodium thiosulfate; low molecular weight polypeptides (i.e. <10 residues); proteins such as human serum albumin, bovine serum albumin, gelatin or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; monosaccharides such as xylose, mannose, fructose and glucose; disaccharides such as lactose, maltose and sucrose; trisaccharides such as raffinose, and polysaccharides such as dextran. Stabilizers are typically present in the range of from 0.1 to 10,000 parts by weight based on the active protein weight.

Non-ionic surfactants or detergents (also known as "wetting agents") may be present to help solubilize the therapeutic agent as well as to protect the therapeutic polypeptide against agitation-induced aggregation, which also permits the formulation to be exposed to shear surface stress without causing denaturation of the polypeptide. Suitable non-ionic surfactants include polysorbates (20, 80, etc.), polyoxamers (184, 188 etc.), Pluronic® polyols, polyoxyethylene sorbitan monoethers (Tween®-20, Tween®-80, etc.).

Additional miscellaneous excipients include bulking agents or fillers (e.g. starch), chelating agents (e.g. EDTA), antioxidants (e.g., ascorbic acid, methionine, vitamin E) and cosolvents.

The active ingredient may also be entrapped in microcapsules prepared, for example, by coascervation techniques or by interfacial polymerization, for example hydroxymethylcellulose, gelatin or poly-(methylmethacylate) microcapsules, in colloidal drug delivery systems

(for example liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, *supra*.

Parenteral formulations to be used for *in vivo* administration must be sterile. This is readily accomplished, for example, by filtration through sterile filtration membranes.

In addition to parenteral formulations, it is also possible to administer the vitamin K-dependent polypeptide in the form of a sustained release formulation, e.g. as described in WO 02/32461.

The invention is further illustrated by the following non-limiting examples.

Example 1

Stabilization of activated protein C

Experiments were carried out to demonstrate the stabilizing effect of Captisol® (a beta-cyclodextrin pharmaceutical excipient) on the degradation as well as aggregation of activated protein C (APC) at a pH above 7. Each of the samples were formulated to final concentrations of 50 mg/ml Captisol® and 100 mg/ ml Captisol®, respectively, and as a control without Captisol®. Storage conditions were either at –20°C or 37°C, and samples were stored for 96 hours.

Four different APC samples were studied:

- Xigris®: recombinant APC commercially available from Eli Lilly, produced in HEK293 cells, and formulated in 16 mM citrate, 150 mM NaCl, 12.5 mg/ml sucrose, pH 6.0;
- recombinant APC having the wild-type sequence ("WT");
- APC variant C01: *156R, *157R, D214N, i.e. with an N-glycosylation site introduced in position 214 and an R-R insertion in position 156-157; and
- APC variant C02: K251N, i.e. with an N-glycosylation site introduced in position 251.

The recombinant WT APC and the APC variants C01 and C02 were produced in CHO cells and were formulated in 20 mM Tris, 150 mM NaCl, pH 7.4.

Samples were analyzed for aggregation, degradation, and enzymatic activity. In all analyses Captisol® was shown to improve the stability of at least one of the samples that were formulated at pH 7.4.

Materials and Methods

Sample preparation

All samples, except Xigris®, were diafiltered to 20 mM Tris, 150 mM NaCl, pH 7.4 using a 6 ml Vivaspin cartridge with a molecular weight cut-off of 10 kDa. Xigris® was maintained in its commercial formulation conditions: 16 mM citrate, 150 mM NaCl, 12.5 mg/ml sucrose, pH 6.0.

A 200 mg/ ml stock solution of Captisol® (CyDex, Overland Park, KS, USA) was added to each of the Captisol® samples, to final concentrations of 100 and 50 mg/ml. The 50 mg/ml samples were adjusted to the same volume as the 100 mg/ml samples with buffer of the corresponding pH. Non-Captisol® samples were diluted with buffer to the same volumes as the Captisol® samples.

The protein concentrations were as follows:

Xigris	1.08 mg/ml
WT	0.84 mg/ml
C01	0.65 mg/ml
C02	0.64 mg/ml

Samples were split in two, and one aliquot was stored at –20°C and the other at 37°C. The latter was frozen at –20°C after 96 hours.

Analyses

Thawed samples were analyzed using SDS-PAGE (for degradation), size-exclusion chromatography (for aggregation), as well as for enzyme activity.

SDS-PAGE:

20 µl of sample was mixed with 10µl Reducing Cocktail (250 µl NuPAGE® LDS Sample Buffer (4x) and 100 µl 0.5M DTT) and heated for 5 minutes at 95°C. Samples were applied on NuPAGE® Novex Bis-Tris 4-12% gels and electrophoresed in an MES buffer system for 35 minutes at a constant 200 V. Gels were incubated in fixing solution (50% (v/v)

methanol, 10% (v/v) acetic acid, 40% (v/v) deionized water), and protein was visualized by staining with Novex Colloidal Blue using the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA).

Size-exclusion chromatography:

A Dionex Summit HPLC system consisting of a pump, degasser, autosampler, and diode-array detector, and controlled by Chromeleon version 6.30 software (Dionex, Sunnyvale, CA, USA), was connected to a Superdex 200 HR 30/10 SEC HPLC column (Amersham Biosciences, Uppsala, Sweden). The buffer used was 50 mM Na_2HPO_4, 500 mM NaCl, pH 7.0, and the flow rate was 1 ml/min. Eluting protein was detected by measurement of OD_{214nm}. 5 µg of sample (between 5 and 8 µl) was injected for each analysis.

Enzyme activity (Amidolytic Assay):

Amidolytic activity was determined using the synthetic peptide substrate SPECTROZYME PCa with the formula H-D-Lys(γ-Cbo)-Pro-Arg-pNA.2AcOH (American Diagnostica Inc, product # 336) at a final concentration of 0.5 mM. Assays were performed in 96 well microtiter plates at 23°C in 50 mM Tris-HCl (pH 8.3), 100 mM NaCl, 5 mM $CaCl_2$ and 0.1% BSA. The rate of hydrolysis was recorded for 3 min at 405 nm as the change in absorbance units/min using a SpectraMax Plus 384 kinetic microplate reader (Molecular Devices, Sunnyvale, CA, USA). Xigris® was used as a standard.

Results and Discussion

SDS-PAGE

When APC is analyzed under the conditions described above, the heavy chain (HC) and the light chain (LC) are separated due to reduction of the disulphide bridge that holds them together. The heavy chain gives rise to several protein bands due to glycosylation heterogeneity; this was especially noticeable in C01, which contains a fully occupied extra glycosylation site at position 214. The light chain normally only gives rise to one band at approximately 22 kDa.

As explained above, protein C is known to degrade at increasing temperature as well as elevated pH, a major degradation pathway being cleavage of the K308-E309 peptide bond in the heavy chain. In these experiments, it was found that degradation in all of the APC variants stored at 37°C was substantially decreased with increasing concentrations of Captisol®

(50 and 100 mg/ml). No formulation effect was seen in the analogous samples that were immediately frozen at –20°C.

The diminished formation of low molecular weight degradation products was primarily seen in WT and C02, and to a lesser extent in Xigris®. Xigris® is formulated at pH 6.0, whereas the other samples had a pH of 7.4, and this could be a cause of the different degradation profiles. The effect of Captisol® on C01 degradation was most apparent in its effect on a heavy chain fragment band migrating just above the light chain band.

Size-exclusion chromatography

Size-exclusion chromatography separates molecules according to their hydrodynamic radius. Under these experimental conditions, APC is in its native conformation, and all interactions, including van der Waal's forces, hydrogen bonds and hydrophobic interactions, are preserved. It is thus possible to monitor aggregation of APC molecules caused not only by covalent interaction but also by these weaker forces.

The results of the size-exclusion chromatography analyses showed that the aggregated forms of APC decrease with increasing amounts of Captisol®, indicating that this excipient prevents the formation of impurities in the form of aggregates. This was seen most clearly for WT APC stored at 37°C.

Enzyme activity

The results of the determinations of enzyme activity are shown in Figures 1-4, which show that the specific amidolytic activity of APC (U/mg) in the absence of Captisol® was lower for samples stored at 37° C (shaded bars) than at –20°C (white bars) for all samples except Xigris®, where the difference was negligible. Addition of Captisol® led to a stabilization (increase) of enzyme activity in WT and C01, whereas the effect was negligible in Xigris® and C02. The effect of Captisol® on samples stored immediately at –20°C was also negligible, except for C02, in which addition of Captisol® resulted in a decrease in specific activity.

No effect of Captisol® was seen on the specific activity of samples that were analyzed immediately after formulation (data not shown).

Example 2

Stabilization of FVII variants

Materials and Methods

Determination of Phospholipid Membrane Binding Affinity

Phospholipid membrane binding affinity may be determined as described in Nelsestuen et al., *Biochemistry*, 1977; 30;10819-10824 or as described in Example 1 in US 6,017,882.

TF-independent Factor X Activation Assay

This assay has been described in detail on page 39826 in Nelsestuen et al., *J Biol Chem*, 2001; 276:39825-39831.

Briefly, the molecule to be assayed is mixed with a source of phospholipid (preferably phosphatidylcholine and phosphatidylserine in a ratio of 8:2) and relipidated Factor X in Tris buffer containing BSA. After a specified incubation time the reaction is stopped by addition of excess EDTA. The concentration of factor Xa is then measured from the absorbance change at 405 nm after addition of a chromogenic substrate (S-2222™, Chromogenix). After correction for background the tissue factor independent activity of rhFVIIa (a_{wt}) is determined as the absorbance change after 10 minutes and the tissue factor independent activity of the FVIIa variant ($a_{variant}$) is also determined as the absorbance change after 10 minutes. The ratio between the activity of the FVIIa variant, in its activated form, and the activity of rhFVIIa is defined as $a_{variant}/a_{wt}$.

Clotting Assay

The clotting activity of the FVIIa or variants thereof may be measured in one-stage assays and the clotting times recorded on a Thrombotrack IV coagulometer (Medinor). Factor VII-depleted human plasma (American Diagnostica) is reconstituted and equilibrated at room temperature for 15-20 minutes. 50 microliters of plasma is then transferred to the coagulometer cups.

FVIIa or variants thereof are diluted in glyoxaline buffer (5.7 mM barbiturate, 4.3 mM sodium citrate, 117 mM NaCl, 1 mg/ml BSA, pH 7.35). The samples are added to the cup in 50 µl portions and incubated at 37°C for 2 minutes.

Thromboplastin (Medinor) is reconstituted with water and $CaCl_2$ is added to a final concentration of 4.5 mM. The reaction is initiated by adding 100 µl of thromboplastin. To measure the clotting activity in the absence of TF the same assay is used without addition of thromboplastin. Data may be analysed using PRISM software.

Whole Blood Assay

The clotting activity of FVIIa or variants thereof may be measured in one-stage assays and the clotting times recorded on a Thrombotrack IV coagulometer (Medinor). 100 µl of FVIIa or variants thereof are diluted in a buffer containing 10 mM glycylglycine, 50 mM NaCl, 37.5 mM $CaCl_2$, pH 7.35 and transferred to the reaction cup. The clotting reaction is initiated by addition of 50 µl blood containing 10% 0.13 M tri-sodium citrate as an anticoagulant. Data may be analysed using Excel or PRISM software.

Amidolytic Assay

The ability of FVII or variants thereof to cleave small peptide substrates can be measured using the chromogenic substrate S-2288™ (D-Ile-Pro-Arg-p-nitroanilide, Chromogenix). FVIIa is diluted to about 10-90 nM in assay buffer (50 mM Na-Hepes pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 0.1% BSA, 1U/ml heparin). Furthermore, soluble TF (sTF) is diluted to 50-450 nM in assay buffer. 120 µl of assay buffer is mixed with 20 µl of the FVIIa sample and 20 µl sTF. After 5 min. of incubation at room temperature with gentle shaking, followed by 10 min. of incubation at 37°C, the reaction is started by addition of the S-2288™ substrate to 1 mM and the absorption at 405 nm is determined at several time points.

ELISA Assay

FVII/FVIIa (or variant) concentrations may be determined by ELISA. Wells of a microtiter plate are coated with an antibody directed against the protease domain using a solution of 2 µg/ml in PBS (100 µl per well). After overnight coating at room temperature, the wells are washed 4 times with THT buffer (100 mM NaCl, 50 mM Tris-HCl pH 7.2, 0.05% Tween®-20). Subsequently, 200 µl of 1% casein (diluted from 2.5% stock using 100 mM NaCl, 50 mM Tris-HCl pH 7.2) is added per well for blocking. After 1 hr of incubation at

room temperature, the wells are emptied, and 100 µl of sample (optionally diluted in dilution buffer (THT + 0.1% casein)) is added. After another incubation of 1 hr at room temperature, the wells are washed 4 times with THT buffer, and 100 µl of a biotin-labelled antibody directed against the EGF-like domain (1 µg/ml) is added. After another 1 hr of incubation at room temperature, followed by 4 more washes with THT buffer, 100 µl of streptavidin-horseradish peroxidase (DAKO A/S, Glostrup, Denmark, diluted 1/10000) is added. After another 1 hr of incubation at room temperature, followed by 4 more washes with THT buffer, 100 µl of TMB (3,3',5,5'-tetramethylbenzidine, Kem-en-Tech A/S, Denmark) is added. After 30 min of incubation at room temperature in the dark, 100 µl of 1 M H_2SO_4 is added and OD_{450nm} is determined. A standard curve is prepared using rhFVIIa (NovoSeven®).

Alternatively, FVII/FVIIa or variants may be quantified via the Gla domain rather than via the protease domain. In this ELISA set-up, wells are coated overnight with an antibody directed against the EGF-like domain and for detection a calcium-dependent biotin-labelled monoclonal anti-Gla domain antibody is used (2 µg/ml, 100 µl per well). 5 mM $CaCl_2$ is added to the THT and dilution buffers.

DSC of formulations containing variant [L280Q]rhFVIIa

Samples of the [L280Q]rhFVIIa variant were analyzed by Differential Scanning Calorimetry (DSC) to study the unfolding (i.e. denaturation) of the protein and, in particular, to determine the melting temperature (T_m) for the protein in each run.

The starting material for DSC analysis was a solution of the protein in 10 mM Gly-Gly buffer containing 50 mM sodium chloride, 10 mM calcium chloride and 0.01% Tween® 80 (adjusted to pH 5.5). A series of solutions were prepared with varying excipients added to give a final protein concentration of 0.25 mg/ml and a final sodium chloride concentration of 86 mM.

The addition of excipient was done by carefully adding a pre-made solution of the excipient in a buffer consisting of 10 mM Gly-Gly buffer containing 140 mM sodium chloride, 10 mM calcium chloride and 0.01% Tween® 80 (adjusted to pH 5.5) to a portion of the thawed bulk solution of the protein. Water for injection was used to serve as blanks for DSC, since the object was only to determine shifts in T_m values. Prior to being subjected to DSC analysis, all solutions were degassed under vacuum for a sufficient time period as described by MicroCal Inc.

The behavior of the protein was evaluated by using a DSC apparatus from MicroCal Inc. (model VP-DSC). The temperature of the solution in question was gradually increased from 15°C to 120°C at a rate of 1.5°C per minute.

Adding 0 mg/ml, 2 mg/ml, 10 mg/ml or 80 mg/ml Captisol® to the original solution resulted in different DSC profiles.

In the first three samples (containing 0, 2, or 10 mg/ml Captisol®), two events occurred as the temperature was increased. The first event was an unfolding reaction (endothermic), and was observed as an upward peak in the scans. The second event was a precipitation (exothermic reaction), and was observed as a downward peak in the scans. The shifts in the observed ΔT_m-values were 0 and + 20°C for samples with 2 and 10 mg/ml Captisol®, respectively. ΔT_m is defined as:

$$\Delta T_m = (T_{m2} - T_{m1})$$

where T_{m1} is the temperature related to maximum of the denaturation peak in the DSC scan of the original solution without additional excipients added (i.e. the solution with 0 mg/ml Captisol®). T_{m2} is the temperature related to the maximum of the denaturation peak in each of the DSC scans of the solutions containing either 2 mg/ml or 10 mg/ml Captisol®.

The DSC profile for the 80 mg/ml Captisol® sample was flat and without a downward peak, indicating an exothermic precipitation. Thus, it was not possible to calculate ΔT_m for this sample.

The observed changes in Tm values are summarized in the table below:

Captisol® conc. (mg/ml)	ΔT_m (°C)
0	0
2	0
10	20
80	*

*) Not possible to determine

These results clearly demonstrate that addition of Captisol® stabilizes an aqueous solution of the Factor VIIa variant.

Activity of formulations containing the [L280Q]rhFVIIa variant upon freezing and thawing.

An excess of the solutions originally made for DSC analysis as described above was transferred into Eppendorf tubes and frozen at –20°C. The samples were later thawed and analyzed for activity in the "Clotting Assay" and the "Amidolytic Assay" described herein

Adding either 0 mg/ml, 2 mg/ml or 10 mg/ml Captisol® to the original solution resulted in rather different activities (see below). The activity of the formulation sample containing 0 mg/ml Captisol® was defined as 100% in both assays.

Captisol® conc. (mg/ml)	Activity (Clotting Assay)		Activity (Amidolytic Assay)
	(U/mg)	%	%
0	18481	100	100
2	58797	318	272
10	37956	205	166

Captisol® was shown not to influence the clotting assay result in itself. This was shown by addition of either 2 or 10 mg/ml Captisol® to the formulation buffer (10 mM Gly-Gly buffer containing 50 mM sodium chloride, 10 mM calcium chloride and 0.01% Tween® 80 - adjusted to pH 5.5). The clotting time for these samples did not differ from the clotting time for the assay buffer (110, 119 and 114 seconds, respectively).

These results clearly demonstrate that addition of Captisol® stabilizes the Factor VIIa variant during freezing and thawing.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be clear to one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention. For example, all the techniques, methods, compositions, apparatus and systems described above may be used in various combinations. All publications, patents, patent applications, or other documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication, patent, patent application, or other document were individually indicated to be incorporated by reference for all purposes.

SEQ ID NO:1

Human protein C (inactive one-chain zymogen)

```
Ala Asn Ser Phe Leu Glu Glu Leu Arg His Ser Ser Leu Glu Arg Glu
 1               5                  10                  15

Cys Ile Glu Glu Ile Cys Asp Phe Glu Glu Ala Lys Glu Ile Phe Gln
            20                  25                  30

Asn Val Asp Asp Thr Leu Ala Phe Trp Ser Lys His Val Asp Gly Asp
        35                  40                  45

Gln Cys Leu Val Leu Pro Leu Glu His Pro Cys Ala Ser Leu Cys Cys
    50                  55                  60

Gly His Gly Thr Cys Ile Asp Gly Ile Gly Ser Phe Ser Cys Asp Cys
65                  70                  75                  80

Arg Ser Gly Trp Glu Gly Arg Phe Cys Gln Arg Glu Val Ser Phe Leu
                85                  90                  95

Asn Cys Ser Leu Asp Asn Gly Gly Cys Thr His Tyr Cys Leu Glu Glu
            100                 105                 110

Val Gly Trp Arg Arg Cys Ser Cys Ala Pro Gly Tyr Lys Leu Gly Asp
        115                 120                 125

Asp Leu Leu Gln Cys His Pro Ala Val Lys Phe Pro Cys Gly Arg Pro
    130                 135                 140

Trp Lys Arg Met Glu Lys Lys Arg Ser His Leu Lys Arg Asp Thr Glu
145                 150                 155                 160

Asp Gln Glu Asp Gln Val Asp Pro Arg Leu Ile Asp Gly Lys Met Thr
                165                 170                 175

Arg Arg Gly Asp Ser Pro Trp Gln Val Val Leu Leu Asp Ser Lys Lys
            180                 185                 190

Lys Leu Ala Cys Gly Ala Val Leu Ile His Pro Ser Trp Val Leu Thr
        195                 200                 205

Ala Ala His Cys Met Asp Glu Ser Lys Lys Leu Leu Val Arg Leu Gly
    210                 215                 220

Glu Tyr Asp Leu Arg Arg Trp Glu Lys Trp Glu Leu Asp Leu Asp Ile
225                 230                 235                 240

Lys Glu Val Phe Val His Pro Asn Tyr Ser Lys Ser Thr Thr Asp Asn
                245                 250                 255

Asp Ile Ala Leu Leu His Leu Ala Gln Pro Ala Thr Leu Ser Gln Thr
            260                 265                 270
```

Ile Val Pro Ile Cys Leu Pro Asp Ser Gly Leu Ala Glu Arg Glu Leu
275 280 285

Asn Gln Ala Gly Gln Glu Thr Leu Val Thr Gly Trp Gly Tyr His Ser
290 295 300

Ser Arg Glu Lys Glu Ala Lys Arg Asn Arg Thr Phe Val Leu Asn Phe
305 310 315 320

Ile Lys Ile Pro Val Val Pro His Asn Glu Cys Ser Glu Val Met Ser
325 330 335

Asn Met Val Ser Glu Asn Met Leu Cys Ala Gly Ile Leu Gly Asp Arg
340 345 350

Gln Asp Ala Cys Glu Gly Asp Ser Gly Gly Pro Met Val Ala Ser Phe
355 360 365

His Gly Thr Trp Phe Leu Val Gly Leu Val Ser Trp Gly Glu Gly Cys
370 375 380

Gly Leu Leu His Asn Tyr Gly Val Tyr Thr Lys Val Ser Arg Tyr Leu
385 390 395 400

Asp Trp Ile His Gly His Ile Arg Asp Lys Glu Ala Pro Gln Lys Ser
405 410 415

Trp Ala Pro

SEQ ID NO:2

Human factor VII

Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu
1 5 10 15

Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys
20 25 30

Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp
35 40 45

Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln
50 55 60

Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn
65 70 75 80

Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly
85 90 95

Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys
100 105 110

Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr
115 120 125

Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg
130 135 140

Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro
145 150 155 160

Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln
165 170 175

Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala
180 185 190

His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu
195 200 205

Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg
210 215 220

Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn
225 230 235 240

His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp
245 250 255

His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr
260 265 270

Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu
275 280 285

Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg
290 295 300

Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser
305 310 315 320

Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser
325 330 335

Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr
340 345 350

Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys
355 360 365

Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile
370 375 380

Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu
385 390 395 400

Leu Arg Ala Pro Phe Pro
405

CLAIMS

1. A composition comprising a vitamin K-dependent polypeptide and a sulfoalkyl ether cyclodextrin derivative.

2. The composition of claim 1, wherein the vitamin K-dependent polypeptide is selected from the group consisting of human protein C, human factor VII, human protein Z, human protein S, human prothrombin, human factor X, human factor IX and human Gas6; and variants thereof comprising 1-15 amino acid modifications relative to the human amino acid sequence.

3. The composition of claim 2, wherein the vitamin K-dependent polypeptide is human factor VIIa (hFVIIa) or a variant thereof that differs from hFVIIa in 1-15 amino acid residues.

4. The composition of claim 2, wherein the vitamin K-dependent polypeptide is human activated protein C (hAPC) or a variant thereof that differs from hAPC in 1-15 amino acid residues.

5. The composition of any of the preceding claims which has a pH of from about 5 to about 8.

6. The composition of claim 4 which has a pH of above about 7.0.

7. The composition of claim 6 which has a pH of from about 7.1 to about 8.5.

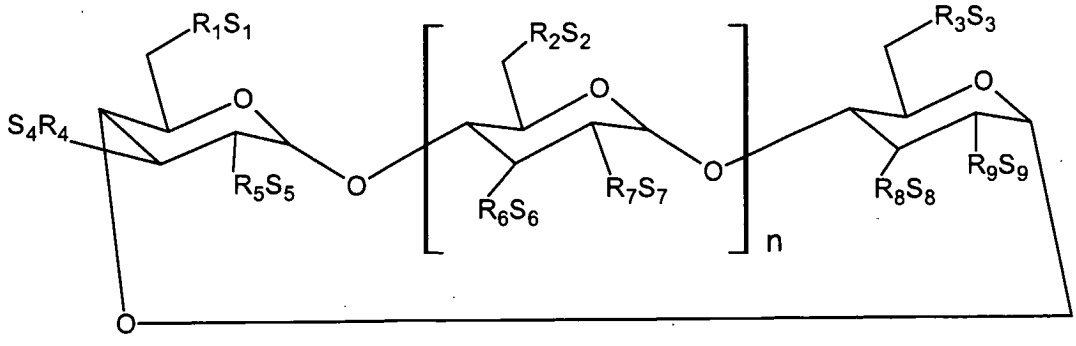
8. The composition of claim 6 which has a pH of from about 7.2 to about 8.0.

9. The composition of claim 6, which has a pH of from about 7.2 to about 7.8.

10. The composition of any of the preceding claims, wherein the polypeptide comprises at least one covalently attached non-polypeptide moiety.

11. The composition of claim 10, wherein the polypeptide comprises at least one introduced glycosylation site.

12. The composition of any of the preceding claims, wherein the sulfoalkyl ether cyclodextrin derivative is a compound of formula (I):



wherein:

n is 4, 5 or 6,

R_1, R_2, R_3, R_4, R_5, R_6, R_7, R_8, and R_9 are each independently -O- or a -O-(C_2-C_6 alkyl)-SO_3- group, wherein at least one of R_1 and R_2 is an -O-(C_2-C_6 alkyl)-SO_3- group, and

S_1, S_2, S_3, S_4, S_5, S_6, S_7, S_8, and S_9 are each independently a pharmaceutically acceptable cation.

13. The composition of claim 12, wherein the sulfoalkyl ether cyclodextrin is a beta cyclodextrin sulfobutyl ether.

14. The composition of claim 13, wherein the beta cyclodextrin sulfobutyl ether is in the form of an alkali metal or alkaline earth metal salt.

15. The composition of claim 13, wherein the beta cyclodextrin sulfobutyl ether is Captisol®.

16. The composition of any of the preceding claims, wherein the sulfoalkyl ether cyclodextrin derivative is present in a concentration from 1 mg/ml to 200 mg/ml.

17. The composition of claim 16, wherein the sulfoalkyl ether cyclodextrin derivative is present in a concentration from 50 mg/ml to 150 mg/ml.

18. The composition of any of the preceding claims, further comprising at least one buffering agent.

19. The composition of any of the preceding claims, further comprising at least one tonicity modifying agent.

20. The composition of any of the preceding claims, further comprising at least one surfactant.

21. The composition of any of the preceding claims, in the form of a liquid formulation.

22. The composition of claim 21, in the form of an aqueous solution or suspension.

23. A method for preparing a composition according to any of the preceding claims, comprising mixing a vitamin K-dependent polypeptide with a sulfoalkyl ether cyclodextrin derivative.

24. A method for increasing the stability of a vitamin K-dependent polypeptide in a pharmaceutical composition, the method comprising incorporating into said composition a sulfoalkyl ether cyclodextrin derivative.

25. The method of claim 24, wherein the vitamin K-dependent polypeptide is selected from the group consisting of human protein C, human factor VII, human protein Z, human protein S, human prothrombin, human factor X, human factor IX and human Gas6; and variants thereof comprising 1-15 amino acid modifications relative to the human amino acid sequence.

26. The method of claim 25, wherein the vitamin K-dependent polypeptide is human factor VIIa (hFVIIa) or a variant thereof that differs from hFVIIa in 1-15 amino acid residues.

27. The method of claim 25, wherein the vitamin K-dependent polypeptide is human activated protein C (hAPC) or a variant thereof that differs from hAPC in 1-15 amino acid residues.

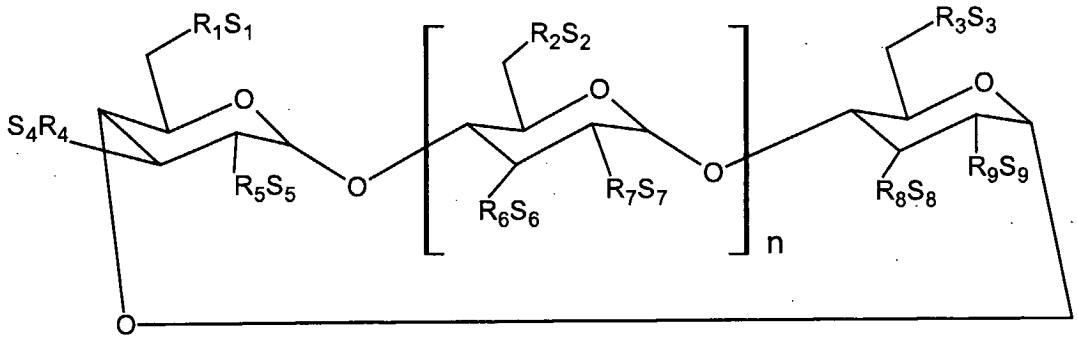
28. The method of claim 27 wherein the composition comprises at least one buffering agent and the pH of the composition is adjusted to above about 7.0.

29. The method of claim 28, wherein the pH of the composition is from about 7.1 to about 8.5.

30. The method of claim 28, wherein the pH of the composition is from about 7.2 to about 8.0.

31. The method of claim 28, wherein the pH of the composition is from about 7.2 to about 7.8.

32. The method of claim 24, wherein the sulfoalkyl ether cyclodextrin derivative is a compound of formula (I):



wherein:

n is 4, 5 or 6,

R_1, R_2, R_3, R_4, R_5, R_6, R_7, R_8, and R_9 are each independently -O- or a -O-(C_2-C_6 alkyl)-SO_3- group, wherein at least one of R_1 and R_2 is an -O-(C_2-C_6 alkyl)-SO_3- group, and

S_1, S_2, S_3, S_4, S_5, S_6, S_7, S_8, and S_9 are each independently a pharmaceutically acceptable cation.

33. The method of claim 32, wherein the sulfoalkyl ether cyclodextrin is a beta cyclodextrin sulfobutyl ether.

34. The method of claim 33, wherein the beta cyclodextrin sulfobutyl ether is in the form of an alkali metal or alkaline earth metal salt.

35. The method of claim 33, wherein the beta cyclodextrin sulfobutyl ether is Captisol®.

36. The method of any of claims 23-35, wherein the sulfoalkyl ether cyclodextrin derivative is present in a concentration from about 1 mg/ml to about 200 mg/ml.

37. A method of treating a condition treatable by administration of factor VIIa, comprising administering to a patient in need thereof a therapeutically effective amount of a composition according to claim 3.

38. The method of claim 37, wherein the condition is a clotting factor deficiency or trauma.

39. A method of treating a condition treatable by administration of activated protein C, comprising administering to a patient in need thereof a therapeutically effective amount of a composition according to claim 4.

40. The method of claim 39, wherein the condition is sepsis or septic shock.

41. A composition according to any of claims 1-22 for use as a medicine.

42. Use of a composition according to claim 3 for the manufacture of a medicament for treatment of a condition treatable by administration of factor VIIa.

43. Use according to claim 42, wherein the condition is a clotting factor deficiency or trauma.

44. Use of a composition according to claim 4 for the manufacture of a medicament for treatment of a condition treatable by administration of activated protein C.

45. Use according to claim 44, wherein the condition is sepsis or septic shock.

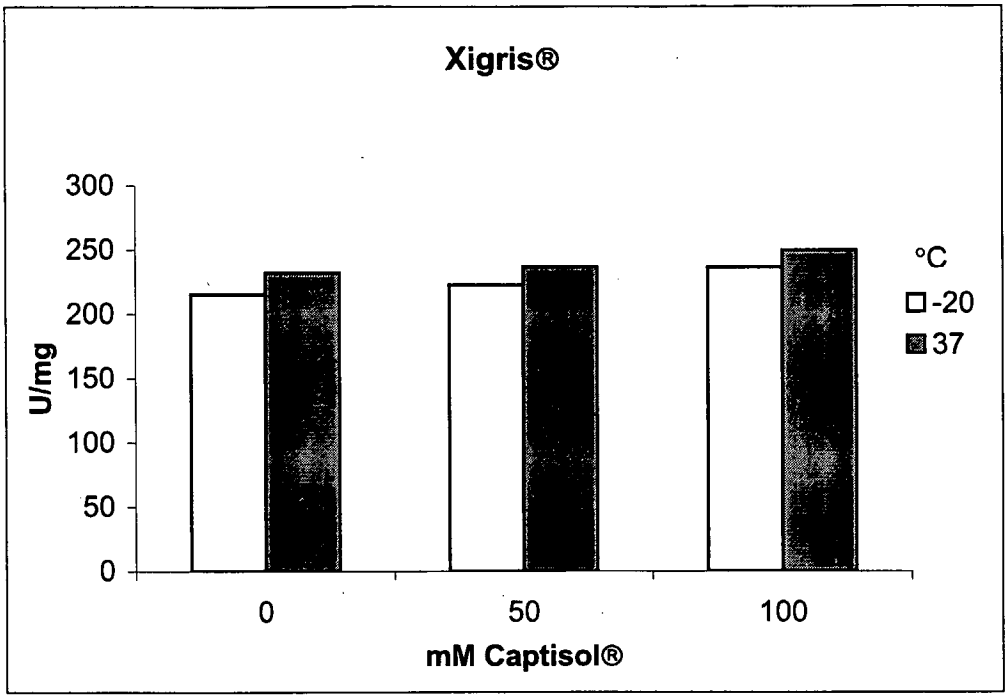

Xigris®
300
250
200
150
100
50
0
U/mg
0
50
100
mM Captisol®
°C
-20
37

Fig. 1

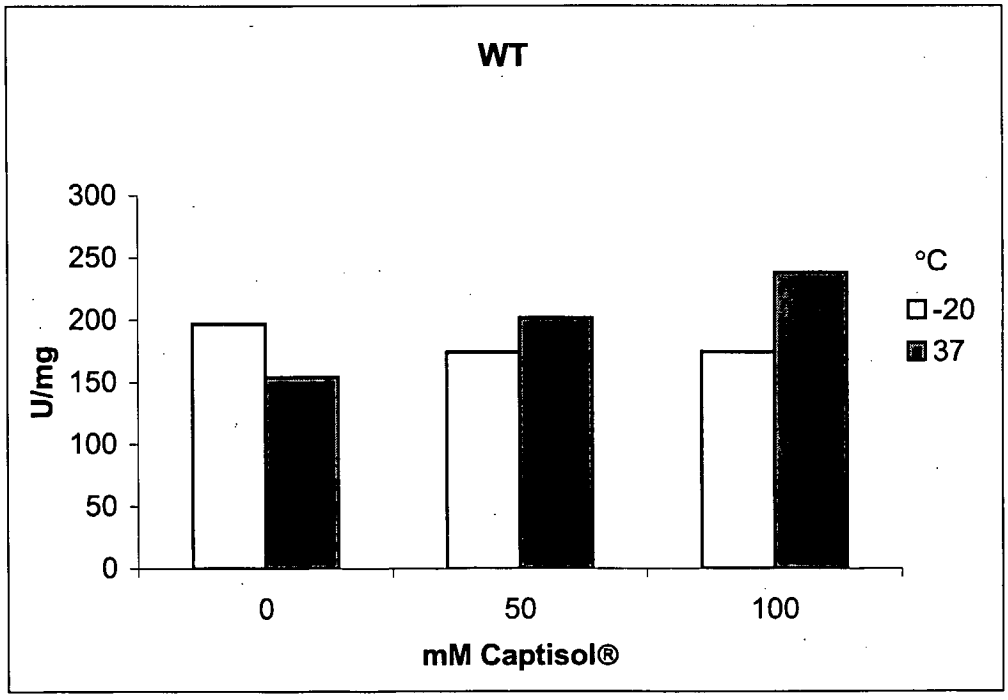

WT
300
250
200
150
100
50
0
U/mg
0
50
100
mM Captisol®
°C
-20
37

Fig. 2

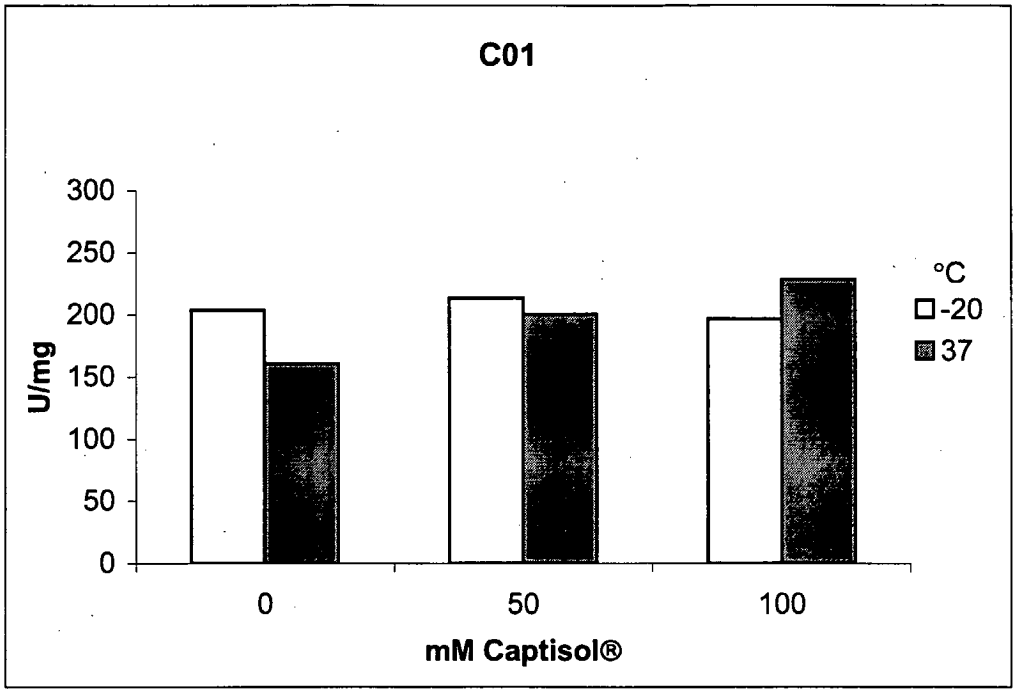

C01
300
250
200
150
100
50
0
U/mg
0
50
100
mM Captisol®
°C
-20
37

Fig. 3

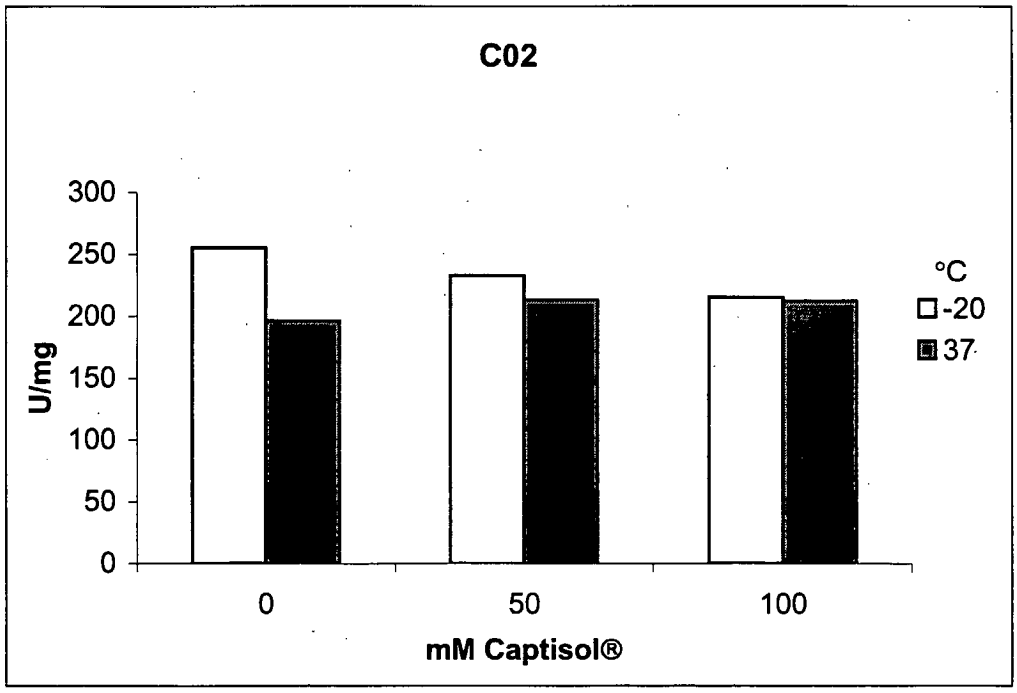

C02
300
250
200
150
100
50
0
U/mg
0
50
100
mM Captisol®
°C
-20
37

Fig. 4

SEQUENCE LISTING

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Maxygen ApS

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35 40 45

Gln Cys Leu Val Leu Pro Leu Glu His Pro Cys Ala Ser Leu Cys Cys
50 55 60

Gly His Gly Thr Cys Ile Asp Gly Ile Gly Ser Phe Ser Cys Asp Cys
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Arg Ser Gly Trp Glu Gly Arg Phe Cys Gln Arg Glu Val Ser Phe Leu
85 90 95

Asn Cys Ser Leu Asp Asn Gly Gly Cys Thr His Tyr Cys Leu Glu Glu
100 105 110

Val Gly Trp Arg Arg Cys Ser Cys Ala Pro Gly Tyr Lys Leu Gly Asp
115 120 125

Asp Leu Leu Gln Cys His Pro Ala Val Lys Phe Pro Cys Gly Arg Pro
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Trp Lys Arg Met Glu Lys Lys Arg Ser His Leu Lys Arg Asp Thr Glu
145 150 155 160

Asp Gln Glu Asp Gln Val Asp Pro Arg Leu Ile Asp Gly Lys Met Thr
165 170 175

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Arg Arg Gly Asp Ser Pro Trp Gln Val Val Leu Leu Asp Ser Lys Lys
            180                 185                 190

Lys Leu Ala Cys Gly Ala Val Leu Ile His Pro Ser Trp Val Leu Thr
        195                 200                 205

Ala Ala His Cys Met Asp Glu Ser Lys Lys Leu Leu Val Arg Leu Gly
    210                 215                 220

Glu Tyr Asp Leu Arg Arg Trp Glu Lys Trp Glu Leu Asp Leu Asp Ile
225                 230                 235                 240

Lys Glu Val Phe Val His Pro Asn Tyr Ser Lys Ser Thr Thr Asp Asn
                245                 250                 255

Asp Ile Ala Leu Leu His Leu Ala Gln Pro Ala Thr Leu Ser Gln Thr
            260                 265                 270

Ile Val Pro Ile Cys Leu Pro Asp Ser Gly Leu Ala Glu Arg Glu Leu
        275                 280                 285

Asn Gln Ala Gly Gln Glu Thr Leu Val Thr Gly Trp Gly Tyr His Ser
    290                 295                 300

Ser Arg Glu Lys Glu Ala Lys Arg Asn Arg Thr Phe Val Leu Asn Phe
305                 310                 315                 320

Ile Lys Ile Pro Val Val Pro His Asn Glu Cys Ser Glu Val Met Ser
                325                 330                 335

Asn Met Val Ser Glu Asn Met Leu Cys Ala Gly Ile Leu Gly Asp Arg
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Gln Asp Ala Cys Glu Gly Asp Ser Gly Gly Pro Met Val Ala Ser Phe
        355                 360                 365

His Gly Thr Trp Phe Leu Val Gly Leu Val Ser Trp Gly Glu Gly Cys
    370                 375                 380

Gly Leu Leu His Asn Tyr Gly Val Tyr Thr Lys Val Ser Arg Tyr Leu
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Asp Trp Ile His Gly His Ile Arg Asp Lys Glu Ala Pro Gln Lys Ser
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Trp Ala Pro
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<210> 2
<211> 406
<212> PRT
<213> Homo sapiens

<400> 2

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            20                  25                  30

Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp
        35                  40                  45

Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln
    50                  55                  60

Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn
65                  70                  75                  80

Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly
                85                  90                  95

Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys
            100                 105                 110

Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr
        115                 120                 125

Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg
    130                 135                 140

Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro
145                 150                 155                 160

Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln
                165                 170                 175

Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala
            180                 185                 190

His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu
        195                 200                 205

Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg
    210                 215                 220

Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn
225                 230                 235                 240

His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp
                245                 250                 255

His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr
            260                 265                 270

Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu
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275 280 285

Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg
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Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser
305 310 315 320

Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser
325 330 335

Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr
340 345 350

Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys
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Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile
370 375 380

Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu
385 390 395 400

Leu Arg Ala Pro Phe Pro
405

INTERNATIONAL SEARCH REPORT

Int nal Application No
PCT/DK2004/000585

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K47/48 A61K38/36 A61K38/48 A61P7/00 A61P31/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, EPO-Internal, WPI Data, PAJ, EMBASE, MEDLINE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2002/146409 A1 (MORRIS JOHN A ET AL) 10 October 2002 (2002-10-10) paragraphs '0006!-'0008!	1-45
Y	WO 03/055512 A (JENSEN MICHAEL BECH ; KORNFELT TROELS (DK); NOVO NORDISK AS (DK); HANS) 10 July 2003 (2003-07-10) page 1-3; claims 1,3,4	1-45
Y	WO 03/055511 A (JENSEN MICHAEL BECH ; KORNFELT TROELS (DK); NOVO NORDISK AS (DK); HANS) 10 July 2003 (2003-07-10) cited in the application page 1 -page 3	1-45

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 November 2004	21/12/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Durrenberger, A

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INTERNATIONAL SEARCH REPORT

Int onal Application No
PCT/DK2004/000585

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5 925 738 A (SINGH MANISH P ET AL) 20 July 1999 (1999-07-20) cited in the application page 1 -page 4, line 11	1-45
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A	UEKAMA KANETO ET AL: "Stabilizing and solubilizing effects of sulfobutyl ether beta-cyclodextrin on prostaglandin E1 analogue" PHARMACEUTICAL RESEARCH (NEW YORK), vol. 18, no. 11, November 2001 (2001-11), pages 1578-1585, XP002308050 ISSN: 0724-8741 the whole document	1-45
T	ANONYMOUS: "Captisol: Solubility and so much more" INTERNET ARTICLE, 'Online! pages 1-8, XP002308080 Retrieved from the Internet: <URL:http://www.captisol.com/New%20Captisol%20Brochure.pdf> 'retrieved on 2004-11-29! the whole document	

Form PCT/ISA/210 (continuation of second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

national application No.
PCT/DK2004/000585

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 37-40 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

formation on patent family members

Int[illegible]nal Application No
PCT/DK2004/000585

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US 2002146409 A1	10-10-2002	WO 02060475 A1	08-08-2002
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		US 2004013644 A1	22-01-2004

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60/503,418 16 September 2003 (16.09.2003) US

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(72) Inventors; and

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(74) Agent: **NOVO NORDISK A/S**; Corporate Patents, Novo Allé, DK-2880 Bagsværd (DK).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: COAGULATION FACTOR VII POLYPEPTIDES

(57) Abstract: The present invention relates to novel coagulation Factor VII polypeptides, polynucleotide constructs encoding such polypeptides, as well as vectors and host cells comprising and expressing the polynucleotide, pharmaceutical compositions, uses and methods of treatment.

WO 2005/024006 A2

COAGULATION FACTOR VII POLYPEPTIDES

FIELD OF THE INVENTION

The present invention relates to novel human coagulation Factor VII polypeptides, as well as polynucleotide constructs encoding such polypeptides, vectors and host cells comprising and expressing the polynucleotide, pharmaceutical compositions comprising Factor VII polypeptides, uses and methods of treatment.

BACKGROUND OF THE INVENTION

Blood coagulation is a process consisting of a complex interaction of various blood components (or factors) that eventually gives rise to a fibrin clot. Generally, the blood components, which participate in what has been referred to as the coagulation "cascade", are enzymatically inactive proteins (proenzymes or zymogens) that are converted to proteolytic enzymes by the action of an activator (which itself is an activated clotting factor). Coagulation factors that have undergone such a conversion are generally referred to as "active factors", and are designated by the addition of the letter "a" to the name of the coagulation factor (e.g. Factor VIIa).

Initiation of the haemostatic process is mediated by the formation of a complex between tissue factor, exposed as a result of injury to the vessel wall, and Factor VIIa. This complex then converts Factors IX and X to their active forms. Factor Xa converts limited amounts of prothrombin to thrombin on the tissue factor-bearing cell. Thrombin activates platelets and Factors V and VIII into Factors Va and VIIIa, both cofactors in the further process leading to the full thrombin burst. This process includes generation of Factor Xa by Factor IXa (in complex with factor VIIIa) and occurs on the surface of activated platelets. Thrombin finally converts fibrinogen to fibrin resulting in formation of a fibrin clot.

Factor VII is a trace plasma glycoprotein that circulates in blood as a single-chain zymogen. The zymogen is catalytically inactive. Single-chain Factor VII may be converted to two-chain Factor VIIa by Factor Xa, Factor XIIa, Factor IXa, Factor VIIa or thrombin in vitro. Factor Xa is believed to be the major physiological activator of Factor VII. The conversion of zymogen Factor VII into the activated two-chain molecule occurs by cleavage of an internal Arg_{152}-Ile_{153} peptide bond.

It is often desirable to stimulate the coagulation cascade in a subject. Factor VIIa has been used to control bleeding disorders that have several causes such as clotting factor deficiencies (e.g. haemophilia A and B or deficiency of coagulation Factors XI or VII) or clotting factor inhibitors. Factor VIIa has also been used to control excessive bleeding occurring

in subjects with a normally functioning blood clotting cascade (no clotting factor deficiencies or inhibitors against any of the coagulation factors). Such bleeding may, for example, be caused by a defective platelet function, thrombocytopenia or von Willebrand's disease. Bleeding is also a major problem in connection with surgery and other forms of tissue damage.

European Patent No. 200,421 (ZymoGenetics) relates to the nucleotide sequence encoding human Factor VII and the recombinant expression of Factor VII in mammalian cells.

Dickinson et al. (*Proc. Natl. Acad. Sci. USA* 93, 14379-14384, 1996) discloses Factor VII polypeptides wherein certain amino acids have been individually replaced by Ala. Iwanaga et al. (*Thromb. Haemost.* (supplement august 1999), 466, abstract 1474) relates to Factor VIIa variants wherein residues 316-320 are deleted or residues 311-322 are replaced with the corresponding residues from trypsin.

There is still a need in the art for improved Factor VII polypeptides having procoagulant activity. In particular, there is a need for Factor VII polypeptides with increased TF independent activity.

SUMMARY OF THE INVENTION

The present invention relates to Factor VII polypeptides with increased TF independent activity, such as novel coagulation Factor VII polypeptides with the same or increased activity compared to wild type Factor VIIa and with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa.

In a first aspect, the present invention relates to a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa with the proviso that the Factor VII polypeptide is not I69A-FVII.

In a second aspect, the invention relates to a composition comprising a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa with the proviso that the Factor VII polypeptide is not I69A-FVII.

In a third aspect, the invention relates to a pharmaceutical composition comprising a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa; and optionally, a pharmaceutically acceptable carrier.

In a further aspect, the invention relates to a polynucleotide construct encoding a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa with the proviso that the Factor VII polypeptide is not I69A-FVII.

In a further aspect, the invention relates to a eukaryotic host cell comprising a polynucleotide construct encoding a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa with the proviso that the Factor VII polypeptide is not I69A-FVII.

In a further aspect, the invention relates to a method for producing a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa with the proviso that the Factor VII polypeptide is not I69A-FVII, the method comprising cultivating a eukaryotic host cell comprising a polynucleotide construct encoding the Factor VII polypeptide in an appropriate growth medium under conditions allowing protein synthesis from said polynucleotide construct and recovering said Factor VII polypeptide from the culture medium.

In a further aspect, the invention relates to a use of a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa; for the preparation of a medicament for the treatment of bleeding episodes or for the enhancement of the normal haemostatic system.

In a further aspect, the invention relates to a method for the treatment of bleeding episodes or bleeding disorders in a subject or for the enhancement of the normal haemostatic system, the method comprising administering to a subject in need thereof a therapeutically or prophylactically effective amount of a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa.

In a further aspect, the invention relates to a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa, wherein said Factor VII polypeptide is selected from:

i) a Factor VII polypeptide comprising one or more amino acid substitution selected from $K18X_1$, $R36X_2$, $S43X_3$, $K62X_4$, $Q64X_5$, $L65X_6$, $I69X_7$, $F71X_8$, $L73X_9$, $P74X_{10}$, $E77X_{11}$, $G78X_{12}$, $R79X_{13}$, $K85X_{14}$, $Q88X_{15}$, $N93X_{16}$, $F275X_{17}$, $R277X_{18}$, $M306X_{19}$,

$T307X_{20}$, $Q308X_{21}$, $D309X_{22}$, or $R379X_{23}$ corresponding to amino acids at positions of SEQ ID NO: 1, which different amino acid decreases the Tissue Factor binding affinity, wherein

X_1 is E, D, A, or F;

X_2 is E, D, K, A, F, or N;

X_3 is E, D, K, R, A, or F;

X_4 is D, A, or F;

X_5 is E, D, K, R, A, F, or N;

X_6 is E, D, K, R, A, or F;

X_7 is C, D, E, F, G, H, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_8 is A, G, H, I, K, L, M, P, R, S, T, V, or W;

X_9 is A, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y;

X_{10} is A, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, or Y;

X_{11} is A, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_{12} is A, D, E, F, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_{13} is A, D, E, F, G, H, I, K, L, M, N, P, Q, S, T, V, W, or Y;

X_{14} is A, D, E, F, G, I, L, M, P, S, T, V, W, or Y;

X_{15} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;

X_{16} is A, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y;

X_{17} is A, D, E, G, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_{18} is A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y;

X_{19} is A, E, F, G, H, I, K, L, P, Q, R, S, T, V, W, or Y;

X_{20} is A, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, V, W, or Y;

X_{21} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;

X_{22} is A, F, G, H, I, K, L, M, N, P, Q, R, V, W, or Y; and

X_{23} is A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y;

and/or

ii) a Factor VII polypeptide, wherein one or more amino acid corresponding to amino acids at positions selected from K18, R36, K62, Q64, L65, I69, F71, L73, P74, E77, G78, R79, K85, Q88, N93, F275, R277, M306, Q308, D309, or R379 of SEQ ID NO: 1 have been substituted with a cysteine amino acid residue, which cysteine amino acid residue decreases the Tissue Factor binding affinity, and which cysteine amino acid residue is optionally conjugated with a chemical group, which chemical group increases the molecular weight of said Factor VII polypeptide;

and/or

iii) a Factor VII polypeptide comprising one or more N-glycosylation site N-Xaa-S/T introduced by amino acid substitutions corresponding to amino acids starting at positions selected from R36, Q64, I69, F71, P74, E77, G78, Q88, N93, F275, M306, T307, or D309 of SEQ ID NO: 1, wherein Xaa is any amino acid except P, which introduced N-glycosylation site decreases the Tissue Factor binding affinity.

In a further aspect, the invention relates to a composition comprising a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa, wherein said Factor VII polypeptide is selected from:

i) a Factor VII polypeptide comprising one or more amino acid substitution selected from $K18X_1$, $R36X_2$, $S43X_3$, $K62X_4$, $Q64X_5$, $L65X_6$, $I69X_7$, $F71X_8$, $L73X_9$, $P74X_{10}$, $E77X_{11}$, $G78X_{12}$, $R79X_{13}$, $K85X_{14}$, $Q88X_{15}$, $N93X_{16}$, $F275X_{17}$, $R277X_{18}$, $M306X_{19}$, $T307X_{20}$, $Q308X_{21}$, $D309X_{22}$, or $R379X_{23}$ corresponding to amino acids at positions of SEQ ID NO: 1, which different amino acid decreases the Tissue Factor binding affinity, wherein

X_1 is E, D, A, or F;

X_2 is E, D, K, A, F, or N;

X_3 is E, D, K, R, A, or F;

X_4 is D, A, or F;

X_5 is E, D, K, R, A, F, or N;

X_6 is E, D, K, R, A, or F;

X_7 is C, D, E, F, G, H, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_8 is A, G, H, I, K, L, M, P, R, S, T, V, or W;

X_9 is A, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y;

X_{10} is A, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, or Y;

X_{11} is A, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_{12} is A, D, E, F, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_{13} is A, D, E, F, G, H, I, K, L, M, N, P, Q, S, T, V, W, or Y;

X_{14} is A, D, E, F, G, I, L, M, P, S, T, V, W, or Y;

X_{15} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;

X_{16} is A, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y;

X_{17} is A, D, E, G, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_{18} is A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y;

X_{19} is A, E, F, G, H, I, K, L, P, Q, R, S, T, V, W, or Y;

X_{20} is A, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, V, W, or Y;

X_{21} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;

X_{22} is A, F, G, H, I, K, L, M, N, P, Q, R, V, W, or Y; and

X_{23} is A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y;

and/or

ii) a Factor VII polypeptide, wherein one or more amino acid corresponding to amino acids at positions selected from K18, R36, K62, Q64, L65, I69, F71, L73, P74, E77, G78, R79, K85, Q88, N93, F275, R277, M306, Q308, D309, or R379 of SEQ ID NO: 1 have been substituted with a cysteine amino acid residue, which cysteine amino acid residue decreases the Tissue Factor binding affinity, and which cysteine amino acid residue is optionally conjugated with a chemical group, which chemical group increases the molecular weight of said Factor VII polypeptide;

and/or

iii) a Factor VII polypeptide comprising one or more N-glycosylation site N-Xaa-S/T introduced by amino acid substitutions corresponding to amino acids starting at positions selected from R36, Q64, I69, F71, P74, E77, G78, Q88, N93, F275, M306, T307, or D309 of SEQ ID NO: 1, wherein Xaa is any amino acid except P, which introduced N-glycosylation site decreases the Tissue Factor binding affinity.

In a further aspect, the invention relates to a pharmaceutical composition comprising a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa, wherein said Factor VII polypeptide is selected from:

i) a Factor VII polypeptide comprising one or more amino acid substitution selected from $K18X_1$, $R36X_2$, $S43X_3$, $K62X_4$, $Q64X_5$, $L65X_6$, $I69X_7$, $F71X_8$, $L73X_9$, $P74X_{10}$, $E77X_{11}$, $G78X_{12}$, $R79X_{13}$, $K85X_{14}$, $Q88X_{15}$, $N93X_{16}$, $F275X_{17}$, $R277X_{18}$, $M306X_{19}$, $T307X_{20}$, $Q308X_{21}$, $D309X_{22}$, or $R379X_{23}$ corresponding to amino acids at positions of SEQ ID NO: 1, which different amino acid decreases the Tissue Factor binding affinity, wherein

X_1 is E, D, A, or F;

X_2 is E, D, K, A, F, or N;

X_3 is E, D, K, R, A, or F;

X_4 is D, A, or F;

X_5 is E, D, K, R, A, F, or N;

X_6 is E, D, K, R, A, or F;

X_7 is C, D, E, F, G, H, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_8 is A, G, H, I, K, L, M, P, R, S, T, V, or W;

X_9 is A, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y;
X_{10} is A, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, or Y;
X_{11} is A, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;
X_{12} is A, D, E, F, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;
X_{13} is A, D, E, F, G, H, I, K, L, M, N, P, Q, S, T, V, W, or Y;
X_{14} is A, D, E, F, G, I, L, M, P, S, T, V, W, or Y;
X_{15} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;
X_{16} is A, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y;
X_{17} is A, D, E, G, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;
X_{18} is A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y;
X_{19} is A, E, F, G, H, I, K, L, P, Q, R, S, T, V, W, or Y;
X_{20} is A, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, V, W, or Y;
X_{21} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;
X_{22} is A, F, G, H, I, K, L, M, N, P, Q, R, V, W, or Y; and
X_{23} is A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y;

and/or

ii) a Factor VII polypeptide, wherein one or more amino acid corresponding to amino acids at positions selected from K18, R36, K62, Q64, L65, I69, F71, L73, P74, E77, G78, R79, K85, Q88, N93, F275, R277, M306, Q308, D309, or R379 of SEQ ID NO: 1 have been substituted with a cysteine amino acid residue, which cysteine amino acid residue decreases the Tissue Factor binding affinity, and which cysteine amino acid residue is optionally conjugated with a chemical group, which chemical group increases the molecular weight of said Factor VII polypeptide;

and/or

iii) a Factor VII polypeptide comprising one or more N-glycosylation site N-Xaa-S/T introduced by amino acid substitutions corresponding to amino acids starting at positions selected from R36, Q64, I69, F71, P74, E77, G78, Q88, N93, F275, M306, T307, or D309 of SEQ ID NO: 1, wherein Xaa is any amino acid except P, which introduced N-glycosylation site decreases the Tissue Factor binding affinity;

and optionally, a pharmaceutically acceptable carrier.

In a further aspect, the invention relates to a use of a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa, wherein said Factor VII polypeptide is selected from:

i) a Factor VII polypeptide comprising one or more amino acid substitution selected from $K18X_1$, $R36X_2$, $S43X_3$, $K62X_4$, $Q64X_5$, $L65X_6$, $I69X_7$, $F71X_8$, $L73X_9$, $P74X_{10}$, $E77X_{11}$, $G78X_{12}$, $R79X_{13}$, $K85X_{14}$, $Q88X_{15}$, $N93X_{16}$, $F275X_{17}$, $R277X_{18}$, $M306X_{19}$, $T307X_{20}$, $Q308X_{21}$, $D309X_{22}$, or $R379X_{23}$ corresponding to amino acids at positions of SEQ ID NO: 1, which different amino acid decreases the Tissue Factor binding affinity, wherein

X_1 is E, D, A, or F;

X_2 is E, D, K, A, F, or N;

X_3 is E, D, K, R, A, or F;

X_4 is D, A, or F;

X_5 is E, D, K, R, A, F, or N;

X_6 is E, D, K, R, A, or F;

X_7 is C, D, E, F, G, H, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_8 is A, G, H, I, K, L, M, P, R, S, T, V, or W;

X_9 is A, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y;

X_{10} is A, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, or Y;

X_{11} is A, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_{12} is A, D, E, F, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_{13} is A, D, E, F, G, H, I, K, L, M, N, P, Q, S, T, V, W, or Y;

X_{14} is A, D, E, F, G, I, L, M, P, S, T, V, W, or Y;

X_{15} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;

X_{16} is A, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y;

X_{17} is A, D, E, G, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_{18} is A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y;

X_{19} is A, E, F, G, H, I, K, L, P, Q, R, S, T, V, W, or Y;

X_{20} is A, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, V, W, or Y;

X_{21} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;

X_{22} is A, F, G, H, I, K, L, M, N, P, Q, R, V, W, or Y; and

X_{23} is A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y;

and/or

ii) a Factor VII polypeptide, wherein one or more amino acid corresponding to amino acids at positions selected from K18, R36, K62, Q64, L65, I69, F71, L73, P74, E77, G78, R79, K85, Q88, N93, F275, R277, M306, Q308, D309, or R379 of SEQ ID NO: 1 have been substituted with a cysteine amino acid residue, which cysteine amino acid residue decreases the Tissue Factor binding affinity, and which cysteine amino

acid residue is optionally conjugated with a chemical group, which chemical group increases the molecular weight of said Factor VII polypeptide;

and/or

iii) a Factor VII polypeptide comprising one or more N-glycosylation site N-Xaa-S/T introduced by amino acid substitutions corresponding to amino acids starting at positions selected from R36, Q64, I69, F71, P74, E77, G78, Q88, N93, F275, M306, T307, or D309 of SEQ ID NO: 1, wherein Xaa is any amino acid except P, which introduced N-glycosylation site decreases the Tissue Factor binding affinity;

for the preparation of a medicament for the treatment of bleeding episodes or for the enhancement of the normal haemostatic system.

In a further aspect, the invention relates to a method for the treatment of bleeding episodes or bleeding disorders in a subject or for the enhancement of the normal haemostatic system, the method comprising administering to a subject in need thereof a therapeutically or prophylactically effective amount of a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa, wherein said Factor VII polypeptide is selected from:

i) a Factor VII polypeptide comprising one or more amino acid substitution selected from $K18X_1$, $R36X_2$, $S43X_3$, $K62X_4$, $Q64X_5$, $L65X_6$, $I69X_7$, $F71X_8$, $L73X_9$, $P74X_{10}$, $E77X_{11}$, $G78X_{12}$, $R79X_{13}$, $K85X_{14}$, $Q88X_{15}$, $N93X_{16}$, $F275X_{17}$, $R277X_{18}$, $M306X_{19}$, $T307X_{20}$, $Q308X_{21}$, $D309X_{22}$, or $R379X_{23}$ corresponding to amino acids at positions of SEQ ID NO: 1, which different amino acid decreases the Tissue Factor binding affinity, wherein

X_1 is E, D, A, or F;

X_2 is E, D, K, A, F, or N;

X_3 is E, D, K, R, A, or F;

X_4 is D, A, or F;

X_5 is E, D, K, R, A, F, or N;

X_6 is E, D, K, R, A, or F;

X_7 is C, D, E, F, G, H, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_8 is A, G, H, I, K, L, M, P, R, S, T, V, or W;

X_9 is A, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y;

X_{10} is A, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, or Y;

X_{11} is A, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_{12} is A, D, E, F, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_{13} is A, D, E, F, G, H, I, K, L, M, N, P, Q, S, T, V, W, or Y;

X_{14} is A, D, E, F, G, I, L, M, P, S, T, V, W, or Y;
X_{15} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;
X_{16} is A, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y;
X_{17} is A, D, E, G, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;
X_{18} is A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y;
X_{19} is A, E, F, G, H, I, K, L, P, Q, R, S, T, V, W, or Y;
X_{20} is A, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, V, W, or Y;
X_{21} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;
X_{22} is A, F, G, H, I, K, L, M, N, P, Q, R, V, W, or Y; and
X_{23} is A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y;

and/or

ii) a Factor VII polypeptide, wherein one or more amino acid corresponding to amino acids at positions selected from K18, R36, K62, Q64, L65, I69, F71, L73, P74, E77, G78, R79, K85, Q88, N93, F275, R277, M306, Q308, D309, or R379 of SEQ ID NO: 1 have been substituted with a cysteine amino acid residue, which cysteine amino acid residue decreases the Tissue Factor binding affinity, and which cysteine amino acid residue is optionally conjugated with a chemical group, which chemical group increases the molecular weight of said Factor VII polypeptide;

and/or

iii) a Factor VII polypeptide comprising one or more N-glycosylation site N-Xaa-S/T introduced by amino acid substitutions corresponding to amino acids starting at positions selected from R36, Q64, I69, F71, P74, E77, G78, Q88, N93, F275, M306, T307, or D309 of SEQ ID NO: 1, wherein Xaa is any amino acid except P, which introduced N-glycosylation site decreases the Tissue Factor binding affinity.

In a further aspect the present invention relates to a composition comprising wild type human FVIIa and a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa.

In a further aspect the present invention relates to a method for the treatment of bleeding episodes in a subject or for the enhancement of the normal haemostatic system, the method comprising administering to a subject in need thereof a therapeutically or prophylactically effective amount of:

a) composition comprising wild type human FVIIa and a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and

substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa; or

b) a first composition comprising wild type human FVIIa and a second composition comprising a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa.

In a further aspect the present invention relates to a process for preparing a composition comprising wild type human FVIIa and a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa, wherein the process comprises the step of: mixing wild type human FVIIa with a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa in an aqueous medium.

In a further aspect the present invention relates to a use of a composition comprising wild type human FVIIa and a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa, for the preparation of a medicament for the treatment of bleeding episodes in a subject or for the enhancement of the normal haemostatic system.

DESCRIPTION OF THE INVENTION

The present invention relates to novel coagulation Factor VII polypeptides with substantially the same or increased activity compared to wild type Factor VIIa and with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa. Increasing evidence suggest that therapeutic treatment with recombinant wild type human coagulation Factor VII works by a TF independent mechanism involving binding of FVII directly to activated thrombocytes and a generation of activated Factor Xa and eventually fibrin locally at the place of tissue damage. The Factor VII polypeptides of the present invention provide an alternative to traditional TF binding Factor VII polypeptides. Factor VII polypeptides, which do not bind to TF may be an advantage in the therapeutic treatment of bleedings, when the exposure of TF is high, e.g. plaque rupture or sepsis.

As used herein, the terms "Factor VII polypeptide " or "FVII polypeptide" means wild-type human Factor VII/VIIa (i.e., a polypeptide having the amino acid sequence disclosed in U.S. Patent No. 4,784,950), as well as variants of Factor VII exhibiting substantially

the same or improved biological activity relative to wild-type Factor VII, Factor VII-related polypeptides as well as Factor VII derivatives and Factor VII conjugates. Unless otherwise indicated, the term "Factor VII" is intended to encompass Factor VII polypeptides in their uncleaved (zymogen) form, as well as those that have been proteolytically processed to yield their respective bioactive forms, which may be designated Factor VIIa. Typically, Factor VII is cleaved between residues 152 and 153 to yield Factor VIIa. Such variants of Factor VII may exhibit different properties relative to human Factor VII, including stability, phospholipid binding, altered specific activity, and the like.

As used herein, "Factor VII-related polypeptides" encompasses polypeptides, including variants, in which the Factor VIIa biological activity has been substantially modified or reduced relative to the activity of wild-type Factor VIIa. These polypeptides include, without limitation, Factor VII or Factor VIIa into which specific amino acid sequence alterations have been introduced that modify or disrupt the bioactivity of the polypeptide.

The term "Factor VII derivative" as used herein, is intended to designate wild-type Factor VII, variants of Factor VII exhibiting substantially the same or improved biological activity relative to wild-type Factor VII and Factor VII-related polypeptides, in which one or more of the amino acids of the parent peptide have been chemically and/or enzymatically modified, e.g. by alkylation, glycosylation, PEGylation, acylation, ester formation or amide formation or the like. This includes but is not limited to PEGylated human Factor VIIa, cysteine-PEGylated human Factor VIIa and variants thereof.

The optional conjugation of a cysteine amino acid residue with the chemical group includes but are not limited to covalent attachment of polyethylene glycol (PEG), monomethoxy-polyethylene glycol, dextran, poly-(N-vinyl pyrrolidone) polyethylene glycol, propylene glycol homopolymers, a polypropylene oxide/ethylene oxide co-polymer, polypropylene glycol, polyoxyethylated polyols (e.g., glycerol) and polyvinyl alcohol, colominic acids or other carbohydrate based polymers, polymers of amino acids, and biotin derivatives. In one embodiment of the present invention, the chemical group is a biocompatible, non-toxic, non-immunogenic and water-soluble polymer. Preferably the chemical group is water-soluble in all proportions.

Methods for attaching PEG groups to cysteine residues is described in Roberts, M.J. et al, Advanced Drug Delivery Reviews 54 (2002) 459-476.

An "N-glycosylation site" has the sequence N-Xaa-S/T, wherein Xaa is any amino acid residue except proline, N is asparagine and S/T is either serine or threonine, such as serine or threonine, such as threonine.

Specific examples of activated PEG polymers particularly preferred for coupling to cysteine residues, include the following linear PEGs : vinylsulfone-PEG (VS-PEG), such as

vinylsulfone-mPEG (VS-mPEG); maleimide-PEG (MAL-PEG), such as maleimide-mPEG (MAL-mPEG) and orthopyridyl-disulfide-PEG (OPSS-PEG), such as orthopyridyl-disulfide-mPEG (OPSS-MPEG). Typically, such PEG or mPEG polymers will have a size of about 2 kDa, such as about 5 kDa, such as about 10 kD, such as about 12 kDa or such as about 20 kDa.

The skilled person will be aware that the activation method and/or conjugation chemi stry to be used depends on the functional groups of the polymer (e. g. being amine, hydroxyl, carboxyl, aldehyde, sulfydryl, succinimidyl, maleimide, vinysulfone or haloacetate).

For conjugated with a chemical group to a cysteine residue (e.g. PEGylation) the FVII polypeptide is usually treated with a reducing agent, such as dithiothreitol (DDT), β-mercaptoethanol, or glutathione (γ-glutamylcysteinylglycine) prior to PEGylation, such as described in Higashi, S. Matsumoto, N., Iwanaga, S. (1997) J.Biol.Chem., 272(41), 25724-25730. The reducing agent is subsequently removed by any conventional method, such as by desalting. Conjugation of PEG to a cysteine residue typically takes place in a suitable buffer at pH 6-9 at temperatures varying from 4°C to 25°C for periods up to 16 hours.

In one embodiment of the present invention, an introduced cysteine residue is conjugated by mixed disulfide bond-formation with a chemical group selected from the list consisting of glutathione (gamma-glutamylcysteinylglycine), gamma-glutamylcysteine and cysteine present in the host cell or in the culture medium during production of the FVII polypeptide.

In one embodiment of the invention the chemical group is selected from the group consisting of: dendrimer, polyalkylene oxide (PAO), polyalkylene glycol (PAG), polyethylene glycol (PEG), polypropylene glycol (PPG), branched PEGs, polyvinyl alcohol (PVA), polycarboxylate, poly-vinylpyrolidone, polyethylene-co-maleic acid anhydride, polystyrene-co-maleic acid anhydride, dextran, carboxymethyl-dextran; serum protein binding-ligands, such as compounds which bind to albumin, such as fatty acids, C5-C24 fatty acid, aliphatic diacid (e.g. C5-C24), a structure (e.g. sialic acid derivatives or mimetics) which inhibits the glycans from binding to receptors (e.g. asialoglycoprotein receptor and mannose receptor), a small organic molecule containing moieties that under physiological conditions alters charge properties, such as carboxylic acids or amines, or neutral sub-stituents that prevent glycan specific recognition such as smaller alkyl substituents (e.g., C1-C5 alkyl), a low molecular organic charged radical (e.g. C1-C25), which may contain one or more carboxylic acids, amines sulfonic, phosphonic acids, or combination thereof; a low molecular neutral hydrophilic molecule (e.g. C1-C25), such as cyclodextrin, or a polyethylene chain which may optionally branched; polyethyleneglycol with a avarage molecular weight of 2-40 KDa; a well defined precission polymer such as a dendrimer with an excact molecular mass ranging from

700 to 20.000 Da, such as between 700-10.000 Da; and a substantially non-imunogenic polypeptide such as albumin or an antibody or part of an antibody optionally containing a Fc-domain.

The term "PEGylated human Factor VIIa" means human Factor VIIa, having a PEG molecule conjugated to a human Factor VIIa polypeptide. It is to be understood, that the PEG molecule may be attached to any part of the Factor VIIa polypeptide including any amino acid residue or carbohydrate moiety of the Factor VIIa polypeptide. The term "cysteine-PEGylated human Factor VIIa" means Factor VIIa having a PEG molecule conjugated to a sulfhydryl group of a cysteine introduced in human Factor VIIa.

The biological activity of Factor VIIa in blood clotting derives from its ability to (i) bind to tissue factor (TF) and (ii) catalyze the proteolytic cleavage of Factor IX or Factor X to produce activated Factor IX or X (Factor IXa or Xa, respectively). For purposes of the invention, Factor VIIa biological activity may be quantified by measuring the ability of a preparation to promote blood clotting using Factor VII-deficient plasma and thromboplastin, as described, e.g., in U.S. Patent No. 5,997,864. In this assay, biological activity is expressed as the reduction in clotting time relative to a control sample and is converted to "Factor VII units" by comparison with a pooled human serum standard containing 1 unit/ml Factor VII activity. Alternatively, Factor VIIa biological activity may be quantified by (i) measuring the ability of Factor VIIa to produce of Factor Xa in a system comprising TF embedded in a lipid membrane and Factor X. (Persson et al., J. Biol. Chem. 272:19919-19924, 1997); (ii) measuring Factor X hydrolysis in an aqueous system; (iii) measuring its physical binding to TF using an instrument based on surface plasmon resonance (Persson, FEBS Letts. 413:359-363, 1997) and (iv) measuring hydrolysis of a synthetic substrate.

Factor VII variants having substantially the same or improved biological activity relative to wild-type Factor VIIa encompass those that exhibit at least about 25%, preferably at least about 50%, more preferably at least about 75% and most preferably at least about 90% of the specific activity of Factor VIIa that has been produced in the same cell type, when tested in one or more of a clotting assay, proteolysis assay, or TF binding assay as described above. Variants of Factor VII, whether exhibiting substantially the same or better bioactivity than wild-type Factor VII, or, alternatively, exhibiting substantially modified or reduced bioactivity relative to wild-type Factor VII, include, without limitation, polypeptides having an amino acid sequence that differs from the sequence of wild-type Factor VII by insertion, deletion, or substitution of one or more amino acids.

The terms "variant" or "variants", as used herein, is intended to designate Factor VII having the sequence of SEQ ID NO:1, wherein one or more amino acids of the parent protein have been substituted by another amino acid and/or wherein one or more amino acids of the

parent protein have been deleted and/or wherein one or more amino acids have been inserted in protein and/or wherein one or more amino acids have been added to the parent protein. Such addition can take place either at the N-terminal end or at the C-terminal end of the parent protein or both. The "variant" or "variants" within this definition still have FVII activity in its activated form. In one embodiment a variant is 70 % identical with the sequence of SEQ ID NO:1. In one embodiment a variant is 80 % identical with the sequence of SEQ ID NO:1. In another embodiment a variant is 90 % identical with the sequence of SEQ ID NO:1. In a further embodiment a variant is 95 % identical with the sequence of SEQ ID NO:1.

Non-limiting examples of Factor VII variants having substantially the same biological activity as wild-type Factor VII include S52A-FVIIa, S60A-FVIIa (Lino et al., Arch. Biochem. Biophys. 352: 182-192, 1998); FVIIa variants exhibiting increased proteolytic stability as disclosed in U.S. Patent No. 5,580,560; Factor VIIa that has been proteolytically cleaved between residues 290 and 291 or between residues 315 and 316 (Mollerup et al., Biotechnol. Bioeng. 48:501-505, 1995); oxidized forms of Factor VIIa (Kornfelt et al., Arch. Biochem. Biophys. 363:43-54, 1999); FVII variants as disclosed in PCT/DK02/00189; and FVII variants exhibiting increased proteolytic stability as disclosed in WO 02/38162 (Scripps Research Institute); FVII variants having a modified Gla-domain and exhibiting an enhanced membrane binding as disclosed in WO 99/20767 (University of Minnesota) and WO 00/66753 (University of Minnesota); and FVII variants as disclosed in WO 01/58935 (Maxygen ApS), WO 03/93465 (Maxygen ApS) and WO 04/029091 (Maxygen ApS).

Non-limiting examples of FVII variants having increased biological activity compared to wild-type FVIIa include FVII variants as disclosed in WO 01/83725, WO 02/22776, WO 02/077218, PCT/DK02/00635, Danish patent application PA 2002 01423, Danish patent application PA 2001 01627; WO 02/38162 (Scripps Research Institute); and FVIIa variants with enhanced activity as disclosed in JP 2001061479 (Chemo-Sero-Therapeutic Res Inst.). Non-limiting examples of Factor VII variants having substantially reduced or modified biological activity relative to wild-type Factor VII include R152E-FVIIa (Wildgoose et al., Biochem 29:3413-3420, 1990), S344A-FVIIa (Kazama et al., J. Biol. Chem. 270:66-72, 1995), FFR-FVIIa (Holst et al., Eur. J. Vasc. Endovasc. Surg. 15:515-520, 1998), and Factor VIIa lacking the Gla domain, (Nicolaisen et al., FEBS Letts. 317:245-249, 1993). Examples of variants of factor VII, factor VII or factor VII-related polypeptides include, without limitation, wild-type Factor VII, L305V-FVII, L305V/M306D/D309S-FVII, L305I-FVII, L305T-FVII, F374P-FVII, V158T/M298Q-FVII, V158D/E296V/M298Q-FVII, K337A-FVII, M298Q-FVII, V158D/M298Q-FVII, L305V/K337A-FVII, V158D/E296V/M298Q/L305V-FVII, V158D/E296V/M298Q/K337A-FVII, V158D/E296V/M298Q/L305V/K337A-FVII, K157A-FVII, E296V-FVII, E296V/M298Q-FVII, V158D/E296V-FVII, V158D/M298K-FVII, and S336G-FVII, L305V/K337A-FVII,

L305V/V158D-FVII, L305V/E296V-FVII, L305V/M298Q-FVII, L305V/V158T-FVII, L305V/K337A/V158T-FVII, L305V/K337A/M298Q-FVII, L305V/K337A/E296V-FVII, L305V/K337A/V158D-FVII, L305V/V158D/M298Q-FVII, L305V/V158D/E296V-FVII, L305V/V158T/M298Q-FVII, L305V/V158T/E296V-FVII, L305V/E296V/M298Q-FVII, L305V/V158D/E296V/M298Q-FVII, L305V/V158T/E296V/M298Q-FVII, L305V/V158T/K337A/M298Q-FVII, L305V/V158T/E296V/K337A-FVII, L305V/V158D/K337A/M298Q-FVII, L305V/V158D/E296V/K337A–FVII, L305V/V158D/E296V/M298Q/K337A-FVII, L305V/V158T/E296V/M298Q/K337A-FVII, S314E/K316H-FVII, S314E/K316Q-FVII, S314E/L305V-FVII, S314E/K337A-FVII, S314E/V158D-FVII, S314E/E296V-FVII, S314E/M298Q-FVII, S314E/V158T-FVII, K316H/L305V-FVII, K316H/K337A-FVII, K316H/V158D-FVII, K316H/E296V-FVII, K316H/M298Q-FVII, K316H/V158T-FVII, K316Q/L305V-FVII, K316Q/K337A-FVII, K316Q/V158D-FVII, K316Q/E296V-FVII, K316Q/M298Q-FVII, K316Q/V158T-FVII, S314E/L305V/K337A-FVII, S314E/L305V/V158D-FVII, S314E/L305V/E296V-FVII, S314E/L305V/M298Q-FVII, S314E/L305V/V158T-FVII, S314E/L305V/K337A/V158T-FVII, S314E/L305V/K337A/M298Q-FVII, S314E/L305V/K337A/E296V-FVII, S314E/L305V/K337A/V158D-FVII, S314E/L305V/V158D/M298Q-FVII, S314E/L305V/V158D/E296V-FVII, S314E/L305V/V158T/M298Q-FVII, S314E/L305V/V158T/E296V-FVII, S314E/L305V/E296V/M298Q-FVII, S314E/L305V/V158D/E296V/M298Q-FVII, S314E/L305V/V158T/E296V/M298Q-FVII, S314E/L305V/V158T/K337A/M298Q-FVII, S314E/L305V/V158T/E296V/K337A-FVII, S314E/L305V/V158D/K337A/M298Q-FVII, S314E/L305V/V158D/E296V/K337A-FVII, S314E/L305V/V158D/E296V/M298Q/K337A-FVII, S314E/L305V/V158T/E296V/M298Q/K337A-FVII, K316H/L305V/K337A-FVII, K316H/L305V/V158D-FVII, K316H/L305V/E296V-FVII, K316H/L305V/M298Q-FVII, K316H/L305V/V158T-FVII, K316H/L305V/K337A/V158T-FVII, K316H/L305V/K337A/M298Q-FVII, K316H/L305V/K337A/E296V-FVII, K316H/L305V/K337A/V158D-FVII, K316H/L305V/V158D/M298Q-FVII, K316H/L305V/V158D/E296V-FVII, K316H/L305V/V158T/M298Q-FVII, K316H/L305V/V158T/E296V-FVII, K316H/L305V/E296V/M298Q-FVII, K316H/L305V/V158D/E296V/M298Q-FVII, K316H/L305V/V158T/E296V/M298Q-FVII, K316H/L305V/V158T/K337A/M298Q-FVII, K316H/L305V/V158T/E296V/K337A-FVII, K316H/L305V/V158D/K337A/M298Q-FVII, K316H/L305V/V158D/E296V/K337A –FVII, K316H/L305V/V158D/E296V/M298Q/K337A-FVII, K316H/L305V/V158T/E296V/M298Q/K337A-FVII, K316Q/L305V/K337A-FVII, K316Q/L305V/V158D-FVII, K316Q/L305V/E296V-FVII, K316Q/L305V/M298Q-FVII, K316Q/L305V/V158T-FVII, K316Q/L305V/K337A/V158T-FVII, K316Q/L305V/K337A/M298Q-

FVII, K316Q/L305V/K337A/E296V-FVII, K316Q/L305V/K337A/V158D-FVII, K316Q/L305V/V158D/M298Q-FVII, K316Q/L305V/V158D/E296V-FVII, K316Q/L305V/V158T/M298Q-FVII, K316Q/L305V/V158T/E296V-FVII, K316Q/L305V/E296V/M298Q-FVII, K316Q/L305V/V158D/E296V/M298Q-FVII, K316Q/L305V/V158T/E296V/M298Q-FVII, K316Q/L305V/V158T/K337A/M298Q-FVII, K316Q/L305V/V158T/E296V/K337A-FVII, K316Q/L305V/V158D/K337A/M298Q-FVII, K316Q/L305V/V158D/E296V/K337A –FVII, K316Q/L305V/V158D/E296V/M298Q/K337A-FVII, K316Q/L305V/V158T/E296V/M298Q/K337A-FVII, F374Y/K337A-FVII, F374Y/V158D-FVII, F374Y/E296V-FVII, F374Y/M298Q-FVII, F374Y/V158T-FVII, F374Y/S314E-FVII, F374Y/L305V-FVII, F374Y/L305V/K337A-FVII, F374Y/L305V/V158D-FVII, F374Y/L305V/E296V-FVII, F374Y/L305V/M298Q-FVII, F374Y/L305V/V158T-FVII, F374Y/L305V/S314E-FVII, F374Y/K337A/S314E-FVII, F374Y/K337A/V158T-FVII, F374Y/K337A/M298Q-FVII, F374Y/K337A/E296V-FVII, F374Y/K337A/V158D-FVII, F374Y/V158D/S314E-FVII, F374Y/V158D/M298Q-FVII, F374Y/V158D/E296V-FVII, F374Y/V158T/S314E-FVII, F374Y/V158T/M298Q-FVII, F374Y/V158T/E296V-FVII, F374Y/E296V/S314E-FVII, F374Y/S314E/M298Q-FVII, F374Y/E296V/M298Q-FVII, F374Y/L305V/K337A/V158D-FVII, F374Y/L305V/K337A/E296V-FVII, F374Y/L305V/K337A/M298Q-FVII, F374Y/L305V/K337A/V158T-FVII, F374Y/L305V/K337A/S314E-FVII, F374Y/L305V/V158D/E296V-FVII, F374Y/L305V/V158D/M298Q-FVII, F374Y/L305V/V158D/S314E-FVII, F374Y/L305V/E296V/M298Q-FVII, F374Y/L305V/E296V/V158T-FVII, F374Y/L305V/E296V/S314E-FVII, F374Y/L305V/M298Q/V158T-FVII, F374Y/L305V/M298Q/S314E-FVII, F374Y/L305V/V158T/S314E-FVII, F374Y/K337A/S314E/V158T-FVII, F374Y/K337A/S314E/M298Q-FVII, F374Y/K337A/S314E/E296V-FVII, F374Y/K337A/S314E/V158D-FVII, F374Y/K337A/V158T/M298Q-FVII, F374Y/K337A/V158T/E296V-FVII, F374Y/K337A/M298Q/E296V-FVII, F374Y/K337A/M298Q/V158D-FVII, F374Y/K337A/E296V/V158D-FVII, F374Y/V158D/S314E/M298Q-FVII, F374Y/V158D/S314E/E296V-FVII, F374Y/V158D/M298Q/E296V-FVII, F374Y/V158T/S314E/E296V-FVII, F374Y/V158T/S314E/M298Q-FVII, F374Y/V158T/M298Q/E296V-FVII, F374Y/E296V/S314E/M298Q-FVII, F374Y/L305V/M298Q/K337A/S314E-FVII, F374Y/L305V/E296V/K337A/S314E-FVII, F374Y/E296V/M298Q/K337A/S314E-FVII, F374Y/L305V/E296V/M298Q/K337A –FVII, F374Y/L305V/E296V/M298Q/S314E-FVII, F374Y/V158D/E296V/M298Q/K337A-FVII, F374Y/V158D/E296V/M298Q/S314E-FVII, F374Y/L305V/V158D/K337A/S314E-FVII, F374Y/V158D/M298Q/K337A/S314E-FVII, F374Y/V158D/E296V/K337A/S314E-FVII,

F374Y/L305V/V158D/E296V/M298Q-FVII, F374Y/L305V/V158D/M298Q/K337A-FVII, F374Y/L305V/V158D/E296V/K337A-FVII, F374Y/L305V/V158D/M298Q/S314E-FVII, F374Y/L305V/V158D/E296V/S314E-FVII, F374Y/V158T/E296V/M298Q/K337A-FVII, F374Y/V158T/E296V/M298Q/S314E-FVII, F374Y/L305V/V158T/K337A/S314E-FVII, F374Y/V158T/M298Q/K337A/S314E-FVII, F374Y/V158T/E296V/K337A/S314E-FVII, F374Y/L305V/V158T/E296V/M298Q-FVII, F374Y/L305V/V158T/M298Q/K337A-FVII, F374Y/L305V/V158T/E296V/K337A-FVII, F374Y/L305V/V158T/M298Q/S314E-FVII, F374Y/L305V/V158T/E296V/S314E-FVII, F374Y/E296V/M298Q/K337A/V158T/S314E-FVII, F374Y/V158D/E296V/M298Q/K337A/S314E-FVII, F374Y/L305V/V158D/E296V/M298Q/S314E-FVII, F374Y/L305V/E296V/M298Q/V158T/S314E-FVII, F374Y/L305V/E296V/M298Q/K337A/V158T-FVII, F374Y/L305V/E296V/K337A/V158T/S314E-FVII, F374Y/L305V/M298Q/K337A/V158T/S314E-FVII, F374Y/L305V/V158D/E296V/M298Q/K337A-FVII, F374Y/L305V/V158D/E296V/K337A/S314E-FVII, F374Y/L305V/V158D/M298Q/K337A/S314E-FVII, F374Y/L305V/E296V/M298Q/K337A/V158T/S314E-FVII, F374Y/L305V/V158D/E296V/M298Q/K337A/S314E-FVII, S52A-Factor VII, S60A-Factor VII; R152E-Factor VII, S344A-Factor VII, Factor VIIa lacking the Gla domain; and P11Q/K33E-FVII, T106N-FVII, K143N/N145T-FVII, V253N-FVII, R290N/A292T-FVII, G291N-FVII, R315N/V317T-FVII, K143N/N145T/R315N/V317T-FVII; and FVII having substitutions, additions or deletions in the amino acid sequence from 233Thr to 240Asn, FVII having substitutions, additions or deletions in the amino acid sequence from 304Arg to 329Cys.

The terminology for amino acid substitutions used is as follows. The first letter represents the amino acid naturally present at a position of human wild type FVII. The following number represents the position in human wild type FVII. The second letter represent the different amino acid substituting for (replacing) the natural amino acid. An example is M298Q, where a methionine at position 298 of human wild type FVII is replaced by a glutamine. In another example, V158T/M298Q, the valine in position 158 of human wild type FVII is replaced by a threonine and the methionine in position 298 of human wild type FVII is replaced by a Glutamine in the same Factor VII polypeptide.

In a further embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at least about 1.25. In one embodiment the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at

least about 2.0. In a further embodiment the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at least about 4.0.

In a further embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at least about 1.25 when tested in a Factor VIIa activity assay. In one embodiment the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at least about 2.0 when tested in a Factor VIIa activity assay. In a further embodiment the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at least about 4.0 when tested in a Factor VIIa activity assay. The Factor VIIa activity may be measured by the assays described under “assays”.

In a further embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at least about 1.25 when tested in the "In Vitro Hydrolysis Assay". In one embodiment the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at least about 2.0 when tested in the "In Vitro Hydrolysis Assay". In a further embodiment the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at least about 4.0 when tested in the "In Vitro Hydrolysis Assay".

In a further embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at least about 1.25 when tested in the "In Vitro Proteolysis Assay". In one embodiment the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at least about 2.0 when tested in the "In Vitro Proteolysis Assay". In a further embodiment the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at least about 4.0 when tested in the "In Vitro Proteolysis Assay". In a further embodiment the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at least about 8.0 when tested in the "In Vitro Proteolysis Assay".

The present invention is also suitable for Factor VII/VIIa variants with increased activity compared to wild type. Factor VII/VIIa variants with increased activity may be found by testing in suitable assays described in the following. These assays can be performed as a simple preliminary in vitro test. Thus, the section “assays” discloses a simple test (entitled "In Vitro Hydrolysis Assay") for the activity of Factor VIIa variants of the invention. Based thereon, Factor VIIa variants which are of particular interest are such variants where the ratio between the activity of the variant and the activity of wild type Factor VII is above 1.0, e.g. at

least about 1.25, preferably at least about 2.0, such as at least about 3.0 or, even more preferred, at least about 4.0 when tested in the "In Vitro Hydrolysis Assay".

The activity of the variants can also be measured using a physiological substrate such as factor X ("In Vitro Proteolysis Assay") (see under "assays"), suitably at a concentration of 100-1000 nM, where the factor Xa generated is measured after the addition of a suitable chromogenic substrate (e.g. S-2765). In addition, the activity assay may be run at physiological temperature.

The ability of the Factor VIIa variants to generate thrombin can also be measured in an assay comprising all relevant coagulation factors and inhibitors at physiological concentrations (minus factor VIII when mimicking hemophilia A conditions) and activated platelets (as described on p. 543 in Monroe et al. (1997) Brit. J. Haematol. 99, 542-547 which is hereby incorporated as reference).

The term "identity" as known in the art, refers to a relationship between the sequences of two or more polypeptide molecules or two or more nucleic acid molecules, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between nucleic acid molecules or between polypeptides, as the case may be, as determined by the number of matches between strings of two or more nucleotide residues or two or more amino acid residues. "Identity" measures the percent of identical matches between the smaller of two or more sequences with gap alignments (if any) addressed by a particular mathematical model or computer program (i.e., "algorithms").

The term "similarity" is a related concept, but in contrast to "identity", refers to a sequence relationship that includes both identical matches and conservative substitution matches. If two polypeptide sequences have, for example, {fraction (10/20)} identical amino acids, and the remainder are all non-conservative substitutions, then the percent identity and similarity would both be 50%. If, in the same example, there are 5 more positions where there are conservative substitutions, then the percent identity remains 50%, but the percent similarity would be 75% ({fraction (15/20)}). Therefore, in cases where there are conservative substitutions, the degree of similarity between two polypeptides will be higher than the percent identity between those two polypeptides.

The term "isolated polypeptide" refers to a polypeptide of the present invention that (1) has been separated from at least about 50 percent of polynucleotides, lipids, carbohydrates or other materials (i.e., contaminants) with which it is naturally associated, (2) is not covalently linked to all or a portion of a polypeptide to which the "isolated polypeptide" is linked in nature, (3) is operably linked covalently to a polypeptide to which it is not covalently linked in nature, or (4) does not occur in nature. Preferably, the isolated polypeptide is substantially free from any other contaminating polypeptides or other contaminants that are

found in its natural environment which would interfere with its therapeutic, diagnostic, prophylactic or research use.

Conservative modifications to the amino acid sequence of SEQ ID NO:1 (and the corresponding modifications to the encoding nucleotides) outside the positions for substitutions claimed will produce FVII polypeptides having functional and chemical characteristics similar to those of naturally occurring FVII polypeptide. In contrast, substantial modifications in the functional and/or chemical characteristics of FVII polypeptides may be accomplished by selecting substitutions in the amino acid sequence of SEQ ID NO:1 that differ significantly in their effect on maintaining (a) the structure of the molecular backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain.

For example, a "conservative amino acid substitution" may involve a substitution of a native amino acid residue with a non-native residue such that there is little or no effect on the polarity or charge of the amino acid residue at that position. Furthermore, any native residue in the polypeptide may also be substituted with almandine, as has been previously described for "alanine scanning mutagenesis" (see, for example, MacLennan et al., 1998, Acta Physiol. Scand. Suppl. 643:55-67; Sasaki et al., 1998, Adv. Biophys. 35:1-24, which discuss alanine scanning mutagenesis).

Identity and similarity of related polypeptides can be readily calculated by known methods. Such methods include, but are not limited to, those described in Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part 1, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M. Stockton Press, New York, 1991; and Carillo et al., SIAM J. Applied Math., 48:1073 (1988).

Preferred methods to determine identity and/or similarity are designed to give the largest match between the sequences tested. Methods to determine identity and similarity are described in publicly available computer programs. Preferred computer program methods to determine identity and similarity between two sequences include, but are not limited to, the GCG program package, including GAP (Devereux et al., Nucl. Acid. Res., 12:387 (1984); Genetics Computer Group, University of Wisconsin, Madison, Wis.), BLASTP, BLASTN, and FASTA (Altschul et al., J. Mol. Biol., 215:403-410 (1990)). The BLASTX program is publicly available from the National Center for Biotechnology Information (NCBI) and other sources (BLAST Manual, Altschul et al. NCB/NLM/NIH Bethesda, Md. 20894; Altschul et al., supra). The well known Smith Waterman algorithm may also be used to determine identity.

Certain alignment schemes for aligning two amino acid sequences may result in the matching of only a short region of the two sequences, and this small aligned region may have very high sequence identity even though there is no significant relationship between the two full length sequences. Accordingly, in a preferred embodiment, the selected alignment method (GAP program) will result in an alignment that spans at least 50 contiguous amino acids of the target polypeptide.

For example, using the computer algorithm GAP (Genetics Computer Group, University of Wisconsin, Madison, Wis.), two polypeptides for which the percent sequence identity is to be determined are aligned for optimal matching of their respective amino acids (the "matched span", as determined by the algorithm). A gap opening penalty (which is calculated as 3.times. the average diagonal; the "average diagonal" is the average of the diagonal of the comparison matrix being used; the "diagonal" is the score or number assigned to each perfect amino acid match by the particular comparison matrix) and a gap extension penalty (which is usually {fraction (1/10)} times the gap opening penalty), as well as a comparison matrix such as PAM 250 or BLOSUM 62 are used in conjunction with the algorithm. A standard comparison matrix (see Dayhoff et al., Atlas of Protein Sequence and Structure, vol. 5, supp.3 (1978) for the PAM 250 comparison matrix; Henikoff et al., Proc. Natl. Acad. Sci USA, 89:10915-10919 (1992) for the BLOSUM 62 comparison matrix) is also used by the algorithm.

Preferred parameters for a polypeptide sequence comparison include the following:

Algorithm: Needleman et al., J. Mol. Biol, 48:443-453 (1970); Comparison matrix: BLOSUM 62 from Henikoff et al., Proc. Natl. Acad. Sci. USA, 89:10915-10919 (1992); Gap Penalty: 12, Gap Length Penalty: 4, Threshold of Similarity: 0.

The GAP program is useful with the above parameters. The aforementioned parameters are the default parameters for polypeptide comparisons (along with no penalty for end gaps) using the GAP algorithm.

Preferred parameters for nucleic acid molecule sequence comparisons include the following: Algorithm: Needleman et al., J. Mol Biol., 48:443-453 (1970); Comparison matrix: matches=+10, mismatch=0, Gap Penalty: 50, Gap Length Penalty: 3.

The GAP program is also useful with the above parameters. The aforementioned parameters are the default parameters for nucleic acid molecule comparisons.

Other exemplary algorithms, gap opening penalties, gap extension penalties, comparison matrices, thresholds of similarity, etc. may be used, including those set forth in the Program Manual, Wisconsin Package, Version 9, September, 1997. The particular choices to be made will be apparent to those of skill in the art and will depend on the specific comparison to be made, such as DNA to DNA, protein to protein, protein to DNA; and additionally,

whether the comparison is between given pairs of sequences (in which case GAP or BestFit are generally preferred) or between one sequence and a large database of sequences (in which case FASTA or BLASTA are preferred).

The term "Tissue Factor binding affinity", as used herein, means the strength of the binding of a FVII polypeptide to human Tissue Factor. The affinity of a FVII polypeptide is measured by the dissociation constant K_d, defined as [FVII] x [TF] / [FVII-TF] where [FVII-TF] is the molar concentration of the FVII/TF complex, [FVII] is the molar concentration of the unbound FVII polypeptide and [TF] is the molar concentration of the unbound human Tissue Factor. The affinity constant K_a is defined by $1/K_d$.

The phrase "substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa", as used herein, means an activity more than 50 % of the activity of recombinant wild type human Factor VIIa. In one embodiment the activity is more than 60 % of the activity of recombinant wild type human Factor VIIa. In one embodiment the activity is more than 70 % of the activity of recombinant wild type human Factor VIIa. In one embodiment the activity is more than 80 % of the activity of recombinant wild type human Factor VIIa. In another embodiment the activity is more than 90 % of the activity of recombinant wild type human Factor VIIa. In a further embodiment the activity is more than 100 % of the activity of recombinant wild type human Factor VIIa. In a further embodiment the activity is more than 120 % of the activity of recombinant wild type human Factor VIIa. In a further embodiment the activity is more than 200 % of the activity of recombinant wild type human Factor VIIa. In a further embodiment the activity is more than 400 % of the activity of recombinant wild type human Factor VIIa.

The term "activity" as used herein means the ability of a Factor VII polypeptide to convert its substrate Factor X to the active Factor Xa. The activity of a Factor VII polypeptide may be measured with the "*In Vitro* Proteolysis Assay".

The phrase "a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa", as used herein, means an binding affinity lower than that of recombinant wild type human Factor VIIa as measured in a TF binding affinity assay, such as the Biosensor assay as described in Example 5. In one embodiment the Tissue Factor binding affinity is less than 90 % of the Tissue Factor binding affinity of recombinant wild type human Factor VIIa. In one embodiment the Tissue Factor binding affinity is less than 80 % of the Tissue Factor binding affinity of recombinant wild type human Factor VIIa. In one embodiment the Tissue Factor binding affinity is less than 70 % of the Tissue Factor binding affinity of recombinant wild type human Factor VIIa. In one embodiment the Tissue Factor binding affinity is less than 60 % of the Tissue Factor binding affinity of recombinant wild type human Factor VIIa. In one embodiment the Tissue Factor binding affinity is less than 50 % of

the Tissue Factor binding affinity of recombinant wild type human Factor VIIa. In one embodiment the Tissue Factor binding affinity is less than 40 % of the Tissue Factor binding affinity of recombinant wild type human Factor VIIa. In one embodiment the Tissue Factor binding affinity is less than 30 % of the Tissue Factor binding affinity of recombinant wild type human Factor VIIa. In one embodiment the Tissue Factor binding affinity is less than 20 % of the Tissue Factor binding affinity of recombinant wild type human Factor VIIa. In one embodiment the Tissue Factor binding affinity is less than 10 % of the Tissue Factor binding affinity of recombinant wild type human Factor VIIa. In one embodiment the Tissue Factor binding affinity is less than 5 % of the Tissue Factor binding affinity of recombinant wild type human Factor VIIa. In one embodiment the Tissue Factor binding affinity is less than 1 % of the Tissue Factor binding affinity of recombinant wild type human Factor VIIa. In one embodiment the Tissue Factor binding affinity is less than 0.1 % of the Tissue Factor binding affinity of recombinant wild type human Factor VIIa. In one embodiment the Tissue Factor binding affinity is less than 0.01 % of the Tissue Factor binding affinity of recombinant wild type human Factor VIIa. In one embodiment the Tissue Factor binding affinity is less than 0.001 % of the Tissue Factor binding affinity of recombinant wild type human Factor VIIa.

The term "polyethylene glycol" or "PEG" means a polyethylene glycol compound or a derivative thereof, with or without coupling agents, coupling or activating moieties (e.g., with thiol, triflate, tresylate, azirdine, oxirane, or preferably with a maleimide moiety). Compounds such as maleimido monomethoxy PEG are exemplary of activated PEG compounds of the invention.

In one embodiment of the invention, the Factor VII polypeptides are Factor VII polypeptides, wherein one or more amino acid corresponding to amino acids at positions selected from K18 , R36, S43, K62, Q64, L65, I69, F71, L73, P74, E77, G78, R79, K85, Q88, N93, F275, R277, M306, T307, Q308, D309, or R379 of SEQ ID NO: 1 have been substituted with a different amino acid, which different amino acid decreases the Tissue Factor binding affinity.

The term “a different amino acid” as used herein means one or more amino acids that are different from that amino acid naturally present at that position. This includes but is not limited to amino acids that can be encoded by a polynucleotide. Preferably the different amino acid is in natural L-form and can be encoded by a polynucleotide. A specific example being L-Glutamic acid (L-Glu).

In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position K18 of

SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position K18 of SEQ ID NO: 1 is selected from the list consisting of E, D, A, and F.

In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position R36 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position R36 of SEQ ID NO: 1 is selected from the list consisting of E, D, A, and F.

In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position S43 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position S43 of SEQ ID NO: 1 is selected from the list consisting of E, D, K, R, A, F, and N. In one embodiment this different amino acid is selected from the list consisting of E, D, K, R, A, and F. In one embodiment this different amino acid is an N.

In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position K62 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position K62 of SEQ ID NO: 1 is selected from the list consisting of E, D, A, and F. In one embodiment this different amino acid is selected from the list consisting of D, A, and F.

In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position Q64 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position Q64 of SEQ ID NO: 1 is selected from the list consisting of E, D, K, R, A, and F.

In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position L65 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position L65 of SEQ ID NO: 1 is selected from the list consisting of E, D, K, R, A, and F.

In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position I69 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position I69 of SEQ ID NO: 1 is selected from the list consisting of E, D, K, R, A, and F. In one embodiment this different amino acid is selected from the list consisting of E, D, K, R, and F.

In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position F71 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position F71 of SEQ ID NO: 1 is selected from the list consisting of E, D, K, R, and A. In one embodiment this different amino acid is selected from the list consisting of K, R, and A.

In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position L73 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position L73 of SEQ ID NO: 1 is selected from the list consisting of E, D, K, R, A, and F.

In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position P74 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position P74 of SEQ ID NO: 1 is selected from the list consisting of E, D, K, R, A, and F.

In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position E77 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position E77 of SEQ ID NO: 1 is selected from the list consisting of K, R, and A.

In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position G78 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position G78 of SEQ ID NO: 1 is selected from the list consisting of E, D, K, R, A, and F.

In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position R79 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position R79 of SEQ ID NO: 1 is selected from the list consisting of E, D, and A.

In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position K85 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position K85 of SEQ ID NO: 1 is selected from the list consisting of E, D, and A.

In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position Q88 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position Q88 of SEQ ID NO: 1 is selected from the list consisting of E, D, K, R, A, and F.

In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position N93 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position N93 of SEQ ID NO: 1 is selected from the list consisting of E, D, K, R, A, and F.

In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position F275 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position F275 of SEQ ID NO: 1 is selected from the list consisting of E, D, K, R, and A.

In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position R277 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position R277 of SEQ ID NO: 1 is selected from the list consisting of E, D, A, and F.

In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position T307 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position T307 of SEQ ID NO: 1 is selected from the list consisting of E, D, K, R, A, F, and N.

In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position Q308 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position Q308 of SEQ ID NO: 1 is selected from the list consisting of E, D, K, R, A, F, and N.

In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position R379 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position R379 of SEQ ID NO: 1 is selected from the list consisting of E, D, A, and F.

In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position M306 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position M306 of SEQ ID NO: 1 is selected from the list consisting of E, D, K, R, A, and F. In one embodiment this different amino acid is selected from the list consisting of E, K, R, A, and F.

In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the amino acid corresponding to the amino acid at a position D309 of SEQ ID NO: 1 has been substituted with a different amino acid. In one embodiment this different amino acid at a position D309 of SEQ ID NO: 1 is selected from the list consisting of K, R, and A.

In a further embodiment, the Factor VII polypeptide of the invention is a Factor VII polypeptide selected from the list consisting of S43N-FVII, K62E-FVII, Q64E-FVII, I69F-FVII, K62E/I69A-FVII, Q64E/I69A-FVII, K62E/Q64E/I69A-FVII, K62E/I69F-FVII, Q64E/I69F-FVII, and K62E/Q64E/I69F-FVII.

In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides further comprising amino acid substitutions selected from the list consisting of L305V, L305V/M306D/D309S, L305I, L305T, F374P, V158T/M298Q, V158D/E296V/M298Q, K337A, M298Q, V158D/M298Q, L305V/K337A, V158D/E296V/M298Q/L305V, V158D/E296V/M298Q/K337A, V158D/E296V/M298Q/L305V/K337A, K157A, E296V, E296V/M298Q, V158D/E296V, V158D/M298K, and S336G, L305V/K337A, L305V/V158D, L305V/E296V, L305V/M298Q, L305V/V158T, L305V/K337A/V158T, L305V/K337A/M298Q, L305V/K337A/E296V, L305V/K337A/V158D, L305V/V158D/M298Q, L305V/V158D/E296V, L305V/V158T/M298Q, L305V/V158T/E296V, L305V/E296V/M298Q, L305V/V158D/E296V/M298Q, L305V/V158T/E296V/M298Q, L305V/V158T/K337A/M298Q, L305V/V158T/E296V/K337A, L305V/V158D/K337A/M298Q, L305V/V158D/E296V/K337A, L305V/V158D/E296V/M298Q/K337A, L305V/V158T/E296V/M298Q/K337A, S314E/K316H, S314E/K316Q, S314E/L305V, S314E/K337A, S314E/V158D, S314E/E296V, S314E/M298Q, S314E/V158T, K316H/L305V, K316H/K337A, K316H/V158D, K316H/E296V, K316H/M298Q, K316H/V158T, K316Q/L305V, K316Q/K337A, K316Q/V158D, K316Q/E296V, K316Q/M298Q, K316Q/V158T, S314E/L305V/K337A, S314E/L305V/V158D, S314E/L305V/E296V, S314E/L305V/M298Q, S314E/L305V/V158T, S314E/L305V/K337A/V158T, S314E/L305V/K337A/M298Q, S314E/L305V/K337A/E296V, S314E/L305V/K337A/V158D, S314E/L305V/V158D/M298Q, S314E/L305V/V158D/E296V, S314E/L305V/V158T/M298Q, S314E/L305V/V158T/E296V, S314E/L305V/E296V/M298Q, S314E/L305V/V158D/E296V/M298Q, S314E/L305V/V158T/E296V/M298Q,

S314E/L305V/V158T/K337A/M298Q, S314E/L305V/V158T/E296V/K337A, S314E/L305V/V158D/K337A/M298Q, S314E/L305V/V158D/E296V/K337A, S314E/L305V/V158D/E296V/M298Q/K337A, S314E/L305V/V158T/E296V/M298Q/K337A, K316H/L305V/K337A, K316H/L305V/V158D, K316H/L305V/E296V, K316H/L305V/M298Q, K316H/L305V/V158T, K316H/L305V/K337A/V158T, K316H/L305V/K337A/M298Q, K316H/L305V/K337A/E296V, K316H/L305V/K337A/V158D, K316H/L305V/V158D/M298Q, K316H/L305V/V158D/E296V, K316H/L305V/V158T/M298Q, K316H/L305V/V158T/E296V, K316H/L305V/E296V/M298Q, K316H/L305V/V158D/E296V/M298Q, K316H/L305V/V158T/E296V/M298Q, K316H/L305V/V158T/K337A/M298Q, K316H/L305V/V158T/E296V/K337A, K316H/L305V/V158D/K337A/M298Q, K316H/L305V/V158D/E296V/K337A , K316H/L305V/V158D/E296V/M298Q/K337A, K316H/L305V/V158T/E296V/M298Q/K337A, K316Q/L305V/K337A, K316Q/L305V/V158D, K316Q/L305V/E296V, K316Q/L305V/M298Q, K316Q/L305V/V158T, K316Q/L305V/K337A/V158T, K316Q/L305V/K337A/M298Q, K316Q/L305V/K337A/E296V, K316Q/L305V/K337A/V158D, K316Q/L305V/V158D/M298Q, K316Q/L305V/V158D/E296V, K316Q/L305V/V158T/M298Q, K316Q/L305V/V158T/E296V, K316Q/L305V/E296V/M298Q, K316Q/L305V/V158D/E296V/M298Q, K316Q/L305V/V158T/E296V/M298Q, K316Q/L305V/V158T/K337A/M298Q, K316Q/L305V/V158T/E296V/K337A, K316Q/L305V/V158D/K337A/M298Q, K316Q/L305V/V158D/E296V/K337A , K316Q/L305V/V158D/E296V/M298Q/K337A, K316Q/L305V/V158T/E296V/M298Q/K337A, F374Y/K337A, F374Y/V158D, F374Y/E296V, F374Y/M298Q, F374Y/V158T, F374Y/S314E, F374Y/L305V, F374Y/L305V/K337A, F374Y/L305V/V158D, F374Y/L305V/E296V, F374Y/L305V/M298Q, F374Y/L305V/V158T, F374Y/L305V/S314E, F374Y/K337A/S314E, F374Y/K337A/V158T, F374Y/K337A/M298Q, F374Y/K337A/E296V, F374Y/K337A/V158D, F374Y/V158D/S314E, F374Y/V158D/M298Q, F374Y/V158D/E296V, F374Y/V158T/S314E, F374Y/V158T/M298Q, F374Y/V158T/E296V, F374Y/E296V/S314E, F374Y/S314E/M298Q, F374Y/E296V/M298Q, F374Y/L305V/K337A/V158D, F374Y/L305V/K337A/E296V, F374Y/L305V/K337A/M298Q, F374Y/L305V/K337A/V158T, F374Y/L305V/K337A/S314E, F374Y/L305V/V158D/E296V, F374Y/L305V/V158D/M298Q, F374Y/L305V/V158D/S314E, F374Y/L305V/E296V/M298Q, F374Y/L305V/E296V/V158T, F374Y/L305V/E296V/S314E, F374Y/L305V/M298Q/V158T, F374Y/L305V/M298Q/S314E, F374Y/L305V/V158T/S314E, F374Y/K337A/S314E/V158T, F374Y/K337A/S314E/M298Q, F374Y/K337A/S314E/E296V, F374Y/K337A/S314E/V158D, F374Y/K337A/V158T/M298Q, F374Y/K337A/V158T/E296V, F374Y/K337A/M298Q/E296V, F374Y/K337A/M298Q/V158D, F374Y/K337A/E296V/V158D, F374Y/V158D/S314E/M298Q, F374Y/V158D/S314E/E296V, F374Y/V158D/M298Q/E296V, F374Y/V158T/S314E/E296V, F374Y/V158T/S314E/M298Q, F374Y/V158T/M298Q/E296V,

F374Y/E296V/S314E/M298Q, F374Y/L305V/M298Q/K337A/S314E, F374Y/L305V/E296V/K337A/S314E, F374Y/E296V/M298Q/K337A/S314E, F374Y/L305V/E296V/M298Q/K337A , F374Y/L305V/E296V/M298Q/S314E, F374Y/V158D/E296V/M298Q/K337A, F374Y/V158D/E296V/M298Q/S314E, F374Y/L305V/V158D/K337A/S314E, F374Y/V158D/M298Q/K337A/S314E, F374Y/V158D/E296V/K337A/S314E, F374Y/L305V/V158D/E296V/M298Q, F374Y/L305V/V158D/M298Q/K337A, F374Y/L305V/V158D/E296V/K337A, F374Y/L305V/V158D/M298Q/S314E, F374Y/L305V/V158D/E296V/S314E, F374Y/V158T/E296V/M298Q/K337A, F374Y/V158T/E296V/M298Q/S314E, F374Y/L305V/V158T/K337A/S314E, F374Y/V158T/M298Q/K337A/S314E, F374Y/V158T/E296V/K337A/S314E, F374Y/L305V/V158T/E296V/M298Q, F374Y/L305V/V158T/M298Q/K337A, F374Y/L305V/V158T/E296V/K337A, F374Y/L305V/V158T/M298Q/S314E, F374Y/L305V/V158T/E296V/S314E, F374Y/E296V/M298Q/K337A/V158T/S314E, F374Y/V158D/E296V/M298Q/K337A/S314E, F374Y/L305V/V158D/E296V/M298Q/S314E, F374Y/L305V/E296V/M298Q/V158T/S314E, F374Y/L305V/E296V/M298Q/K337A/V158T, F374Y/L305V/E296V/K337A/V158T/S314E, F374Y/L305V/M298Q/K337A/V158T/S314E, F374Y/L305V/V158D/E296V/M298Q/K337A, F374Y/L305V/V158D/E296V/K337A/S314E, F374Y/L305V/V158D/M298Q/K337A/S314E, F374Y/L305V/E296V/M298Q/K337A/V158T/S314E, F374Y/L305V/V158D/E296V/M298Q/K337A/S314E, S52A, S60A; R152E, S344A, P11Q/K33E, T106N, K143N/N145T, V253N, R290N/A292T, G291N, R315N/V317T, and K143N/N145T/R315N/V317T; or having substitutions, additions or deletions in the amino acid sequence from 233Thr to 240Asn, or having substitutions, additions or deletions in the amino acid sequence from 304Arg to 329Cys.

In a further embodiment, the Factor VII polypeptides of the invention are Factor VII polypeptides, wherein the dissociation constant K_d of said Factor VII polypeptide is higher than 5 nM, such as higher than 7 nM, such as higher than 10 nM, such as higher than 20 nM, such as higher than 30 nM, such as higher than 50 nM, such as higher than 100 nM, such as higher than 200 nM, such as higher than 300 nM, such as higher than 400 nM, such as higher than 500 nM, such as higher than 1 μM.

In a further embodiment, the Factor VII polypeptides of the invention comprises one or more amino acid substitution selected from $K18X_1$, $R36X_2$, $S43X_3$, $K62X_4$, $Q64X_5$, $L65X_6$, $I69X_7$, $F71X_8$, $L73X_9$, $P74X_{10}$, $E77X_{11}$, $G78X_{12}$, $R79X_{13}$, $K85X_{14}$, $Q88X_{15}$, $N93X_{16}$, $F275X_{17}$, $R277X_{18}$, $M306X_{19}$, $T307X_{20}$, $Q308X_{21}$, $D309X_{22}$, or $R379X_{23}$ corresponding to amino acids at positions of SEQ ID NO: 1, which different amino acid decreases the Tissue Factor binding affinity, wherein

X_1 is E, D, A, or F;

X_2 is E, D, K, A, F, or N;

X_3 is E, D, K, R, A, or F;

X_4 is D, A, or F;

X_5 is E, D, K, R, A, F, or N;

X_6 is E, D, K, R, A, or F;

X_7 is C, D, E, F, G, H, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_8 is A, G, H, I, K, L, M, P, R, S, T, V, or W;

X_9 is A, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y;

X_{10} is A, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, or Y;

X_{11} is A, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_{12} is A, D, E, F, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_{13} is A, D, E, F, G, H, I, K, L, M, N, P, Q, S, T, V, W, or Y;

X_{14} is A, D, E, F, G, I, L, M, P, S, T, V, W, or Y;

X_{15} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;

X_{16} is A, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y;

X_{17} is A, D, E, G, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_{18} is A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y;

X_{19} is A, E, F, G, H, I, K, L, P, Q, R, S, T, V, W, or Y;

X_{20} is A, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, V, W, or Y;

X_{21} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;

X_{22} is A, F, G, H, I, K, L, M, N, P, Q, R, V, W, or Y; and

X_{23} is A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y.

In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein one or more amino acid corresponding to amino acids at positions selected from K18, R36, K62, Q64, L65, I69, F71, L73, P74, E77, G78, R79, K85, Q88, N93, F275, R277, M306, Q308, D309, or R379 of SEQ ID NO: 1 have been substituted with a cysteine amino acid residue, which cysteine amino acid residue decreases the Tissue Factor binding affinity, and which cysteine amino acid residue is optionally conjugated with a chemical group, which chemical group increases the molecular weight of said Factor VII polypeptide. In one embodiment K18 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment R36 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment K62 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment Q64 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment L65 of SEQ ID NO: 1 has been sub-

stituted with a cysteine amino acid residue. In one embodiment I69 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment F71 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment L73 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment P74 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment E77 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment G78 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment R79 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment K85 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment Q88 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment N93 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment F275 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment R277 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment M306 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment Q308 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment D309 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue. In one embodiment R379 of SEQ ID NO: 1 has been substituted with a cysteine amino acid residue.

In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide comprising one or more N-glycosylation site N-Xaa-S/T introduced by amino acid substitutions corresponding to amino acids starting at positions selected from R36, Q64, I69, F71, P74, E77, G78, Q88, N93, F275, M306, T307, or D309 of SEQ ID NO: 1, wherein Xaa is any amino acid except P, which introduced N-glycosylation site decreases the Tissue Factor binding affinity. In one embodiment an N-glycosylation site N-Xaa-S/T is introduced at a position starting at R36 of SEQ ID NO: 1. In one embodiment an N-glycosylation site N-Xaa-S/T is introduced at a position starting at R36 of SEQ ID NO: 1. In one embodiment an N-glycosylation site N-Xaa-S/T is introduced at a position starting at Q64 of SEQ ID NO: 1. In one embodiment an N-glycosylation site N-Xaa-S/T is introduced at a position starting at I69 of SEQ ID NO: 1. In one embodiment an N-glycosylation site N-Xaa-S/T is introduced at a position starting at F71 of SEQ ID NO: 1. In one embodiment an N-glycosylation site N-Xaa-S/T is introduced at a position starting at P74 of SEQ ID NO: 1. In one embodiment an N-glycosylation site N-Xaa-S/T is introduced at a position starting at E77 of SEQ ID NO: 1. In one embodiment an N-glycosylation site N-Xaa-S/T is introduced at a position starting at G78 of SEQ ID NO: 1.In one embodiment an N-glycosylation site N-Xaa-S/T is introduced at a position starting at Q88 of SEQ ID NO: 1.In one embodiment an N-glycosylation site N-

Xaa-S/T is introduced at a position starting at N93 of SEQ ID NO: 1.In one embodiment an N-glycosylation site N-Xaa-S/T is introduced at a position starting at F275 of SEQ ID NO: 1. In one embodiment an N-glycosylation site N-Xaa-S/T is introduced at a position starting at M306 of SEQ ID NO: 1.In one embodiment an N-glycosylation site N-Xaa-S/T is introduced at a position starting at T307 of SEQ ID NO: 1.In one embodiment an N-glycosylation site N-Xaa-S/T is introduced at a position starting at D309 of SEQ ID NO: 1.

In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position K18 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of E, D, A, and F.

In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position R36 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of E, D, K, A, F, and N.

In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position S43 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of E, D, K, R, A, and F.

In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position K62 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of D, A, and F.

In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position Q64 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of E, D, K, R, A, F, and N.

In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position L65 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of E, D, K, R, A, and F.

In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position I69 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of C, D, E, F, G, H, K, L, M, N, P, Q, R, S, T, V, W, and Y.

In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position F71 of

SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, G, H, I, K, L, M, P, R, S, T, V, and W.

In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position L73 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, and Y.

In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position P74 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, and Y.

In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position E77 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, and Y.

In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position G78 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, D, E, F, H, I, K, L, M, N, P, Q, R, S, T, V, W, and Y.

In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position R79 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, D, E, F, G, H, I, K, L, M, N, P, Q, S, T, V, W, and Y.

In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position K85 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, D, E, F, G, I, L, M, P, S, T, V, W, and Y.

In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position Q88 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, and Y.

In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position N93 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, and Y.

In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position F275 of

SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, D, E, G, I, K, L, M, N, P, Q, R, S, T, V, W, and Y.

In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position R277 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, and Y.

In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position M306 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, E, F, G, H, I, K, L, P, Q, R, S, T, V, W, and Y.

In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position T307 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, V, W, and Y.

In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position Q308 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, and Y.

In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position D309 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, F, G, H, I, K, L, M, N, P, Q, R, V, W, and Y.

In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, wherein the amino acid corresponding to the amino acid at a position R379 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y.

In a further embodiment, the Factor VII polypeptides of the invention is a Factor VII polypeptide, which is selected from the list consisting of FVII-(S43N), FVII-(K62E), FVII-(Q64E), FVII-(I69F), FVII-(K62E/I69A), FVII-(Q64E/I69A), FVII-(Q62E/Q64E/I69A), FVII-(K62E/I69F), FVII-(Q64E/I69F), FVII-(K62E/Q64E/I69F), FVII-(S43C), FVII-(I69C), FVII-(Q64C), FVII-(M306C), FVII-(R277C), FVII-(I69N/F71T), FVII-(F71N/L73T), FVII-(R277N), and FVII-(D309N/L311T).

In one embodiment the polynucleotide construct of the invention is a vector.

The term "a polynucleotide" denotes a single- or double-stranded polymer of deoxy-ribonucleotide or ribonucleotide bases read from the 5' to the 3' end. Polynucleotides include RNA and DNA, and may be isolated from natural sources, synthesized in vitro, or prepared

from a combination of natural and synthetic molecules. The length of a polynucleotide molecule is given herein in terms of nucleotides (abbreviated "nt") or base pairs (abbreviated "bp"). The term "nucleotides" is used for both single- and double-stranded molecules where the context permits. When the term is applied to double-stranded molecules it is used to denote overall length and will be understood to be equivalent to the term "base pairs". It will be recognized by those skilled in the art that the two strands of a double-stranded polynucleotide may differ slightly in length and that the ends thereof may be staggered as a result of enzymatic cleavage; thus all nucleotides within a double-stranded polynucleotide molecule may not be paired. Such unpaired ends will in general not exceed 20 nt in length.

The term "vector", as used herein, means any nucleic acid entity capable of the amplification in a host cell. Thus, the vector may be an autonomously replicating vector, i.e. a vector, which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated. The choice of vector will often depend on the host cell into which it is to be introduced. Vectors include, but are not limited to plasmid vectors, phage vectors, viruses or cosmid vectors. Vectors usually contain a replication origin and at least one selectable gene, i.e., a gene which encodes a product which is readily detectable or the presence of which is essential for cell growth.

In one embodiment, the eukaryotic host cell of the invention is of mammalian origin.

In a further embodiment, the eukaryotic host cell of the invention is selected from the group consisting of CHO cells, BHK cells or HEK cells.

The term "a eukaryotic host cell", as used herein, represent any cell, including hybrid cells, in which heterologous DNA can be expressed. Typical host cells includes, but are not limited to insect cells, yeast cells, mammalian cells, including human cells, such as BHK, CHO, HEK, and COS cells. In practicing the present invention, the host cells being cultivated are preferably mammalian cells, more preferably an established mammalian cell line, including, without limitation, CHO (e.g., ATCC CCL 61), COS-1 (e.g., ATCC CRL 1650), baby hamster kidney (BHK) and HEK293 (e.g., ATCC CRL 1573; Graham et al., *J. Gen. Virol.* 36:59-72, 1977) cell lines.

A preferred BHK cell line is the tk$^-$ ts13 BHK cell line (Waechter and Baserga, *Proc.Natl.Acad.Sci.USA* 79:1106-1110, 1982), hereinafter referred to as BHK 570 cells. The BHK 570 cell line is available from the American Type Culture Collection, 12301 Parklawn Dr., Rockville, MD 20852, under ATCC accession number CRL 10314. A tk$^-$ ts13 BHK cell line is also available from the ATCC under accession number CRL 1632.

Other suitable cell lines include, without limitation, Rat Hep I (Rat hepatoma; ATCC CRL 1600), Rat Hep II (Rat hepatoma; ATCC CRL 1548), TCMK (ATCC CCL 139), Human lung (ATCC HB 8065), NCTC 1469 (ATCC CCL 9.1) and DUKX cells (Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA* 77:4216-4220, 1980). Also useful are 3T3 cells, Namalwa cells, myelomas and fusions of myelomas with other cells. In one embodiment the eucaryotic host cell is of mammalian origin. In a further embodiment the eucaryotic host cell is selected from the group consisting of CHO cells, BHK cells or HEK cells.

The term "treatment", as used herein, means the administration of an effective amount of a therapeutically active compound of the invention with the purpose of preventing any symptoms or disease state to develop or with the purpose of curing or easing such symptoms or disease states already developed. The term "treatment" is thus meant to include prophylactic treatment.

The term "enhancement of the normal haemostatic system" means an enhancement of the ability to generate thrombin.

As used herein the term "bleeding disorders" reflects any defect, congenital, acquired or induced, of cellular or molecular origin that is manifested in bleedings. Examples are clotting factor deficiencies (e.g. haemophilia A and B or deficiency of coagulation Factors XI or VII), clotting factor inhibitors, defective platelet function, thrombocytopenia or von Willebrand's disease.

The term "bleeding episodes" is meant to include uncontrolled and excessive bleeding which is a major problem both in connection with surgery and other forms of tissue damage. Uncontrolled and excessive bleeding may occur in subjects having a normal coagulation system and subjects having coagulation or bleeding disorders. Clotting factor deficiencies (haemophilia A and B, deficiency of coagulation factors XI or VII) or clotting factor inhibitors may be the cause of bleeding disorders. Excessive bleedings also occur in subjects with a normally functioning blood clotting cascade (no clotting factor deficiencies or -inhibitors against any of the coagulation factors) and may be caused by a defective platelet function, thrombocytopenia or von Willebrand's disease. In such cases, the bleedings may be similar to those bleedings caused by haemophilia because the haemostatic system, as in haemophilia, lacks or has abnormal essential clotting "compounds" (such as platelets or von Willebrand factor protein) that causes major bleedings. In subjects who experience extensive tissue damage in association with surgery or vast trauma, the normal haemostatic mechanism may be overwhelmed by the demand of immediate haemostasis and they may develop bleeding in spite of a normal haemostatic mechanism. Achieving satisfactory haemostasis also is a problem when bleedings occur in organs such as the brain, inner ear region and eyes with limited possibility for surgical haemostasis. The same problem may arise in the

process of taking biopsies from various organs (liver, lung, tumour tissue, gastrointestinal tract) as well as in laparoscopic surgery. Common for all these situations is the difficulty to provide haemostasis by surgical techniques (sutures, clips, etc.) which also is the case when bleeding is diffuse (haemorrhagic gastritis and profuse uterine bleeding). Acute and profuse bleedings may also occur in subjects on anticoagulant therapy in whom a defective haemostasis has been induced by the therapy given. Such subjects may need surgical interventions in case the anticoagulant effect has to be counteracted rapidly. Radical retropubic prostatectomy is a commonly performed procedure for subjects with localized prostate cancer. The operation is frequently complicated by significant and sometimes massive blood loss. The considerable blood loss during prostatectomy is mainly related to the complicated anatomical situation, with various densely vascularized sites that are not easily accessible for surgical haemostasis, and which may result in diffuse bleeding from a large area. Another situation that may cause problems in the case of unsatisfactory haemostasis is when subjects with a normal haemostatic mechanism are given anticoagulant therapy to prevent thromboembolic disease. Such therapy may include heparin, other forms of proteoglycans, warfarin or other forms of vitamin K-antagonists as well as aspirin and other platelet aggregation inhibitors.

In one embodiment of the invention, the bleeding is associated with haemophilia A or B. In another embodiment, the bleeding is associated with haemophilia with aquired inhibitors. In another embodiment, the bleeding is associated with thrombocytopenia. In another embodiment, the bleeding is associated with von Willebrand's disease. In another embodiment, the bleeding is associated with severe tissue damage. In another embodiment, the bleeding is associated with severe trauma. In another embodiment, the bleeding is associated with surgery. In another embodiment, the bleeding is associated with laparoscopic surgery. In another embodiment, the bleeding is associated with haemorrhagic gastritis. In another embodiment, the bleeding is profuse uterine bleeding. In another embodiment, the bleeding is occurring in organs with a limited possibility for mechanical haemostasis. In another embodiment, the bleeding is occurring in the brain, inner ear region or eyes. In another embodiment, the bleeding is associated with the process of taking biopsies. In another embodiment, the bleeding is associated with anticoagulant therapy.

The term "subject" as used herein is intended to mean any animal, in particular mammals, such as humans, and may, where appropriate, be used interchangeably with the term "patient".

The terminology for amino acid substitutions used herein is as follows. The first letter represent the amino acid naturally present at a position of SEQ ID NO: 1. The following number represent the position in SEQ ID NO: 1. The second letter represent the different

amino acid substituting for the natural amino acid. An example is S43N, where a serine at position 43 of SEQ ID NO: 1 is replaced by an Asparagine. In another example, K62E/I69A, the lysine in position 62 of SEQ ID NO: 1 is replaced by a glutamic acid and the isoleucine in position 69 of SEQ ID NO: 1 is replaced by an alanine in the same Factor VII polypeptide. I69A-FVII thus means human wild type FVII, wherein a isoleucine in position 69 of SEQ ID NO: 1 has been replaced by an alanine.

In one aspect the present invention relates to a composition comprising wild type human FVIIa and a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa.

In a further aspect the present invention relates to a method for the treatment of bleeding episodes in a subject or for the enhancement of the normal haemostatic system, the method comprising administering to a subject in need thereof a therapeutically or prophylactically effective amount of:

a) composition comprising wild type human FVIIa and a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa; or

b) a first composition comprising wild type human FVIIa and a second composition comprising a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa.

In a further aspect the present invention relates to a process for preparing a composition comprising wild type human FVIIa and a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa, wherein the process comprises the step of: mixing wild type human FVIIa with a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa in an aqueous medium.

In a further aspect the present invention relates to a use of a composition comprising wild type human FVIIa and a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa, for the preparation of a medicament for the treatment of bleeding episodes in a subject or for the enhancement of the normal haemostatic system.

In a further aspect the present invention relates to the use of a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa; in the therapeutic treatment of bleedings, where the exposure of TF is high, such as indications where there is a risk of plaque rupture, plaque disruption after percutaneous transluminal coronary angioplasty (PTCA) procedures, sepsis, or other bleeding complications, where inflammatory conditions where increased quantities of tissue factor is seen. In one embodiment the bleedings, where the exposure of TF is high is a coagulation disorder, such as disseminated intravascular coagulation (DIC).

The present invention also encompasses methods and compositions that provide combination therapies in which the Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa is administered with another procoagulant compound, antifibrinolytic compound or regulator of anticoagulant compounds. Suitable compounds include, without limitation, FVIII (such as Refacto® (Genetics Institute), Kogenate FS® (Bayer), Monoclate-P® (Aventis Behring)), Factor XIII (see, e.g., WO 01/85198); inhibitors of tissue factor pathway inhibitor (TFPI inhibitors) (see, e.g., WO 01/85199); Factor IX (see, e.g., WO 02/062376); thrombin activatable fibrinolysis inhibitor (TAFI) (see, e.g., PCT/DK02/00734; PAI-1 (see, e.g., PCT/DK02/00735; Factor V (see, e.g., PCT/DK02/00736); protein C inhibitors (see, e.g., PCT/DK02/00737); thrombomodulin (see, e.g., PCT/DK02/00738); protein S inhibitors (see, e.g., PCT/DK02/00739); tissue plasminogen activator inhibitors (see, e.g., PCT/DK02/00740); alpha2-antiplasmin (see, e.g., PCT/DK02/00741); aprotinin (see, e.g., PCT/DK02/00742); tranexamic acid (see, e.g., PCT/DK02/00751); epsilon-aminocaproic acid (see, e.g., PCT/DK02/00752); prothrombin, thrombin, Factor VII, Factor X, Factor XI and fibrinogen, artificial oxygen carriers (such as POLYHEME®, Northfield lab), colloids (such as Hextend, BioTime, Inc), Desmopressin (such as DDAVP® Tablets (desmopressin acetate) Adventis Pharmaceuticals), activated prothrombin complex concentrates (i.e., Bebulin, Proplex-T, Profiline, Autoplex, FEIBA), Clopidogrel, Ticlopidine, Glycoprotein IIB/IIIA antagonists (Abciximab), LMWH, Warfarin. Streptokinase, Tissue plasminogen activator (tPA)/ tPA mutants.

In one embodiment the Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa is as disclosed in any of international patent applications WO 99/20767, WO 00/66753, WO 01/58935, WO 03/93465 and WO 04/029091, each of which is hereby incorporated by reference in its entirety.

In the present specification, amino acids are represented using abbreviations, as indicated in table 1, approved by IUPAC-IUB Commission on Biochemical Nomenclature (CBN). Amino acid and the like having isomers represented by name or the following abbreviations are in natural L-form unless otherwise indicated. Further, the left and right ends of an amino acid sequence of a peptide are, respectively, the N- and C-termini unless otherwise specified.

Table 1: Abbreviations for amino acids:

Amino acid	Tree-letter code	One-letter code
Glycine	Gly	G
Proline	Pro	P
Alanine	Ala	A
Valine	Val	V
Leucine	Leu	L
Isoleucine	Ile	I
Methionine	Met	M
Cysteine	Cys	C
Phenylalanine	Phe	F
Tyrosine	Tyr	Y
Tryptophan	Trp	W
Histidine	His	H
Lysine	Lys	K
Arginine	Arg	R
Glutamine	Gln	Q
Asparagine	Asn	N
Glutamic Acid	Glu	E
Aspartic Acid	Asp	D
Serine	Ser	S
Threonine	Thr	T

The invention also relates to a method of preparing human Factor VII polypeptides as mentioned above. The human Factor VII polypeptides are preferably produced by recombinant DNA techniques. To this end, DNA sequences encoding human Factor VII may be isolated by preparing a genomic or cDNA library and screening for DNA sequences coding for all or part of the protein by hybridization using synthetic oligonucleotide probes in accordance with standard techniques (cf. Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989). For the present purpose, the DNA sequence encoding the protein is preferably of human origin, i.e. derived from a human genomic DNA or cDNA library.

The DNA sequences encoding the human Factor VII polypeptides may also be prepared synthetically by established standard methods, e.g. the phosphoamidite method described by Beaucage and Caruthers, *Tetrahedron Letters* 22 (1981), 1859 - 1869, or the method described by Matthes et al., *EMBO Journal* 3 (1984), 801 - 805. According to the

phosphoamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in suitable vectors.

The DNA sequences may also be prepared by polymerase chain reaction using specific primers, for instance as described in US 4,683,202, Saiki et al., *Science* 239 (1988), 487 - 491, or Sambrook et al., *supra*.

The DNA sequences encoding the human Factor VII polypeptides are usually inserted into a recombinant vector which may be any vector, which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector, which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

The vector is preferably an expression vector in which the DNA sequence encoding the human Factor VII polypeptides is operably linked to additional segments required for transcription of the DNA. In general, the expression vector is derived from plasmid or viral DNA, or may contain elements of both. The term, "operably linked" indicates that the segments are arranged so that they function in concert for their intended purposes, e.g. transcription initiates in a promoter and proceeds through the DNA sequence coding for the polypeptide.

The promoter may be any DNA sequence, which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell.

Examples of suitable promoters for directing the transcription of the DNA encoding the human Factor VII polypeptide in mammalian cells are the SV40 promoter (Subramani et al., *Mol. Cell Biol.* 1 (1981), 854 -864), the MT-1 (metallothionein gene) promoter (Palmiter et al., *Science* 222 (1983), 809 - 814), the CMV promoter (Boshart et al., *Cell* 41:521-530, 1985) or the adenovirus 2 major late promoter (Kaufman and Sharp, *Mol. Cell. Biol*, 2:1304-1319, 1982).

An example of a suitable promoter for use in insect cells is the polyhedrin promoter (US 4,745,051; Vasuvedan et al., *FEBS Lett.* 311, (1992) 7 - 11), the P10 promoter (J.M. Vlak et al., *J. Gen. Virology* 69, 1988, pp. 765-776), the *Autographa californica* polyhedrosis virus basic protein promoter (EP 397 485), the baculovirus immediate early gene 1 promoter (US 5,155,037; US 5,162,222), or the baculovirus 39K delayed-early gene promoter (US 5,155,037; US 5,162,222).

Examples of suitable promoters for use in yeast host cells include promoters from yeast glycolytic genes (Hitzeman et al., *J. Biol. Chem.* 255 (1980), 12073 - 12080; Alber and

Kawasaki, *J. Mol. Appl. Gen.* 1 (1982), 419 - 434) or alcohol dehydrogenase genes (Young et al., in *Genetic Engineering of Microorganisms for Chemicals* (Hollaender et al, eds.), Plenum Press, New York, 1982), or the TPI1 (US 4,599,311) or ADH2-4c (Russell et al., *Nature* 304 (1983), 652 - 654) promoters.

Examples of suitable promoters for use in filamentous fungus host cells are, for instance, the ADH3 promoter (McKnight et al., *The EMBO J.* 4 (1985), 2093 - 2099) or the tpiA promoter. Examples of other useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral α-amylase, *A. niger* acid stable α-amylase, *A. niger* or *A. awamori* glucoamylase (gluA), *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase. Preferred are the TAKA-amylase and gluA promoters. Suitable promoters are mentioned in, e.g. EP 238 023 and EP 383 779.

The DNA sequences encoding the human Factor VII polypeptides may also, if necessary, be operably connected to a suitable terminator, such as the human growth hormone terminator (Palmiter et al., *Science* 222, 1983, pp. 809-814) or the TPI1 (Alber and Kawasaki, *J. Mol. Appl. Gen.* 1, 1982, pp. 419-434) or ADH3 (McKnight et al., *The EMBO J.* 4, 1985, pp. 2093-2099) terminators. The vector may also contain a set of RNA splice sites located downstream from the promoter and upstream from the insertion site for the Factor VII sequence itself. Preferred RNA splice sites may be obtained from adenovirus and/or immunoglobulin genes. Also contained in the expression vectors is a polyadenylation signal located downstream of the insertion site. Particularly preferred polyadenylation signals include the early or late polyadenylation signal from SV40 (Kaufman and Sharp, ibid.), the polyadenylation signal from the adenovirus 5 Elb region, the human growth hormone gene terminator (DeNoto et al. *Nuc. Acids Res.* 9:3719-3730, 1981) or the polyadenylation signal from the human Factor VII gene or the bovine Factor VII gene. The expression vectors may also include a noncoding viral leader sequence, such as the adenovirus 2 tripartite leader, located between the promoter and the RNA splice sites; and enhancer sequences, such as the SV40 enhancer.

The recombinant vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. An example of such a sequence (when the host cell is a mammalian cell) is the SV40 origin of replication.

When the host cell is a yeast cell, suitable sequences enabling the vector to replicate are the yeast plasmid 2μ replication genes REP 1-3 and origin of replication.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or the *Schizosaccharomyces pombe* TPI gene (described by P.R. Russell, *Gene* 40,

1985, pp. 125-130), or one which confers resistance to a drug, e.g. ampicillin, kanamycin, tetracyclin, chloramphenicol, neomycin, hygromycin or methotrexate. For filamentous fungi, selectable markers include *amdS, pyrG, argB, niaD* or *sC*.

To direct the human Factor VII polypeptides of the present invention into the secretory pathway of the host cells, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence) may be provided in the recombinant vector. The secretory signal sequence is joined to the DNA sequences encoding the human Factor VII polypeptides in the correct reading frame. Secretory signal sequences are commonly positioned 5' to the DNA sequence encoding the peptide. The secretory signal sequence may be that, normally associated with the protein or may be from a gene encoding another secreted protein.

For secretion from yeast cells, the secretory signal sequence may encode any signal peptide, which ensures efficient direction of the expressed human Factor VII polypeptides into the secretory pathway of the cell. The signal peptide may be naturally occurring signal peptide, or a functional part thereof, or it may be a synthetic peptide. Suitable signal peptides have been found to be the α-factor signal peptide (cf. US 4,870,008), the signal peptide of mouse salivary amylase (cf. O. Hagenbuchle et al., *Nature* 289, 1981, pp. 643-646), a modified carboxypeptidase signal peptide (cf. L.A. Valls et al., *Cell* 48, 1987, pp. 887-897), the yeast BAR1 signal peptide (cf. WO 87/02670), or the yeast aspartic protease 3 (YAP3) signal peptide (cf. M. Egel-Mitani et al., *Yeast* 6, 1990, pp. 127-137).

For efficient secretion in yeast, a sequence encoding a leader peptide may also be inserted downstream of the signal sequence and upstream of the DNA sequence encoding the human Factor VII polypeptides. The function of the leader peptide is to allow the expressed peptide to be directed from the endoplasmic reticulum to the Golgi apparatus and further to a secretory vesicle for secretion into the culture medium (i.e. exportation of the human Factor VII polypeptides across the cell wall or at least through the cellular membrane into the periplasmic space of the yeast cell). The leader peptide may be the yeast alpha-factor leader (the use of which is described in e.g. US 4,546,082, US 4,870,008, EP 16 201, EP 123 294, EP 123 544 and EP 163 529). Alternatively, the leader peptide may be a synthetic leader peptide, which is to say a leader peptide not found in nature. Synthetic leader peptides may, for instance, be constructed as described in WO 89/02463 or WO 92/11378.

For use in filamentous fungi, the signal peptide may conveniently be derived from a gene encoding an *Aspergillus* sp. amylase or glucoamylase, a gene encoding a *Rhizomucor miehei* lipase or protease or a *Humicola lanuginosa* lipase. The signal peptide is preferably derived from a gene encoding *A. oryzae* TAKA amylase, *A. niger* neutral α-amylase, *A. niger* acid-stable amylase, or *A. niger* glucoamylase. Suitable signal peptides are disclosed in, e.g. EP 238 023 and EP 215 594.

For use in insect cells, the signal peptide may conveniently be derived from an insect gene (cf. WO 90/05783), such as the lepidopteran *Manduca sexta* adipokinetic hormone precursor signal peptide (cf. US 5,023,328).

The procedures used to ligate the DNA sequences coding for the human Factor VII polypeptides, the promoter and optionally the terminator and/or secretory signal sequence, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989).

Methods of transfecting mammalian cells and expressing DNA sequences introduced in the cells are described in e.g. Kaufman and Sharp, *J. Mol. Biol.* 159 (1982), 601 - 621; Southern and Berg, *J. Mol. Appl. Genet.* 1 (1982), 327 - 341; Loyter et al., *Proc. Natl. Acad. Sci. USA* 79 (1982), 422 - 426; Wigler et al., *Cell* 14 (1978), 725; Corsaro and Pearson, *Somatic Cell Genetics* 7 (1981), 603, Graham and van der Eb, *Virology* 52 (1973), 456; and Neumann et al., *EMBO J.* 1 (1982), 841 - 845.

Selectable markers may be introduced into the cell on a separate plasmid at the same time as the gene of interest, or they may be introduced on the same plasmid. If on the same plasmid, the selectable marker and the gene of interest may be under the control of different promoters or the same promoter, the latter arrangement producing a dicistronic message. Constructs of this type are known in the art (for example, Levinson and Simonsen, U.S. Pat. No. 4,713,339). It may also be advantageous to add additional DNA, known as "carrier DNA," to the mixture that is introduced into the cells.

After the cells have taken up the DNA, they are grown in an appropriate growth medium, typically 1-2 days, to begin expressing the gene of interest. As used herein the term "appropriate growth medium" means a medium containing nutrients and other components required for the growth of cells and the expression of the human Factor VII polypeptides of interest. Media generally include a carbon source, a nitrogen source, essential amino acids, essential sugars, vitamins, salts, phospholipids, protein and growth factors. For production of gamma-carboxylated proteins, the medium will contain vitamin K, preferably at a concentration of about 0.1 μg/ml to about 5 μg/ml. Drug selection is then applied to select for the growth of cells that are expressing the selectable marker in a stable fashion. For cells that have been transfected with an amplifiable selectable marker the drug concentration may be increased to select for an increased copy number of the cloned sequences, thereby increasing expression levels. Clones of stably transfected cells are then screened for expression of the human Factor VII polypeptide of interest.

The host cell into which the DNA sequences encoding the human Factor VII polypep-

tides is introduced may be any cell, which is capable of producing the posttranslational modified human Factor VII polypeptides and includes yeast, fungi and higher eucaryotic cells.

Examples of mammalian cell lines for use in the present invention are the COS-1 (ATCC CRL 1650), baby hamster kidney (BHK) and 293 (ATCC CRL 1573; Graham et al., *J. Gen. Virol.* 36:59-72, 1977) cell lines. A preferred BHK cell line is the tk^- ts13 BHK cell line (Waechter and Baserga, *Proc. Natl. Acad. Sci. USA* 79:1106-1110, 1982, incorporated herein by reference), hereinafter referred to as BHK 570 cells. The BHK 570 cell line has been deposited with the American Type Culture Collection, 12301 Parklawn Dr., Rockville, Md. 20852, under ATCC accession number CRL 10314. A tk^- ts13 BHK cell line is also available from the ATCC under accession number CRL 1632. In addition, a number of other cell lines may be used within the present invention, including Rat Hep I (Rat hepatoma; ATCC CRL 1600), Rat Hep II (Rat hepatoma; ATCC CRL 1548), TCMK (ATCC CCL 139), Human lung (ATCC HB 8065), NCTC 1469 (ATCC CCL 9.1), CHO (ATCC CCL 61) and DUKX cells (Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA* 77:4216-4220, 1980).

Examples of suitable yeasts cells include cells of *Saccharomyces* spp. or *Schizosaccharomyces* spp., in particular strains of *Saccharomyces cerevisiae* or *Saccharomyces kluyveri*. Methods for transforming yeast cells with heterologous DNA and producing heterologous polypeptides there from are described, e.g. in US 4,599,311, US 4,931,373, US 4,870,008, 5,037,743, and US 4,845,075, all of which are hereby incorporated by reference. Transformed cells are selected by a phenotype determined by a selectable marker, commonly drug resistance or the ability to grow in the absence of a particular nutrient, e.g. leucine. A preferred vector for use in yeast is the POT1 vector disclosed in US 4,931,373. The DNA sequences encoding the human Factor VII polypeptides may be preceded by a signal sequence and optionally a leader sequence, e.g. as described above. Further examples of suitable yeast cells are strains of *Kluyveromyces*, such as *K. lactis*, *Hansenula*, e.g. *H. polymorpha*, or *Pichia*, e.g. *P. pastoris* (cf. Gleeson et al., *J. Gen. Microbiol.* 132, 1986, pp. 3459-3465; US 4,882,279).

Examples of other fungal cells are cells of filamentous fungi, e.g. *Aspergillus* spp., *Neurospora* spp., *Fusarium* spp. or *Trichoderma* spp., in particular strains of *A. oryzae*, *A. nidulans* or *A. niger*. The use of *Aspergillus* spp. for the expression of proteins is described in, e.g., EP 272 277, EP 238 023, EP 184 438 The transformation of *F. oxysporum* may, for instance, be carried out as described by Malardier et al., 1989, *Gene* 78: 147-156. The transformation of *Trichoderma* spp. may be performed for instance as described in EP 244 234.

When a filamentous fungus is used as the host cell, it may be transformed with the DNA construct of the invention, conveniently by integrating the DNA construct in the host chromosome to obtain a recombinant host cell. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of

the DNA constructs into the host chromosome may be performed according to conventional methods, e.g. by homologous or heterologous recombination.

Transformation of insect cells and production of heterologous polypeptides therein may be performed as described in US 4,745,051; US 4,879,236; US 5,155,037; 5,162,222; EP 397,485) all of which are incorporated herein by reference. The insect cell line used as the host may suitably be a *Lepidoptera* cell line, such as *Spodoptera frugiperda* cells or *Trichoplusia ni* cells (cf. US 5,077,214). Culture conditions may suitably be as described in, for instance, WO 89/01029 or WO 89/01028, or any of the aforementioned references.

The transformed or transfected host cell described above is then cultured in a suitable nutrient medium under conditions permitting expression of the human Factor VII polypeptide after which all or part of the resulting peptide may be recovered from the culture. The medium used to culture the cells may be any conventional medium suitable for growing the host cells, such as minimal or complex media containing appropriate supplements. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. in catalogues of the American Type Culture Collection). The human Factor VII polypeptide produced by the cells may then be recovered from the culture medium by conventional procedures including separating the host cells from the medium by centrifugation or filtration, precipitating the proteinaqueous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulphate, purification by a variety of chromatographic procedures, e.g. ion exchange chromatography, gelfiltration chromatography, affinity chromatography, or the like, dependent on the type of polypeptide in question.

For the preparation of recombinant human Factor VII polypeptides, a cloned wild-type Factor VII DNA sequence is used. This sequence may be modified to encode a desired Factor VII variant. The complete nucleotide and amino acid sequences for human Factor VII are known. See U.S. Pat. No. 4,784,950, which is incorporated herein by reference, where the cloning and expression of recombinant human Factor VII is described. The bovine Factor VII sequence is described in Takeya et al., *J. Biol. Chem*, 263:14868-14872 (1988), which is incorporated by reference herein.

The amino acid sequence alterations may be accomplished by a variety of techniques. Modification of the DNA sequence may be by site-specific mutagenesis. Techniques for site-specific mutagenesis are well known in the art and are described by, for example, Zoller and Smith (*DNA* 3:479-488, 1984). Thus, using the nucleotide and amino acid sequences of Factor VII, one may introduce the alterations of choice.

DNA sequences for use within the present invention will typically encode a pre-pro peptide at the amino-terminus of the Factor VII protein to obtain proper post-translational processing (e.g. gamma-carboxylation of glutamic acid residues) and secretion from the host

cell. The pre-pro peptide may be that of Factor VII or another vitamin K-dependent plasma protein, such as factor IX, factor X, prothrombin, protein C or protein S. As will be appreciated by those skilled in the art, additional modifications can be made in the amino acid sequence of Factor VII where those modifications do not significantly impair the ability of the protein to act as a coagulation factor. For example, Factor VII in the catalytic triad can also be modified in the activation cleavage site to inhibit the conversion of zymogen Factor VII into its activated two-chain form, as generally described in U.S. Pat. No. 5,288,629, incorporated herein by reference.

Within the present invention, transgenic animal technology may be employed to produce the human Factor VII polypeptide. It is preferred to produce the proteins within the mammary glands of a host female mammal. Expression in the mammary gland and subsequent secretion of the protein of interest into the milk overcomes many difficulties encountered in isolating proteins from other sources. Milk is readily collected, available in large quantities, and well characterized biochemically. Furthermore, the major milk proteins are present in milk at high concentrations (typically from about 1 to 15 g/l). From a commercial point of view, it is clearly preferable to use as the host a species that has a large milk yield. While smaller animals such as mice and rats can be used (and are preferred at the proof of principle stage), within the present invention it is preferred to use livestock mammals including, but not limited to, pigs, goats, sheep and cattle. Sheep are particularly preferred due to such factors as the previous history of transgenesis in this species, milk yield, cost and the ready availability of equipment for collecting sheep milk. See WIPO Publication WO 88/00239 for a comparison of factors influencing the choice of host species. It is generally desirable to select a breed of host animal that has been bred for dairy use, such as East Friesland sheep, or to introduce dairy stock by breeding of the transgenic line at a later date. In any event, animals of known, good health status should be used.

To obtain expression in the mammary gland, a transcription promoter from a milk protein gene is used. Milk protein genes include those genes encoding caseins (see U.S. Pat. No. 5,304,489, incorporated herein by reference), beta-lactoglobulin, alpha-lactalbumin, and whey acidic protein. The beta-lactoglobulin (BLG) promoter is preferred. In the case of the ovine beta-lactoglobulin gene, a region of at least the proximal 406 bp of 5' flanking sequence of the gene will generally be used, although larger portions of the 5' flanking sequence, up to about 5 kbp, are preferred, such as about 4.25 kbp DNA segment encompassing the 5' flanking promoter and non-coding portion of the beta-lactoglobulin gene. See Whitelaw et al., *Biochem J.* 286: 31-39 (1992). Similar fragments of promoter DNA from other species are also suitable.

Other regions of the beta-lactoglobulin gene may also be incorporated in constructs, as may genomic regions of the gene to be expressed. It is generally accepted in the art that constructs lacking introns, for example, express poorly in comparison with those that contain such DNA sequences (see Brinster et al., *Proc. Natl. Acad. Sci. USA* 85: 836-840 (1988); Palmiter et al., *Proc. Natl. Acad. Sci. USA* 88: 478-482 (1991); Whitelaw et al., *Transgenic Res.* 1: 3-13 (1991); WO 89/01343; and WO 91/02318, each of which is incorporated herein by reference). In this regard, it is generally preferred, where possible, to use genomic sequences containing all or some of the native introns of a gene encoding the protein or polypeptide of interest, thus the further inclusion of at least some introns from, e.g, the beta-lactoglobulin gene, is preferred. One such region is a DNA segment which provides for intron splicing and RNA polyadenylation from the 3' non-coding region of the ovine beta-lactoglobulin gene. When substituted for the natural 3' non-coding sequences of a gene, this ovine beta-lactoglobulin segment can both enhance and stabilize expression levels of the protein or polypeptide of interest. Within other embodiments, the region surrounding the initiation ATG of the sequence encoding the human Factor VII polypeptide is replaced with corresponding sequences from a milk specific protein gene. Such replacement provides a putative tissue-specific initiation environment to enhance expression. It is convenient to replace the entire pre-pro sequence of the human Factor VII polypeptide and 5' non-coding sequences with those of, for example, the BLG gene, although smaller regions may be replaced.

For expression of a human Factor VII polypeptide in transgenic animals, a DNA segment encoding the human Factor VII polypeptide is operably linked to additional DNA segments required for its expression to produce expression units. Such additional segments include the above-mentioned promoter, as well as sequences which provide for termination of transcription and polyadenylation of mRNA. The expression units will further include a DNA segment encoding a secretory signal sequence operably linked to the segment encoding the human Factor VII polypeptide. The secretory signal sequence may be a native secretory signal sequence of the human Factor VII polypeptide or may be that of another protein, such as a milk protein. See, for example, von Heinje, *Nuc. Acids Res.* 14: 4683-4690 (1986); and Meade et al., U.S. Pat. No. 4,873,316, which are incorporated herein by reference.

Construction of expression units for use in transgenic animals is conveniently carried out by inserting a sequence encoding the human Factor VII polypeptide into a plasmid or phage vector containing the additional DNA segments, although the expression unit may be constructed by essentially any sequence of ligations. It is particularly convenient to provide a vector containing a DNA segment encoding a milk protein and to replace the coding sequence for the milk protein with that of the human Factor VII polypeptide, thereby creating a

gene fusion that includes the expression control sequences of the milk protein gene. In any event, cloning of the expression units in plasmids or other vectors facilitates the amplification of the human Factor VII polypeptide. Amplification is conveniently carried out in bacterial (e.g. E. coli) host cells, thus the vectors will typically include an origin of replication and a selectable marker functional in bacterial host cells.

The expression unit is then introduced into fertilized eggs (including early-stage embryos) of the chosen host species. Introduction of heterologous DNA can be accomplished by one of several routes, including microinjection (e.g. U.S. Pat. No. 4,873,191), retroviral infection (Jaenisch, *Science* 240: 1468-1474 (1988)) or site-directed integration using embryonic stem (ES) cells (reviewed by Bradley et al., *Bio/Technology* 10: 534-539 (1992)). The eggs are then implanted into the oviducts or uteri of pseudopregnant females and allowed to develop. Offspring carrying the introduced DNA in their germ line can pass the DNA on to their progeny in the normal, Mendelian fashion, allowing the development of transgenic herds.

General procedures for producing transgenic animals are known in the art. See, for example, Hogan et al., *Manipulating the Mouse Embryo: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1986; Simons et al., *Bio/Technology* 6: 179-183 (1988); Wall et al., Biol. Reprod. 32: 645-651 (1985); Buhler et al., *Bio/Technology* 8: 140-143 (1990); Ebert et al., *Bio/Technology* 9: 835-838 (1991); Krimpenfort et al., *Bio/Technology* 9: 844-847 (1991); Wall et al., *J. Cell. Biochem.* 49: 113-120 (1992); U.S. Pat. Nos. 4,873,191 and 4,873,316; WIPO publications WO 88/00239, WO 90/05188, WO 92/11757; and GB 87/00458, which are incorporated herein by reference. Techniques for introducing foreign DNA sequences into mammals and their germ cells were originally developed in the mouse. See, e.g., Gordon et al., *Proc. Natl. Acad. Sci. USA* 77: 7380-7384 (1980); Gordon and Ruddle, *Science* 214: 1244-1246 (1981); Palmiter and Brinster, *Cell* 41: 343-345 (1985); and Brinster et al., *Proc. Natl. Acad. Sci. USA* 82: 4438-4442 (1985). These techniques were subsequently adapted for use with larger animals, including livestock species (see e.g., WIPO publications WO 88/00239, WO 90/05188, and WO 92/11757; and Simons et al., *Bio/Technology* 6: 179-183 (1988). To summarize, in the most efficient route used to date in the generation of transgenic mice or livestock, several hundred linear molecules of the DNA of interest are injected into one of the pro-nuclei of a fertilized egg according to established techniques. Injection of DNA into the cytoplasm of a zygote can also be employed. Production in transgenic plants may also be employed. Expression may be generalized or directed to a particular organ, such as a tuber. See, Hiatt, *Nature* 344:469-479 (1990); Edelbaum et al., *J. Interferon Res.* 12:449-453 (1992); Sijmons et al., *Bio/Technology* 8:217-221 (1990); and European Patent Office Publication EP 255,378.

Factor VII produced according to the present invention may be purified by affinity chromatography on an anti-Factor VII antibody column. It is preferred that the immunoadsorption column comprise a high-specificity monoclonal antibody. The use of calcium-dependent monoclonal antibodies, as described by Wakabayashi et al., *J. Biol. Chem,* 261:11097-11108, (1986) and Thim et al., *Biochem.* 27: 7785-7793, (1988), incorporated by reference herein, is particularly preferred. Additional purification may be achieved by conventional chemical purification means, such as high performance liquid chromatography. Other methods of purification, including barium citrate precipitation, are known in the art, and may be applied to the purification of the Factor VII described herein (see, generally, Scopes, R., *Protein Purification*, Springer-Verlag, N.Y., 1982). Substantially pure Factor VII of at least about 90 to 95% homogeneity is preferred, and 98 to 99% or more homogeneity most preferred, for pharmaceutical uses. Once purified, partially or to homogeneity as desired, the Factor VII may then be used therapeutically.

Conversion of single-chain Factor VII to active two-chain Factor VIIa may be achieved using factor XIIa as described by Hedner and Kisiel (1983, *J. Clin. Invest.* 71: 1836-1841), or with other proteases having trypsin-like specificity (Kisiel and Fujikawa, *Behring Inst. Mitt.* 73: 29-42, 1983). Alternatively Factor VII may be autoactivated by passing it through an ion-exchange chromatography column, such as mono Q.RTM. (Pharmacia Fire Chemicals) or the like (Bjoern et al., 1986, *Research Disclosures* 269:564-565). The Factor VII molecules of the present invention and pharmaceutical compositions thereof are particularly useful for administration to humans to treat a variety of conditions involving intravascular coagulation.

The invention also provides suitable assays for selecting preferred Factor VIIa polypeptides and Factor VIIa derivatives according to the invention. These assays can be performed as a simple preliminary *in vitro* test.

Thus, Example 3 herein discloses a simple test (entitled "*In Vitro* Hydrolysis Assay") for the activity of Factor VIIa polypeptides of the invention. Based thereon, Factor VIIa polypeptides which are of particular interest are such polypeptides where the ratio between the activity of the variant and the activity of native human Factor VII shown in Fig. 1 is about 1.0 or higher, when tested in the "*In Vitro* Hydrolysis Assay" defined herein.

The activity of the polypeptides can also be measured using a physiological substrate such as factor X ("*In Vitro* Proteolysis Assay") (see Example 4), suitably at a concentration of 100-1000 nM, where the factor Xa generated is measured after the addition of a suitable chromogenic substrate (e.g. S-2765). In addition, the activity assay may be run at physiological temperature.

The ability of the procoagulant Factor VIIa polypeptides to generate thrombin can also be measured in an assay comprising all relevant coagulation factors and inhibitors at physiological concentrations (minus factor VIII when mimicking haemophilia A conditions) and activated platelets (as described on p. 543 in Monroe et al. (1997) *Brit. J. Haematol.* 99, 542-547 which is hereby incorporated as reference).

The procoagulant Factor VII polypeptides according to the present invention may be used to control bleeding disorders which have several causes such as clotting factor deficiencies (e.g. haemophilia A and B or deficiency of coagulation factors XI or VII) or clotting factor inhibitors, or they may be used to control excessive bleeding occurring in subjects with a normally functioning blood clotting cascade (no clotting factor deficiencies or inhibitors against any of the coagulation factors). The bleedings may be caused by a defective platelet function, thrombocytopenia or von Willebrand's disease. They may also be seen in subjects in whom an increased fibrinolytic activity has been induced by various stimuli.

In subjects who experience extensive tissue damage in association with surgery or vast trauma, the haemostatic mechanism may be overwhelmed by the demand of immediate haemostasis and they may develop bleedings in spite of a normal haemostatic mechanism. Achieving satisfactory haemostasis is also a problem when bleedings occur in organs such as the brain, inner ear region and eyes and may also be a problem in cases of diffuse bleedings (haemorrhagic gastritis and profuse uterine bleeding) when it is difficult to identify the source. The same problem may arise in the process of taking biopsies from various organs (liver, lung, tumour tissue, gastrointestinal tract) as well as in laparoscopic surgery. These situations share the difficulty of providing haemostasis by surgical techniques (sutures, clips, etc.). Acute and profuse bleedings may also occur in subjects on anticoagulant therapy in whom a defective haemostasis has been induced by the therapy given. Such subjects may need surgical interventions in case the anticoagulant effect has to be counteracted rapidly. Another situation that may cause problems in the case of unsatisfactory haemostasis is when subjects with a normal haemostatic mechanism are given anticoagulant therapy to prevent thromboembolic disease. Such therapy may include heparin, other forms of proteoglycans, warfarin or other forms of vitamin K-antagonists as well as aspirin and other platelet aggregation inhibitors.

A systemic activation of the coagulation cascade may lead to disseminated intravascular coagulation (DIC). However, such complications have not been seen in subjects treated with high doses of recombinant Factor VIIa because of a localised haemostatic process of the kind induced by the complex formation between Factor VIIa and TF exposed at the site of vessel wall injury. The procoagulant Factor VII polypeptides ac-

cording to the invention may thus also be used in their activated form to control such excessive bleedings associated with a normal haemostatic mechanism.

For treatment in connection with deliberate interventions, the procoagulant Factor VII polypeptides of the invention will typically be administered within about 24 hours prior to performing the intervention, and for as much as 7 days or more thereafter. Administration as a coagulant can be by a variety of routes as described herein.

The dose of the Factor VII polypeptides ranges from about 0.05 mg to 500 mg/day, preferably from about 1 mg to 200 mg/day, and more preferably from about 10 mg to about 175 mg/day for a 70 kg subject as loading and maintenance doses, depending on the severity of the condition.

The pharmaceutical compositions are primarily intended for parenteral administration for prophylactic and/or therapeutic treatment. Preferably, the pharmaceutical compositions are administered parenterally, i.e., intravenously, subcutaneously, or intramuscularly, or it may be administered by continuous or pulsatile infusion. The compositions for parenteral administration comprise the Factor VII polypeptide of the invention in combination with, preferably dissolved in, a pharmaceutically acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, such as water, buffered water, 0.4% saline, 0.3% glycine and the like. The Factor VII polypeptides of the invention can also be formulated into liposome preparations for delivery or targeting to the sites of injury. Liposome preparations are generally described in, e.g., U.S. 4,837,028, U.S. 4,501,728, and U.S. 4,975,282. The compositions may be sterilised by conventional, well-known sterilisation techniques. The resulting aqueous solutions may be packaged for use or filtered under aseptic conditions and lyophilised, the lyophilised preparation being combined with a sterile aqueous solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, etc.

The concentration of Factor VII polypeptide in these formulations can vary widely, i.e., from less than about 0.5% by weight, usually at or at least about 1% by weight to as much as 15 or 20% by weight and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

Thus, a typical pharmaceutical composition for intravenous infusion could be made up to contain 250 ml of sterile Ringer's solution and 10 mg of the Factor VII polypeptide. Actual methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art and are described in more detail in, for example,

Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing Company, Easton, PA (1990).

The compositions containing the Factor VII polypeptides of the present invention can be administered for prophylactic and/or therapeutic treatments. In therapeutic applications, compositions are administered to a subject already suffering from a disease, as described above, in an amount sufficient to cure, alleviate or partially arrest the disease and its complications. An amount adequate to accomplish this is defined as "therapeutically effective amount". As will be understood by the person skilled in the art amounts effective for this purpose will depend on the severity of the disease or injury as well as the weight and general state of the subject. In general, however, the effective amount will range from about 0.05 mg up to about 500 mg of the Factor VII polypeptide per day for a 70 kg subject, with dosages of from about 1.0 mg to about 200 mg of the Factor VII polypeptide per day being more commonly used.

It must be kept in mind that the materials of the present invention may generally be employed in serious disease or injury states, that is, life threatening or potentially life threatening situations. In such cases, in view of the minimisation of extraneous substances and general lack of immunogenicity of human Factor VII polypeptides in humans, it is possible and may be felt desirable by the treating physician to administer a substantial excess of these variant Factor VII compositions.

In prophylactic applications, compositions containing the Factor VII polypeptide of the invention are administered to a subject susceptible to or otherwise at risk of a disease state or injury to enhance the subject's own coagulative capability. Such an amount is defined to be a "prophylactically effective dose." In prophylactic applications, the precise amounts once again depend on the subject's state of health and weight, but the dose generally ranges from about 0.05 mg to about 500 mg per day for a 70-kilogram subject, more commonly from about 1.0 mg to about 200 mg per day for a 70-kilogram subject.

Single or multiple administrations of the compositions can be carried out with dose levels and patterns being selected by the treating physician. For ambulatory subjects requiring daily maintenance levels, the Factor VII polypeptides may be administered by continuous infusion using e.g. a portable pump system.

Local delivery of the Factor VII polypeptide of the present invention, such as, for example, topical application may be carried out, for example, by means of a spray, perfusion, double balloon catheters, stent, incorporated into vascular grafts or stents, hydrogels used to coat balloon catheters, or other well established methods. In any event, the pharmaceutical compositions should provide a quantity of Factor VII polypeptide sufficient to effectively treat the subject.

Inactivated Factor VII polypeptides of the present invention are able to bind to cell-surface tissue factor. For example, DEGR-Factor VIIa binds cell-surface tissue factor with an equivalent or higher affinity than wild-type Factor VIIa. DEGR-Factor VIIa, however, has no enzymatic activity, yet it binds to tissue factor and acts as a competitive antagonist for wild-type Factor VIIa, thereby inhibiting the subsequent steps in the extrinsic pathway of coagulation leading to the generation of thrombin.

Inactivated Factor VII polypeptides are particularly useful for administration to humans to treat a variety of conditions involving intravascular coagulation. For example, although deep vein thrombosis and pulmonary embolism can be treated with conventional anticoagulants, the inactivated Factor VII polypeptides described herein may be used to prevent the occurrence of thromboembolic complications in identified high risk patients, such as those undergoing surgery or those with congestive heart failure. In addition, inactivated Factor VII polypeptides may act as an antagonist for tissue factor-mediated induction of coagulation, thus blocking the production of thrombin and the subsequent deposition of fibrin. As such, inactivated Factor VII polypeptides may be useful for inhibiting tissue factor activity resulting in, for example, the inhibition of blood coagulation, thrombosis or platelet deposition.

The inactivated Factor VII polypeptides may be particularly useful in the treatment of intimal hyperplasia, restenosis due to acute vascular injury, deep venous thrombosis, arterial thrombosis, post surgical thrombosis, coronary artery bypass graft (CABG), percutaneous transdermal coronary angioplastry (PTCA), stroke, cancer, tumour metastasis, angiogenesis, ischemia/reperfusion, rheumatoid arthritis, thrombolysis, arteriosclerosis and restenosis following angioplastry, acute and chronic indications such as inflammation, septic chock, septicemia, hypotension, adult respiratory distress syndrome (ARDS), disseminated intravascular coagulopathy (DIC), pulmonary embolism, platelet deposition, myocardial infarction, or the prophylactic treatment of mammals with atherosclerotic vessels at risk for thrombosis. Acute vascular injuries are those which occur rapidly (i.e. over days to months), in contrast to chronic vascular injuries (e.g. atherosclerosis) which develop over a lifetime. Acute vascular injuries often result from surgical procedures such as vascular reconstruction, wherein the techniques of angioplasty, endarterectomy, atherectomy, vascular graft emplacement or the like are employed. Hyperplasia may also occur as a delayed response in response to, e.g., graft emplacement or organ transplantation. Since inactivated Factor VII polypeptides is more selective than heparin, generally binding only tissue factor which has been exposed at sites of injury, and because inactivated Factor VII polypeptides does not destroy other coagulation proteins, it will be more effective and less likely to cause bleeding complications than heparin when used prophylactically for the prevention of deep vein thrombosis.

Inactivated Factor VII polypeptides which maintain tissue factor binding inhibit platelet accumulation at the site of vascular injury by blocking the production of thrombin and the subsequent deposition of fibrin.

Due to the ability of DEGR-Factor VII to block thrombin generation and limit platelet deposition at sites of acute vascular injury, inactivated Factor VII polypeptides which maintain tissue factor binding activity but lack Factor VIIa enzymatic activity can be used to inhibit vascular restenosis.

Compositions comprising inactivated Factor VII polypeptides are particularly useful in methods for treating patients when formulated into pharmaceutical compositions, where they may be given to individuals suffering from a variety of disease states to treat coagulation-related conditions. Such inactivated Factor VII polypeptides, capable of binding tissue factor but having a substantially reduced ability to catalyze activation of other factors in the clotting cascade, may possess a longer plasma half-life and thus a correspondingly longer period of anticoagulative activity when compared to other anticoagulants. Among the medical indications for the subject compositions are those commonly treated with anticoagulants, such as, for example, deep vein thrombosis, pulmonary embolism, stroke, disseminated intravascular coagulation (DIC), fibrin deposition in lungs and kidneys associated with gram-negative endotoxemia, and myocardial infarction. The compositions can be used to inhibit vascular restenosis as occurs following mechanical vascular injury, such as injury caused by balloon angioplasty, endarterectomy, reductive atherectomy, stent placement, laser therapy or rotablation, or as occurs secondary to vascular grafts, stents, bypass grafts or organ transplants. The compositions can thus be used to inhibit platelet deposition and associated disorders. Thus, a method of inhibiting coagulation, vascular restenosis or platelet deposition, for example, comprises administering to a patient a composition comprising inactivated Factor VII polypeptides, such as that having at least one amino acid substitution in a catalytic triad of Ser344, Asp242 and His193, in an amount sufficient to effectively inhibit coagulation, vascular restenosis or platelet deposition. The methods also find use in the treatment of acute closure of a coronary artery in an individual (e.g. acute myocardial infarction), which comprises administering the inactivated Factor VII polypeptides, which includes DEGR-Factor VII and FFR-Factor VII, in conjunction with tissue plasminogen activator or streptokinase, and can accelerate tPA induced thrombolysis. The inactivated Factor VII polypeptides is given prior to, in conjunction with, or shortly following administration of a thrombolytic agent, such as tissue plasminogen activator.

Compositions of inactivated Factor VII polypeptides will also have substantial utility in the prevention of cardiogenic emboli and in the treatment of thrombotic strokes. Because of its low potential for causing bleeding complications and its selectivity, Factor VII polypep-

tides can be given to stroke victims and may prevent the extension of the occluding arterial thrombus. The amount of Factor VII polypeptides administered will vary with each patient depending on the nature and severity of the stroke, but doses will generally be in the range of those suggested below.

Inactivated Factor VII polypeptides and compositions thereof can also be used to inhibit deleterious events associated with ischemic reperfusion. Severe ischemia to a tissue, organ or limb may be due to a decrease in blood flow and may be associated with trauma, surgical manipulation, or lowered blood pressure. One of the complications associated with severe ischemia is the up-regulation of tissue factor in the arterial system. This increased expression of tissue factor is believed to stimulate a procoagulant response, primarily in the capillary bed. Following reperfusion to the ischemic tissue, thrombi can be generated which may be either occlusive or non-occlusive. The generation of thrombi in the arterial bed, and the deposition of platelets along the thrombus, lead to the secondary generation of ischemia to the tissue. The generation of the thrombi and the presence of platelets can then cause the generation and release of multiple bioactive factors, including those generated from the coagulation pathway, such as thrombin and Factor X, as well as factors released from activated platelets. In turn, these factors may induce the generation of additional factors by the underlying endothelial and smooth muscle cells, or by adjacent mononuclear cells, such as TNF-alpha and IL-1. These factors, in turn, can then activate the endothelial cells leading to the up-regulation of various adhesion molecules associated with monocyte and neutrophil binding. The binding and transmigration of monocytes and neutrophils, the release of bioactive compounds by these cells, including the generation of free-oxygen radicals, can exacerbate the level of endothelial cell activation and damage. Ultimately, if the cascade of events goes unchecked, this can lead to systemic complications and the potential to stimulate multiple organ failure. By blocking tissue factor according to the present invention by administering a specific inhibitor for tissue factor/Factor VII binding (e.g., FFR-FVIIa), and thereby blocking the initiation of the extrinsic pathway of coagulation, the initiation of the cascade of events may be prevented, thereby eliminating, or minimizing the deleterious events associated with ischemia/reperfusion.

The dose of inactivated Factor VII polypeptides for prevention of deep vein thrombosis is in the range of about 50 μg to 500 mg/day, more typically 1 mg to 200 mg/day, and more preferably 10 to about 175 mg/day for a 70 kg patient, and administration should begin at least about 6 hours prior to surgery and continue at least until the patient becomes ambulatory. The dose of inactivated Factor VII polypeptides in the treatment for restenosis will vary with each patient but will generally be in the range of those suggested above.

Compositions comprising inactivated Factor VII polypeptides will typically be administered within about 24 hours prior to performing an intervention, and for as much as 7 days or more thereafter. Administration can be by a variety of routes as further described herein. The compositions comprising inactivated Factor VII polypeptides can also be administered systemically or locally for the placement of vascular grafts (e.g., by coating synthetic or modified natural arterial vascular grafts), at sites of anastomosis, surgical endarterectomy (typically carotid artery endarterectomy), bypass grafts, and the like.

In the treatment of established deep vein thrombosis and/or pulmonary embolism, the dose of Factor VII polypeptides ranges from about 50 μg to 500 mg/day, more typically 1 mg to 200 mg/day, and more preferably 10 mg to about 175 mg/day for a 70 kg patient as loading and maintenance doses, depending on the weight of the patient and the severity of the condition. Because of the lower likelihood of bleeding complications from infusions of inactivated Factor VII polypeptides, inactivated Factor VII polypeptides can replace or lower the dose of heparin during or after surgery in conjunction with thrombectomies or embolectomies.

In cases of acute bacteremia, endotoxemia or DIC, the patient is given a loading dose of a Factor VII polypeptide of at least about 50 μg to 500 mg/day, more typically 1 mg to 200 mg/day, and more preferably 10 mg to about 175 mg/day for a 70 kg patient, with maintenance doses thereafter in the range of 50 μg to 500 mg/day, typically 1 mg to 200 mg/day for a 70 kg patient.

Preferably, the Factor VII polypeptide has a half-life ($t_{1/2}$) which is enhanced relative to the half-life of the unconjugated Factor VII from which it was derived. Preferably, the half-life of the Factor VII polypeptide is enhanced by at least 1.5-fold to 2-fold, more preferably by about 2-fold to 3-fold, even more preferably by about 5-fold to 10-fold, optimally about 100-fold, usually about 6-fold relative to the half-life of the unmodified parent Factor VII.

General methods of attaching polyethylene glycol to proteins are disclosed within U.S. Pat. No. 4,179,337 issued Dec. 18, 1979 (incorporated herein by reference to disclose methods of attaching polyethylene glycol to proteins). Further, other methods of attaching polyethylene glycol are disclosed within U.S. Pat. No. 5,122,614 issued Jun. 16, 1992, also incorporated herein by reference to disclose methods of attaching polyethylene glycol to proteins. Maleimido-PEG is perhaps the most useful reagent for cysteine-PEGylation, but other chemistries are available for specific cysteine modification.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention is described in further detail in the examples with reference to the appended drawings wherein

Fig. 1 The structure of correctly processed human coagulation Factor VII, amino acids 1 to 406, with gamma carboxylated Glu-residues (γ) and glycosylation (✱). The arrow at amino acid residue 152 shows the site where single-chain Factor VII is cleaved to be converted to activated two-chain Factor VII (FVIIa).

Fig. 2 The full amino acid sequence of native (wild type) human coagulation Factor VII (SEQ ID NO:1).

The present invention is further illustrated by the following examples which, however, are not to be construed as limiting the scope of protection. The features disclosed in the foregoing description and in the following examples may, both separately and in any combination thereof, be material for realizing the invention in diverse forms thereof.

EXAMPLES

Example 1

Construction of DNA encoding FVII-(S43N), FVII-(K62E), FVII-(Q64E), FVII-(I69F), FVII-(K62E/I69A), FVII-(Q64E/I69A), FVII-(Q62E/Q64E/I69A), FVII-(K62E/I69F), FVII-(Q64E/I69F), FVII-(K62E/Q64E/I69F), FVII-(S43C), FVII-(I69C), FVII-(Q64C), FVII-(M306C), FVII-(R277C), FVII-(I69N/F71T), FVII-(F71N/L73T), FVII-(R277N), and FVII-(D309N/L311T):

DNA constructs encoding FVII-(S43N), FVII-(K62E), FVII-(Q64E), FVII-(I69F), FVII-(K62E/I69A), FVII-(Q64E/I69A), FVII-(Q62E/Q64E/I69A), FVII-(K62E/I69F), FVII-(Q64E/I69F), FVII-(K62E/Q64E/I69F), FVII-(S43C), FVII-(I69C), FVII-(Q64C), FVII-(M306C), FVII-(R277C), FVII-(I69N/F71T), FVII-(F71N/L73T), FVII-(R277N), and FVII-(D309N/L311T) were prepared by site-directed mutagenesis using a supercoiled, double stranded DNA vector with insert of human FVII and two synthetic primers containing the desired mutation. The following primers were used:

For FVII-(S43N):
5'- GGACGAAGCTGTTCTGGATTAACTACAGTGATGGGGACCAG-3' (SEQ ID NO:2)
5'- CTGGTCCCCATCACTGTAGTTAATCCAGAACAGCTTCGTCC-3'(SEQ ID NO:3)

For FVII-(K62E):

5'- GGGGGCTCCTGCGAGGACCAGCTCCAG-3'(SEQ ID NO:4)

5'- CTGGAGCTGGTCCTCGCAGGAGCCCCC-3'(SEQ ID NO:5)

For FVII-(Q64E):

5'- GGGCTCCTGCAAGGACGAGCTCCAGTCCTATATCTGC-3'(SEQ ID NO:6)

5'- GCAGATATAGGACTGGAGCTCGTCCTTGCAGGAGCCC-3'(SEQ ID NO:7)

For FVII-(I69F):

5'- CCAGCTCCAGTCCTATTTCTGCTTCTGCCTCCC-3'(SEQ ID NO:8)

5'- GGGAGGCAGAAGCAGAAATAGGACTGGAGCTGG-3'(SEQ ID NO:9)

For FVII-(K62E/I69A):

5'- GGGGGCTCCTGCGAGGACCAGCTCCAGTCCTATGCCTGCTTCTGCCTC-3'(SEQ ID NO:10)

5'- GAGGCAGAAGCAGGCATAGGACTGGAGCTGGTCCTCGCAGGAGCCCCC-3'(SEQ ID NO:11)

For FVII-(Q64E/I69A):

5'- GGGCTCCTGCAAGGACGAGCTCCAGTCCTATGCCTGCTTCTGCCTCC-3'(SEQ ID NO:12)

5'- GGAGGCAGAAGCAGGCATAGGACTGGAGCTCGTCCTTGCAGGAGCCC-3'(SEQ ID NO:13)

For FVII-(K62E/Q64E/I69A):

5'- GGGGGCTCCTGCGAGGACGAGCTCCAGTCCTATGCCTGCTTCTGCCTCC-3'(SEQ ID NO:14)

5'- GGAGGCAGAAGCAGGCATAGGACTGGAGCTCGTCCTCGCAGGAGCCCCC-3'(SEQ ID NO:15)

For FVII-(S43C):

5'-GGACGAAGCTGTTCTGGATTTGCTACAGTGATGGGGAC-3'(SEQ ID NO:16)

5'-GTCCCCATCACTGTAGCAAATCCAGAACAGCTTCGTCC-3'(SEQ ID NO:17)

For FVII-(I69C)

5'-CCAGCTCCAGTCCTATTGCTGCTTCTGCCTCCCTG-3'(SEQ ID NO:18)

5'-CAGGGAGGCAGAAGCAGCAATAGGACTGGAGCTGG-3'(SEQ ID NO:19)

For FVII-(Q64C)
5'-GGGCTCCTGCAAGGACTGCCTCCAGTCCTATATCTG-3'(SEQ ID NO:20)
5'-CAGATATAGGACTGGAGGCAGTCCTTGCAGGAGCCC-3'(SEQ ID NO:21)

For FVII-(R277C)
5'-GGACGCTGGCCTTCGTGTGCTTCTCATTGGTCAGCG-3'(SEQ ID NO:22)
5'-CGCTGACCAATGAGAAGCACACGAAGGCCAGCGTCC-3'(SEQ ID NO:23)

For FVII-(M306C)
5'-CAACGTGCCCCGGCTGTGCACCCAGGACTGCCTGC-3'(SEQ ID NO:24)
5'-GCAGGCAGTCCTGGGTGCACAGCCGGGGCACGTTG-3'(SEQ ID NO:25)

FVII-(I69N/F71T):
5'-CCAGCTCCAGTCCTATAACTGCACCTGCCTCCCTGCCTTCG-3'(SEQ ID NO:26)
5'-CGAAGGCAGGGAGGCAGGTGCAGTTATAGGACTGGAGCTGG-3'(SEQ ID NO:27)

FVII-(F71N/L73T):
5'-CCAGTCCTATATCTGCAACTGCACCCCTGCCTTCGAGGGCCG-3'(SEQ ID NO:28)
5'-CGGCCCTCGAAGGCAGGGGTGCAGTTGCAGATATAGGACTGG-3'(SEQ ID NO:29)

FVII-(R277N)
5'-GGACGCTGGCCTTCGTGAACTTCTCATTGGTCAGCGG-3'(SEQ ID NO:30)
5'-CCGCTGACCAATGAGAAGTTCACGAAGGCCAGCGTCC-3'(SEQ ID NO:31)

FVII-(D309N/L311T)
5'-CGGCTGATGACCCAGAACTGCACCCAGCAGTCACGGAAGG-3'(SEQ ID NO:32)
5'-CCTTCCGTGACTGCTGGGTGCAGTTCTGGGTCATCAGCCG-3'(SEQ ID NO:33)

The oligonucleotide primers, each complementary to opposite strands of the vector insert, were extended during temperature cycling by means of Pfu DNA polymerase. On incorporation of the primers, a mutated plasmid containing staggered nicks was generated. Following temperature cycling, the product was treated with DpnI which is specific for methylated and hemimethylated DNA to digest the parental DNA template and to select for mutation-containing synthesized DNA.

Procedures for preparing a DNA construct using polymerase chain reaction using specific primers are well known to persons skilled in the art (cf. *PCR Protocols*, 1990, Academic ress, San Diego, California, USA).

Example 2

Preparation of FVII-(S43N), FVII-(K62E), FVII-(Q64E), FVII-(I69F), FVII-(K62E/I69A), FVII-(Q64E/I69A), FVII-(Q62E/Q64E/I69A), FVII-(K62E/I69F), FVII-(Q64E/I69F), FVII-(K62E/Q64E/I69F), FVII-(S43C), FVII-(I69C), FVII-(Q64C), FVII-(M306C), FVII-(R277C), FVII-(I69N/F71T), FVII-(F71N/L73T), FVII-(R277N), and FVII-(D309N/L311T).

BHK cells were transfected essentially as previously described (Thim et al. (1988) *Biochemistry* 27, 7785-7793; Persson and Nielsen (1996) *FEBS Lett.* 385, 241-243) to obtain expression of the variant FVII polypeptide. The Factor VII polypeptide was purified as follows:

Conditioned medium was loaded onto a 50-ml column of Q Sepharose Fast Flow (Pharmacia Biotech) after addition of 5 mM EDTA, and 10 mM Tris, adjustment of pH to 8.0 and adjustment of the conductivity to 10-11 mS/cm by adding water. Elution of the protein was accomplished by a gradient from 10 mM Tris, 50 mM NaCl, pH 8.0 to 10 mM Tris, 50 mM NaCl, 25 mM $CaCl_2$, pH 8.0. The fractions containing the variant FVII polypeptide were pooled, and applied to a 25-ml column containing the monoclonal antibody F1A2 (Novo Nordisk, Bagsværd, Denmark) coupled to CNBr-activated Sepharose 4B (Pharmacia Biotech). The column was equilibrated with 50 mM Hepes, pH 7.5, containing 10 mM $CaCl_2$, and 100 mM NaCl. After washing with equilibration buffer and equilibration buffer containing 2 M NaCl, bound material was eluted with equilibration buffer containing 10 mM EDTA instead of $CaCl_2$. Before use or storage, excess $CaCl_2$ over EDTA was added or the variant FVII polypeptide was transferred to a Ca^{2+}-containing buffer. The yield of each step was followed by factor VII ELISA measurements and the purified protein was analysed by SDS-PAGE.

Example 3

***In Vitro* Hydrolysis Assay**

Native (wild-type) Factor VIIa and Factor VIIa variant (both hereafter referred to as "Factor VIIa") are assayed in parallel to directly compare their specific activities. The assay is carried out in a microtiter plate (MaxiSorp, Nunc, Denmark). The chromogenic substrate D-Ile-Pro-Arg-*p*-nitroanilide (S-2288, Chromogenix, Sweden), final concentration 1 mM, is added to Factor VIIa (final concentration 100 nM) in 50 mM Hepes, pH 7.4, containing 0.1 M NaCl, 5 mM $CaCl_2$ and 1 mg/ml bovine serum albumin. The absorbance at 405 nm is measured continuously in a SpectraMax™ 340 plate reader (Molecular Devices, USA). The absorbance developed during a 20-minute incubation, after subtraction of the absorbance in a blank well

containing no enzyme, is used to calculate the ratio between the activities of variant and wild-type Factor VIIa:

$$\text{Ratio} = (A_{405\,nm}\ \text{Factor VIIa variant})/(A_{405\,nm}\ \text{Factor VIIa wild-type}).$$

Example 4

***In Vitro* Proteolysis Assay**

Native (wild-type) Factor VIIa and Factor VIIa variant (both hereafter referred to as "Factor VIIa") are assayed in parallel to directly compare their specific activities. The assay is carried out in a microtiter plate (MaxiSorp, Nunc, Denmark). Factor VIIa (10 nM) and Factor X (0.8 microM) in 100 microL 50 mM Hepes, pH 7.4, containing 0.1 M NaCl, 5 mM $CaCl_2$ and 1 mg/ml bovine serum albumin, are incubated for 15 min. Factor X cleavage is then stopped by the addition of 50 microL 50 mM Hepes, pH 7.4, containing 0.1 M NaCl, 20 mM EDTA and 1 mg/ml bovine serum albumin. The amount of Factor Xa generated is measured by addition of the chromogenic substrate Z-D-Arg-Gly-Arg-*p*-nitroanilide (S-2765, Chromogenix, Sweden), final concentration 0.5 mM. The absorbance at 405 nm is measured continuously in a SpectraMax™ 340 plate reader (Molecular Devices, USA). The absorbance developed during 10 minutes, after subtraction of the absorbance in a blank well containing no FVIIa, is used to calculate the ratio between the proteolytic activities of variant and wild-type Factor VIIa:

$$\text{Ratio} = (A_{405\,nm}\ \text{Factor VIIa variant})/(A_{405\,nm}\ \text{Factor VIIa wild-type}).$$

Example 5

TF binding affinity assay - Biosensor assay:

FVII polypeptides are tested on the Biacore instrument by passing a standard solution of the FVII polypeptide over a chip with immobilized TF. This is followed by different concentrations of sTF in 10 mM hepes pH 7.4 containing 150 mM NaCl, 10 mM $CaCl_2$ and 0.0003 % polysorbate 20. Kd's are calculated from the sensorgrams using the integrated Biacore evaluation software.

CLAIMS

1. A Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa, wherein said Factor VII polypeptide is selected from:

i) a Factor VII polypeptide comprising one or more amino acid substitution selected from $K18X_1$, $R36X_2$, $S43X_3$, $K62X_4$, $Q64X_5$, $L65X_6$, $I69X_7$, $F71X_8$, $L73X_9$, $P74X_{10}$, $E77X_{11}$, $G78X_{12}$, $R79X_{13}$, $K85X_{14}$, $Q88X_{15}$, $N93X_{16}$, $F275X_{17}$, $R277X_{18}$, $M306X_{19}$, $T307X_{20}$, $Q308X_{21}$, $D309X_{22}$, or $R379X_{23}$ corresponding to amino acids at positions of SEQ ID NO: 1, which different amino acid decreases the Tissue Factor binding affinity, wherein

X_1 is E, D, A, or F;

X_2 is E, D, K, A, F, or N;

X_3 is E, D, K, R, A, or F;

X_4 is D, A, or F;

X_5 is E, D, K, R, A, F, or N;

X_6 is E, D, K, R, A, or F;

X_7 is C, D, E, F, G, H, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_8 is A, G, H, I, K, L, M, P, R, S, T, V, or W;

X_9 is A, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y;

X_{10} is A, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, or Y;

X_{11} is A, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_{12} is A, D, E, F, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_{13} is A, D, E, F, G, H, I, K, L, M, N, P, Q, S, T, V, W, or Y;

X_{14} is A, D, E, F, G, I, L, M, P, S, T, V, W, or Y;

X_{15} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;

X_{16} is A, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y;

X_{17} is A, D, E, G, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_{18} is A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y;

X_{19} is A, E, F, G, H, I, K, L, P, Q, R, S, T, V, W, or Y;

X_{20} is A, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, V, W, or Y;

X_{21} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;

X_{22} is A, F, G, H, I, K, L, M, N, P, Q, R, V, W, or Y; and

X_{23} is A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y;

and/or

ii) a Factor VII polypeptide, wherein one or more amino acid corresponding to amino acids at positions selected from K18, R36, K62, Q64, L65, I69, F71, L73, P74, E77, G78, R79, K85, Q88, N93, F275, R277, M306, Q308, D309, or R379 of SEQ ID NO: 1 have been substituted with a cysteine amino acid residue, which cysteine amino acid residue decreases the Tissue Factor binding affinity, and which cysteine amino acid residue is optionally conjugated with a chemical group, which chemical group increases the molecular weight of said Factor VII polypeptide;

and/or

iii) a Factor VII polypeptide comprising one or more N-glycosylation site N-Xaa-S/T introduced by amino acid substitutions corresponding to amino acids starting at positions selected from R36, Q64, I69, F71, P74, E77, G78, Q88, N93, F275, M306, T307, or D309 of SEQ ID NO: 1, wherein Xaa is any amino acid except P, which introduced N-glycosylation site decreases the Tissue Factor binding affinity.

2. The Factor VII polypeptide according to claim 1 comprising one or more amino acid substitution selected from $K18X_1$, $R36X_2$, $S43X_3$, $K62X_4$, $Q64X_5$, $L65X_6$, $I69X_7$, $F71X_8$, $L73X_9$, $P74X_{10}$, $E77X_{11}$, $G78X_{12}$, $R79X_{13}$, $K85X_{14}$, $Q88X_{15}$, $N93X_{16}$, $F275X_{17}$, $R277X_{18}$, $M306X_{19}$, $T307X_{20}$, $Q308X_{21}$, $D309X_{22}$, or $R379X_{23}$ corresponding to amino acids at positions of SEQ ID NO: 1, which different amino acid decreases the Tissue Factor binding affinity, wherein

X_1 is E, D, A, or F;

X_2 is E, D, K, A, F, or N;

X_3 is E, D, K, R, A, or F;

X_4 is D, A, or F;

X_5 is E, D, K, R, A, F, or N;

X_6 is E, D, K, R, A, or F;

X_7 is C, D, E, F, G, H, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_8 is A, G, H, I, K, L, M, P, R, S, T, V, or W;

X_9 is A, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y;

X_{10} is A, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, or Y;

X_{11} is A, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_{12} is A, D, E, F, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_{13} is A, D, E, F, G, H, I, K, L, M, N, P, Q, S, T, V, W, or Y;

X_{14} is A, D, E, F, G, I, L, M, P, S, T, V, W, or Y;

X_{15} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;

X_{16} is A, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y;

X_{17} is A, D, E, G, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;
X_{18} is A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y;
X_{19} is A, E, F, G, H, I, K, L, P, Q, R, S, T, V, W, or Y;
X_{20} is A, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, V, W, or Y;
X_{21} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;
X_{22} is A, F, G, H, I, K, L, M, N, P, Q, R, V, W, or Y; and
X_{23} is A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y.

3. The Factor VII polypeptide according to any of claim 1 or 2, wherein one or more amino acid corresponding to amino acids at positions selected from K18, R36, K62, Q64, L65, I69, F71, L73, P74, E77, G78, R79, K85, Q88, N93, F275, R277, M306, Q308, D309, or R379 of SEQ ID NO: 1 have been substituted with a cysteine amino acid residue, which cysteine amino acid residue decreases the Tissue Factor binding affinity, and which cysteine amino acid residue is optionally conjugated with a chemical group, which chemical group increases the molecular weight of said Factor VII polypeptide.

4. The Factor VII polypeptide according to any one of claims 1-3 comprising one or more N-glycosylation site N-Xaa-S/T introduced by amino acid substitutions corresponding to amino acids starting at positions selected from R36, Q64, I69, F71, P74, E77, G78, Q88, N93, F275, M306, T307, or D309 of SEQ ID NO: 1, wherein Xaa is any amino acid except P, which introduced N-glycosylation site decreases the Tissue Factor binding affinity.

5. The Factor VII polypeptide according to any one of claims 1-4, wherein the amino acid corresponding to the amino acid at a position K18 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of E, D, A, and F.

6. The Factor VII polypeptide according to any one of claims 1-5, wherein the amino acid corresponding to the amino acid at a position R36 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of E, D, K, A, F, and N.

7. The Factor VII polypeptide according to any one of claims 1-6, wherein the amino acid corresponding to the amino acid at a position S43 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of E, D, K, R, A, and F.

8. The Factor VII polypeptide according to any one of claims 1-7, wherein the amino acid corresponding to the amino acid at a position K62 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of D, A, and F.

9. The Factor VII polypeptide according to any one of claims 1-8, wherein the amino acid corresponding to the amino acid at a position Q64 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of E, D, K, R, A, F, and N.

10. The Factor VII polypeptide according to any one of claims 1-9, wherein the amino acid corresponding to the amino acid at a position L65 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of E, D, K, R, A, and F.

11. The Factor VII polypeptide according to any one of claims 1-10, wherein the amino acid corresponding to the amino acid at a position I69 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of C, D, E, F, G, H, K, L, M, N, P, Q, R, S, T, V, W, and Y.

12. The Factor VII polypeptide according to any one of claims 1-11, wherein the amino acid corresponding to the amino acid at a position F71 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, G, H, I, K, L, M, P, R, S, T, V, and W.

13. The Factor VII polypeptide according to any one of claims 1-12, wherein the amino acid corresponding to the amino acid at a position L73 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, and Y.

14. The Factor VII polypeptide according to any one of claims 1-13, wherein the amino acid corresponding to the amino acid at a position P74 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, and Y.

15. The Factor VII polypeptide according to any one of claims 1-14, wherein the amino acid corresponding to the amino acid at a position E77 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, and Y.

16. The Factor VII polypeptide according to any one of claims 1-15, wherein the amino acid corresponding to the amino acid at a position G78 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, D, E, F, H, I, K, L, M, N, P, Q, R, S, T, V, W, and Y.

17. The Factor VII polypeptide according to any one of claims 1-16, wherein the amino acid corresponding to the amino acid at a position R79 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, D, E, F, G, H, I, K, L, M, N, P, Q, S, T, V, W, and Y.

18. The Factor VII polypeptide according to any one of claims 1-17, wherein the amino acid corresponding to the amino acid at a position K85 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, D, E, F, G, I, L, M, P, S, T, V, W, and Y.

19. The Factor VII polypeptide according to any one of claims 1-18, wherein the amino acid corresponding to the amino acid at a position Q88 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, and Y.

20. The Factor VII polypeptide according to any one of claims 1-19, wherein the amino acid corresponding to the amino acid at a position N93 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, and Y.

21. The Factor VII polypeptide according to any one of claims 1-20, wherein the amino acid corresponding to the amino acid at a position F275 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, D, E, G, I, K, L, M, N, P, Q, R, S, T, V, W, and Y.

22. The Factor VII polypeptide according to any one of claims 1-21, wherein the amino acid corresponding to the amino acid at a position R277 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, and Y.

23. The Factor VII polypeptide according to any one of claims 1-22, wherein the amino acid corresponding to the amino acid at a position M306 of SEQ ID NO: 1 has been substituted

with an amino acid selected from the list consisting of A, E, F, G, H, I, K, L, P, Q, R, S, T, V, W, and Y.

24. The Factor VII polypeptide according to any one of claims 1-23, wherein the amino acid corresponding to the amino acid at a position T307 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, V, W, and Y.

25. The Factor VII polypeptide according to any one of claims 1-24, wherein the amino acid corresponding to the amino acid at a position Q308 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, and Y.

26. The Factor VII polypeptide according to any one of claims 1-25, wherein the amino acid corresponding to the amino acid at a position D309 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, F, G, H, I, K, L, M, N, P, Q, R, V, W, and Y.

27. The Factor VII polypeptide according to any one of claims 1-26, wherein the amino acid corresponding to the amino acid at a position R379 of SEQ ID NO: 1 has been substituted with an amino acid selected from the list consisting of A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y.

28. The Factor VII polypeptide according to claim 1, which is selected from the list consisting of FVII-(S43N), FVII-(K62E), FVII-(Q64E), FVII-(I69F), FVII-(K62E/I69A), FVII-(Q64E/I69A), FVII-(Q62E/Q64E/I69A), FVII-(K62E/I69F), FVII-(Q64E/I69F), FVII-(K62E/Q64E/I69F), FVII-(S43C), FVII-(I69C), FVII-(Q64C), FVII-(M306C), FVII-(R277C), FVII-(I69N/F71T), FVII-(F71N/L73T), FVII-(R277N), and FVII-(D309N/L311T).

29. The Factor VII polypeptide according to any one of claims 1-27, further comprising amino acid substitutions selected from the list consisting of L305V, L305V/M306D/D309S, L305I, L305T, F374P, V158T/M298Q, V158D/E296V/M298Q, K337A, M298Q, V158D/M298Q, L305V/K337A, V158D/E296V/M298Q/L305V, V158D/E296V/M298Q/K337A, V158D/E296V/M298Q/L305V/K337A, K157A, E296V, E296V/M298Q, V158D/E296V, V158D/M298K, and S336G, L305V/K337A, L305V/V158D, L305V/E296V, L305V/M298Q, L305V/V158T, L305V/K337A/V158T, L305V/K337A/M298Q, L305V/K337A/E296V,

L305V/K337A/V158D, L305V/V158D/M298Q, L305V/V158D/E296V, L305V/V158T/M298Q, L305V/V158T/E296V, L305V/E296V/M298Q, L305V/V158D/E296V/M298Q, L305V/V158T/E296V/M298Q, L305V/V158T/K337A/M298Q, L305V/V158T/E296V/K337A, L305V/V158D/K337A/M298Q, L305V/V158D/E296V/K337A, L305V/V158D/E296V/M298Q/K337A, L305V/V158T/E296V/M298Q/K337A, S314E/K316H, S314E/K316Q, S314E/L305V, S314E/K337A, S314E/V158D, S314E/E296V, S314E/M298Q, S314E/V158T, K316H/L305V, K316H/K337A, K316H/V158D, K316H/E296V, K316H/M298Q, K316H/V158T, K316Q/L305V, K316Q/K337A, K316Q/V158D, K316Q/E296V, K316Q/M298Q, K316Q/V158T, S314E/L305V/K337A, S314E/L305V/V158D, S314E/L305V/E296V, S314E/L305V/M298Q, S314E/L305V/V158T, S314E/L305V/K337A/V158T, S314E/L305V/K337A/M298Q, S314E/L305V/K337A/E296V, S314E/L305V/K337A/V158D, S314E/L305V/V158D/M298Q, S314E/L305V/V158D/E296V, S314E/L305V/V158T/M298Q, S314E/L305V/V158T/E296V, S314E/L305V/E296V/M298Q, S314E/L305V/V158D/E296V/M298Q, S314E/L305V/V158T/E296V/M298Q, S314E/L305V/V158T/K337A/M298Q, S314E/L305V/V158T/E296V/K337A, S314E/L305V/V158D/K337A/M298Q, S314E/L305V/V158D/E296V/K337A, S314E/L305V/V158D/E296V/M298Q/K337A, S314E/L305V/V158T/E296V/M298Q/K337A, K316H/L305V/K337A, K316H/L305V/V158D, K316H/L305V/E296V, K316H/L305V/M298Q, K316H/L305V/V158T, K316H/L305V/K337A/V158T, K316H/L305V/K337A/M298Q, K316H/L305V/K337A/E296V, K316H/L305V/K337A/V158D, K316H/L305V/V158D/M298Q, K316H/L305V/V158D/E296V, K316H/L305V/V158T/M298Q, K316H/L305V/V158T/E296V, K316H/L305V/E296V/M298Q, K316H/L305V/V158D/E296V/M298Q, K316H/L305V/V158T/E296V/M298Q, K316H/L305V/V158T/K337A/M298Q, K316H/L305V/V158T/E296V/K337A, K316H/L305V/V158D/K337A/M298Q, K316H/L305V/V158D/E296V/K337A , K316H/L305V/V158D/E296V/M298Q/K337A, K316H/L305V/V158T/E296V/M298Q/K337A, K316Q/L305V/K337A, K316Q/L305V/V158D, K316Q/L305V/E296V, K316Q/L305V/M298Q, K316Q/L305V/V158T, K316Q/L305V/K337A/V158T, K316Q/L305V/K337A/M298Q, K316Q/L305V/K337A/E296V, K316Q/L305V/K337A/V158D, K316Q/L305V/V158D/M298Q, K316Q/L305V/V158D/E296V, K316Q/L305V/V158T/M298Q, K316Q/L305V/V158T/E296V, K316Q/L305V/E296V/M298Q, K316Q/L305V/V158D/E296V/M298Q, K316Q/L305V/V158T/E296V/M298Q, K316Q/L305V/V158T/K337A/M298Q, K316Q/L305V/V158T/E296V/K337A, K316Q/L305V/V158D/K337A/M298Q, K316Q/L305V/V158D/E296V/K337A , K316Q/L305V/V158D/E296V/M298Q/K337A, K316Q/L305V/V158T/E296V/M298Q/K337A, F374Y/K337A, F374Y/V158D, F374Y/E296V, F374Y/M298Q, F374Y/V158T, F374Y/S314E, F374Y/L305V, F374Y/L305V/K337A, F374Y/L305V/V158D, F374Y/L305V/E296V,

F374Y/L305V/M298Q, F374Y/L305V/V158T, F374Y/L305V/S314E, F374Y/K337A/S314E, F374Y/K337A/V158T, F374Y/K337A/M298Q, F374Y/K337A/E296V, F374Y/K337A/V158D, F374Y/V158D/S314E, F374Y/V158D/M298Q, F374Y/V158D/E296V, F374Y/V158T/S314E, F374Y/V158T/M298Q, F374Y/V158T/E296V, F374Y/E296V/S314E, F374Y/S314E/M298Q, F374Y/E296V/M298Q, F374Y/L305V/K337A/V158D, F374Y/L305V/K337A/E296V, F374Y/L305V/K337A/M298Q, F374Y/L305V/K337A/V158T, F374Y/L305V/K337A/S314E, F374Y/L305V/V158D/E296V, F374Y/L305V/V158D/M298Q, F374Y/L305V/V158D/S314E, F374Y/L305V/E296V/M298Q, F374Y/L305V/E296V/V158T, F374Y/L305V/E296V/S314E, F374Y/L305V/M298Q/V158T, F374Y/L305V/M298Q/S314E, F374Y/L305V/V158T/S314E, F374Y/K337A/S314E/V158T, F374Y/K337A/S314E/M298Q, F374Y/K337A/S314E/E296V, F374Y/K337A/S314E/V158D, F374Y/K337A/V158T/M298Q, F374Y/K337A/V158T/E296V, F374Y/K337A/M298Q/E296V, F374Y/K337A/M298Q/V158D, F374Y/K337A/E296V/V158D, F374Y/V158D/S314E/M298Q, F374Y/V158D/S314E/E296V, F374Y/V158D/M298Q/E296V, F374Y/V158T/S314E/E296V, F374Y/V158T/S314E/M298Q, F374Y/V158T/M298Q/E296V, F374Y/E296V/S314E/M298Q, F374Y/L305V/M298Q/K337A/S314E, F374Y/L305V/E296V/K337A/S314E, F374Y/E296V/M298Q/K337A/S314E, F374Y/L305V/E296V/M298Q/K337A , F374Y/L305V/E296V/M298Q/S314E, F374Y/V158D/E296V/M298Q/K337A, F374Y/V158D/E296V/M298Q/S314E, F374Y/L305V/V158D/K337A/S314E, F374Y/V158D/M298Q/K337A/S314E, F374Y/V158D/E296V/K337A/S314E, F374Y/L305V/V158D/E296V/M298Q, F374Y/L305V/V158D/M298Q/K337A, F374Y/L305V/V158D/E296V/K337A, F374Y/L305V/V158D/M298Q/S314E, F374Y/L305V/V158D/E296V/S314E, F374Y/V158T/E296V/M298Q/K337A, F374Y/V158T/E296V/M298Q/S314E, F374Y/L305V/V158T/K337A/S314E, F374Y/V158T/M298Q/K337A/S314E, F374Y/V158T/E296V/K337A/S314E, F374Y/L305V/V158T/E296V/M298Q, F374Y/L305V/V158T/M298Q/K337A, F374Y/L305V/V158T/E296V/K337A, F374Y/L305V/V158T/M298Q/S314E, F374Y/L305V/V158T/E296V/S314E, F374Y/E296V/M298Q/K337A/V158T/S314E, F374Y/V158D/E296V/M298Q/K337A/S314E, F374Y/L305V/V158D/E296V/M298Q/S314E, F374Y/L305V/E296V/M298Q/V158T/S314E, F374Y/L305V/E296V/M298Q/K337A/V158T, F374Y/L305V/E296V/K337A/V158T/S314E, F374Y/L305V/M298Q/K337A/V158T/S314E, F374Y/L305V/V158D/E296V/M298Q/K337A, F374Y/L305V/V158D/E296V/K337A/S314E, F374Y/L305V/V158D/M298Q/K337A/S314E, F374Y/L305V/E296V/M298Q/K337A/V158T/S314E, F374Y/L305V/V158D/E296V/M298Q/K337A/S314E, S52A, S60A; R152E, S344A, P11Q/K33E, T106N, K143N/N145T, V253N, R290N/A292T, G291N, R315N/V317T, and K143N/N145T/R315N/V317T; or having substitutions, additions or deletions in the amino

acid sequence from 233Thr to 240Asn, or having substitutions, additions or deletions in the amino acid sequence from 304Arg to 329Cys.

30. The Factor VII polypeptide according to any one of claims 1-29, wherein the dissociation constant K_d of said Factor VII polypeptide is higher than 5 nM, such as higher than 7 nM, such as higher than 10 nM, such as higher than 20 nM, such as higher than 30 nM, such as higher than 50 nM, such as higher than 100 nM, such as higher than 200 nM, such as higher than 300 nM, such as higher than 400 nM, such as higher than 500 nM, such as higher than 1 μM.

31. A composition comprising a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa, wherein said Factor VII polypeptide is selected from:

i) a Factor VII polypeptide comprising one or more amino acid substitution selected from $K18X_1$, $R36X_2$, $S43X_3$, $K62X_4$, $Q64X_5$, $L65X_6$, $I69X_7$, $F71X_8$, $L73X_9$, $P74X_{10}$, $E77X_{11}$, $G78X_{12}$, $R79X_{13}$, $K85X_{14}$, $Q88X_{15}$, $N93X_{16}$, $F275X_{17}$, $R277X_{18}$, $M306X_{19}$, $T307X_{20}$, $Q308X_{21}$, $D309X_{22}$, or $R379X_{23}$ corresponding to amino acids at positions of SEQ ID NO: 1, which different amino acid decreases the Tissue Factor binding affinity, wherein

X_1 is E, D, A, or F;

X_2 is E, D, K, A, F, or N;

X_3 is E, D, K, R, A, or F;

X_4 is D, A, or F;

X_5 is E, D, K, R, A, F, or N;

X_6 is E, D, K, R, A, or F;

X_7 is C, D, E, F, G, H, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_8 is A, G, H, I, K, L, M, P, R, S, T, V, or W;

X_9 is A, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y;

X_{10} is A, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, or Y;

X_{11} is A, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_{12} is A, D, E, F, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_{13} is A, D, E, F, G, H, I, K, L, M, N, P, Q, S, T, V, W, or Y;

X_{14} is A, D, E, F, G, I, L, M, P, S, T, V, W, or Y;

X_{15} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;

X_{16} is A, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y;

X_{17} is A, D, E, G, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;
X_{18} is A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y;
X_{19} is A, E, F, G, H, I, K, L, P, Q, R, S, T, V, W, or Y;
X_{20} is A, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, V, W, or Y;
X_{21} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;
X_{22} is A, F, G, H, I, K, L, M, N, P, Q, R, V, W, or Y; and
X_{23} is A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y;

and/or

ii) a Factor VII polypeptide, wherein one or more amino acid corresponding to amino acids at positions selected from K18, R36, K62, Q64, L65, I69, F71, L73, P74, E77, G78, R79, K85, Q88, N93, F275, R277, M306, Q308, D309, or R379 of SEQ ID NO: 1 have been substituted with a cysteine amino acid residue, which cysteine amino acid residue decreases the Tissue Factor binding affinity, and which cysteine amino acid residue is optionally conjugated with a chemical group, which chemical group increases the molecular weight of said Factor VII polypeptide;

and/or

iii) a Factor VII polypeptide comprising one or more N-glycosylation site N-Xaa-S/T introduced by amino acid substitutions corresponding to amino acids starting at positions selected from R36, Q64, I69, F71, P74, E77, G78, Q88, N93, F275, M306, T307, or D309 of SEQ ID NO: 1, wherein Xaa is any amino acid except P, which introduced N-glycosylation site decreases the Tissue Factor binding affinity.

32. A composition comprising a Factor VII polypeptide according to any one of the claims 1-30.

33. A pharmaceutical composition comprising a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa, wherein said Factor VII polypeptide is selected from:

i) a Factor VII polypeptide comprising one or more amino acid substitution selected from $K18X_1$, $R36X_2$, $S43X_3$, $K62X_4$, $Q64X_5$, $L65X_6$, $I69X_7$, $F71X_8$, $L73X_9$, $P74X_{10}$, $E77X_{11}$, $G78X_{12}$, $R79X_{13}$, $K85X_{14}$, $Q88X_{15}$, $N93X_{16}$, $F275X_{17}$, $R277X_{18}$, $M306X_{19}$, $T307X_{20}$, $Q308X_{21}$, $D309X_{22}$, or $R379X_{23}$ corresponding to amino acids at positions of SEQ ID NO: 1, which different amino acid decreases the Tissue Factor binding affinity, wherein

X_1 is E, D, A, or F;

X_2 is E, D, K, A, F, or N;

X_3 is E, D, K, R, A, or F;

X_4 is D, A, or F;

X_5 is E, D, K, R, A, F, or N;

X_6 is E, D, K, R, A, or F;

X_7 is C, D, E, F, G, H, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_8 is A, G, H, I, K, L, M, P, R, S, T, V, or W;

X_9 is A, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y;

X_{10} is A, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, or Y;

X_{11} is A, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_{12} is A, D, E, F, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_{13} is A, D, E, F, G, H, I, K, L, M, N, P, Q, S, T, V, W, or Y;

X_{14} is A, D, E, F, G, I, L, M, P, S, T, V, W, or Y;

X_{15} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;

X_{16} is A, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y;

X_{17} is A, D, E, G, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_{18} is A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y;

X_{19} is A, E, F, G, H, I, K, L, P, Q, R, S, T, V, W, or Y;

X_{20} is A, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, V, W, or Y;

X_{21} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;

X_{22} is A, F, G, H, I, K, L, M, N, P, Q, R, V, W, or Y; and

X_{23} is A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y;

and/or

ii) a Factor VII polypeptide, wherein one or more amino acid corresponding to amino acids at positions selected from K18, R36, K62, Q64, L65, I69, F71, L73, P74, E77, G78, R79, K85, Q88, N93, F275, R277, M306, Q308, D309, or R379 of SEQ ID NO: 1 have been substituted with a cysteine amino acid residue, which cysteine amino acid residue decreases the Tissue Factor binding affinity, and which cysteine amino acid residue is optionally conjugated with a chemical group, which chemical group increases the molecular weight of said Factor VII polypeptide;

and/or

iii) a Factor VII polypeptide comprising one or more N-glycosylation site N-Xaa-S/T introduced by amino acid substitutions corresponding to amino acids starting at positions selected from R36, Q64, I69, F71, P74, E77, G78, Q88, N93, F275, M306,

T307, or D309 of SEQ ID NO: 1, wherein Xaa is any amino acid except P, which introduced N-glycosylation site decreases the Tissue Factor binding affinity; and optionally, a pharmaceutically acceptable carrier.

34. A pharmaceutical composition comprising a Factor VII polypeptide according to any one of the claims 1-30; and optionally, a pharmaceutically acceptable carrier.

35. A polynucleotide construct encoding a Factor VII polypeptide according to any one of the claims 1-30.

36. The polynucleotide construct according to claim 35, which is a vector.

37. A eukaryotic host cell comprising the polynucleotide construct according to any one of the claims 35-36.

38. The eukaryotic host cell according to claim 37, which is of mammalian origin.

39. The eucaryotic host cell according to claim 38, wherein said cell is selected from the group consisting of CHO cells, BHK cells or HEK cells.

40. A method for producing the Factor VII polypeptide according to any one of the claims 1-30, the method comprising cultivating a eukaryotic host cell according to any one of the claims 37-39 in an appropriate growth medium under conditions allowing protein synthesis from said polynucleotide construct and recovering said Factor VII polypeptide from the culture medium.

41. Use of a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa, wherein said Factor VII polypeptide is selected from:

i) a Factor VII polypeptide comprising one or more amino acid substitution selected from $K18X_1$, $R36X_2$, $S43X_3$, $K62X_4$, $Q64X_5$, $L65X_6$, $I69X_7$, $F71X_8$, $L73X_9$, $P74X_{10}$, $E77X_{11}$, $G78X_{12}$, $R79X_{13}$, $K85X_{14}$, $Q88X_{15}$, $N93X_{16}$, $F275X_{17}$, $R277X_{18}$, $M306X_{19}$, $T307X_{20}$, $Q308X_{21}$, $D309X_{22}$, or $R379X_{23}$ corresponding to amino acids at positions of SEQ ID NO: 1, which different amino acid decreases the Tissue Factor binding affinity, wherein

X_1 is E, D, A, or F;

X_2 is E, D, K, A, F, or N;

X_3 is E, D, K, R, A, or F;

X_4 is D, A, or F;

X_5 is E, D, K, R, A, F, or N;

X_6 is E, D, K, R, A, or F;

X_7 is C, D, E, F, G, H, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_8 is A, G, H, I, K, L, M, P, R, S, T, V, or W;

X_9 is A, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y;

X_{10} is A, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, or Y;

X_{11} is A, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_{12} is A, D, E, F, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_{13} is A, D, E, F, G, H, I, K, L, M, N, P, Q, S, T, V, W, or Y;

X_{14} is A, D, E, F, G, I, L, M, P, S, T, V, W, or Y;

X_{15} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;

X_{16} is A, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y;

X_{17} is A, D, E, G, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_{18} is A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y;

X_{19} is A, E, F, G, H, I, K, L, P, Q, R, S, T, V, W, or Y;

X_{20} is A, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, V, W, or Y;

X_{21} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;

X_{22} is A, F, G, H, I, K, L, M, N, P, Q, R, V, W, or Y; and

X_{23} is A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y;

and/or

ii) a Factor VII polypeptide, wherein one or more amino acid corresponding to amino acids at positions selected from K18, R36, K62, Q64, L65, I69, F71, L73, P74, E77, G78, R79, K85, Q88, N93, F275, R277, M306, Q308, D309, or R379 of SEQ ID NO: 1 have been substituted with a cysteine amino acid residue, which cysteine amino acid residue decreases the Tissue Factor binding affinity, and which cysteine amino acid residue is optionally conjugated with a chemical group, which chemical group increases the molecular weight of said Factor VII polypeptide;

and/or

iii) a Factor VII polypeptide comprising one or more N-glycosylation site N-Xaa-S/T introduced by amino acid substitutions corresponding to amino acids starting at positions selected from R36, Q64, I69, F71, P74, E77, G78, Q88, N93, F275, M306,

T307, or D309 of SEQ ID NO: 1, wherein Xaa is any amino acid except P, which introduced N-glycosylation site decreases the Tissue Factor binding affinity;

for the preparation of a medicament for the treatment of bleeding episodes or for the enhancement of the normal haemostatic system.

42. Use of a Factor VII polypeptide according to any one of the claims 1-30 for the preparation of a medicament for the treatment of bleeding episodes or for the enhancement of the normal haemostatic system.

43. A method for the treatment of bleeding episodes or bleeding disorders in a subject or for the enhancement of the normal haemostatic system, the method comprising administering to a subject in need thereof a therapeutically or prophylactically effective amount of a Factor VII polypeptide with a Tissue Factor binding affinity lower than recombinant wild type human Factor VIIa and substantially the same activity or increased activity compared to recombinant wild type human Factor VIIa, wherein said Factor VII polypeptide is selected from:

i) a Factor VII polypeptide comprising one or more amino acid substitution selected from $K18X_1$, $R36X_2$, $S43X_3$, $K62X_4$, $Q64X_5$, $L65X_6$, $I69X_7$, $F71X_8$, $L73X_9$, $P74X_{10}$, $E77X_{11}$, $G78X_{12}$, $R79X_{13}$, $K85X_{14}$, $Q88X_{15}$, $N93X_{16}$, $F275X_{17}$, $R277X_{18}$, $M306X_{19}$, $T307X_{20}$, $Q308X_{21}$, $D309X_{22}$, or $R379X_{23}$ corresponding to amino acids at positions of SEQ ID NO: 1, which different amino acid decreases the Tissue Factor binding affinity, wherein

X_1 is E, D, A, or F;

X_2 is E, D, K, A, F, or N;

X_3 is E, D, K, R, A, or F;

X_4 is D, A, or F;

X_5 is E, D, K, R, A, F, or N;

X_6 is E, D, K, R, A, or F;

X_7 is C, D, E, F, G, H, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_8 is A, G, H, I, K, L, M, P, R, S, T, V, or W;

X_9 is A, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y;

X_{10} is A, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, or Y;

X_{11} is A, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_{12} is A, D, E, F, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y;

X_{13} is A, D, E, F, G, H, I, K, L, M, N, P, Q, S, T, V, W, or Y;

X_{14} is A, D, E, F, G, I, L, M, P, S, T, V, W, or Y;

X_{15} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;

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X_{20} is A, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, V, W, or Y;
X_{21} is A, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y;
X_{22} is A, F, G, H, I, K, L, M, N, P, Q, R, V, W, or Y; and
X_{23} is A, D, E, F, G, H, I, K, L, M, P, Q, S, T, V, W, or Y;

and/or

ii) a Factor VII polypeptide, wherein one or more amino acid corresponding to amino acids at positions selected from K18, R36, K62, Q64, L65, I69, F71, L73, P74, E77, G78, R79, K85, Q88, N93, F275, R277, M306, Q308, D309, or R379 of SEQ ID NO: 1 have been substituted with a cysteine amino acid residue, which cysteine amino acid residue decreases the Tissue Factor binding affinity, and which cysteine amino acid residue is optionally conjugated with a chemical group, which chemical group increases the molecular weight of said Factor VII polypeptide;

and/or

iii) a Factor VII polypeptide comprising one or more N-glycosylation site N-Xaa-S/T introduced by amino acid substitutions corresponding to amino acids starting at positions selected from R36, Q64, I69, F71, P74, E77, G78, Q88, N93, F275, M306, T307, or D309 of SEQ ID NO: 1, wherein Xaa is any amino acid except P, which introduced N-glycosylation site decreases the Tissue Factor binding affinity.

44. A method for the treatment of bleeding episodes or bleeding disorders in a subject or for the enhancement of the normal haemostatic system, the method comprising administering to a subject in need thereof a therapeutically or prophylactically effective amount of a Factor VII polypeptide according to any one of the claims 1-30.

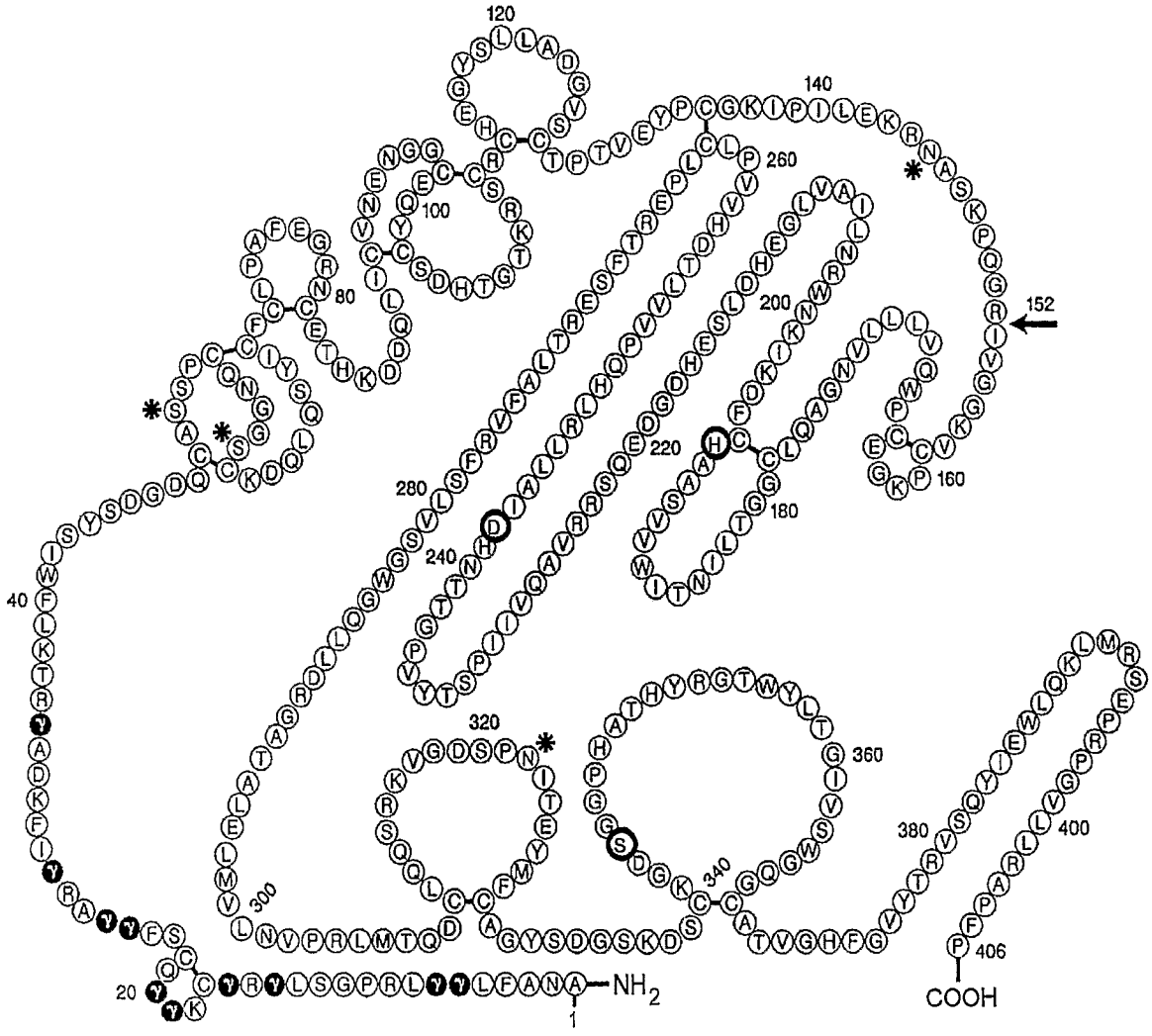

120
140
260
100
80
152
200
220
280
240
160
180
40
320
360
300
380
400
20
1
406
NH2
COOH

Fig. 1

2/3

SEQ ID NO: 1 (The amino acid sequence of native human coagulation Factor VII):

```
Ala-Asn-Ala-Phe-Leu-GLA-GLA-Leu-Arg-Pro-Gly-Ser-Leu-GLA-Arg-GLA-Cys-Lys-
                  5                  10                  15

GLA-GLA-Gln-Cys-Ser-Phe-GLA-GLA-Ala-Arg-GLA-Ile-Phe-Lys-Asp-Ala-GLA-Arg-
     20                  25                  30                  35

Thr-Lys-Leu-Phe-Trp-Ile-Ser-Tyr-Ser-Asp-Gly-Asp-Gln-Cys-Ala-Ser-Ser-Pro-
             40                  45                  50

Cys-Gln-Asn-Gly-Gly-Ser-Cys-Lys-Asp-Gln-Leu-Gln-Ser-Tyr-Ile-Cys-Phe-Cys-
 55                  60                  65                  70

Leu-Pro-Ala-Phe-Glu-Gly-Arg-Asn-Cys-Glu-Thr-His-Lys-Asp-Asp-Gln-Leu-Ile-
         75                  80                  85                  90

Cys-Val-Asn-Glu-Asn-Gly-Gly-Cys-Glu-Gln-Tyr-Cys-Ser-Asp-His-Thr-Gly-Thr-
                 95                 100                 105

Lys-Arg-Ser-Cys-Arg-Cys-His-Glu-Gly-Tyr-Ser-Leu-Leu-Ala-Asp-Gly-Val-Ser-
    110                 115                 120                 125

Cys-Thr-Pro-Thr-Val-Glu-Tyr-Pro-Cys-Gly-Lys-Ile-Pro-Ile-Leu-Glu-Lys-Arg-
            130                 135                 140

Asn-Ala-Ser-Lys-Pro-Gln-Gly-Arg-Ile-Val-Gly-Gly-Lys-Val-Cys-Pro-Lys-Gly-
145                 150                 155                 160

Glu-Cys-Pro-Trp-Gln-Val-Leu-Leu-Leu-Val-Asn-Gly-Ala-Gln-Leu-Cys-Gly-Gly-
        165                 170                 175                 180

Thr-Leu-Ile-Asn-Thr-Ile-Trp-Val-Val-Ser-Ala-Ala-His-Cys-Phe-Asp-Lys-Ile-
                185                 190                 195

Lys-Asn-Trp-Arg-Asn-Leu-Ile-Ala-Val-Leu-Gly-Glu-His-Asp-Leu-Ser-Glu-His-
    200                 205                 210                 215
```

Fig. 2

```
Asp-Gly-Asp-Glu-Gln-Ser-Arg-Arg-Val-Ala-Gln-Val-Ile-Ile-Pro-Ser-Thr-Tyr-
            220                 225                 230

Val-Pro-Gly-Thr-Thr-Asn-His-Asp-Ile-Ala-Leu-Leu-Arg-Leu-His-Gln-Pro-Val-
235                 240                 245                 250

Val-Leu-Thr-Asp-His-Val-Val-Pro-Leu-Cys-Leu-Pro-Glu-Arg-Thr-Phe-Ser-Glu-
        255                 260                 265                 270

Arg-Thr-Leu-Ala-Phe-Val-Arg-Phe-Ser-Leu-Val-Ser-Gly-Trp-Gly-Gln-Leu-Leu-
                275                 280                 285

Asp-Arg-Gly-Ala-Thr-Ala-Leu-Glu-Leu-Met-Val-Leu-Asn-Val-Pro-Arg-Leu-Met-
    290                 295                 300                 305

Thr-Gln-Asp-Cys-Leu-Gln-Gln-Ser-Arg-Lys-Val-Gly-Asp-Ser-Pro-Asn-Ile-Thr-
            310                 315                 320

Glu-Tyr-Met-Phe-Cys-Ala-Gly-Tyr-Ser-Asp-Gly-Ser-Lys-Asp-Ser-Cys-Lys-Gly-
325                 330                 335                 340

Asp-Ser-Gly-Gly-Pro-His-Ala-Thr-His-Tyr-Arg-Gly-Thr-Trp-Tyr-Leu-Thr-Gly-
        345                 350                 355                 360

Ile-Val-Ser-Trp-Gly-Gln-Gly-Cys-Ala-Thr-Val-Gly-His-Phe-Gly-Val-Tyr-Thr-
                365                 370                 375

Arg-Val-Ser-Gln-Tyr-Ile-Glu-Trp-Leu-Gln-Lys-Leu-Met-Arg-Ser-Glu-Pro-Arg-
    380                 385                 390                 395

Pro-Gly-Val-Leu-Leu-Arg-Ala-Pro-Phe-Pro
            400                 405 406
```

Fig. 2

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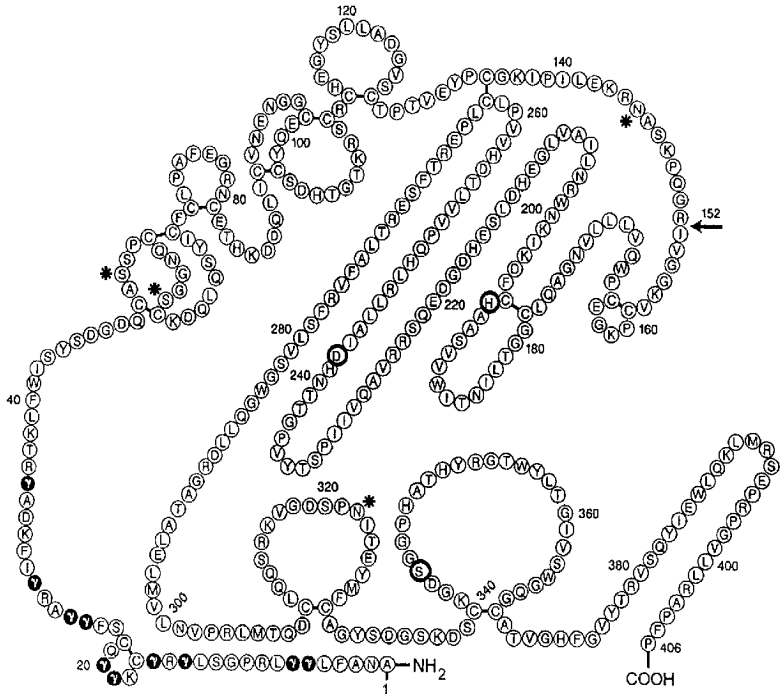
(74) Agent: **NOVO NORDISK A/S**; Corporate Patents, Novo Allé, DK-2880 Bagsværd (DK).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

[Continued on next page]

(54) Title: COAGULATION FACTOR VII POLYPEPTIDES



(57) Abstract: The present invention relates to novel coagulation Factor VII polypeptides, polynucleotide constructs encoding such polypeptides, as well as vectors and host cells comprising and expressing the polynucleotide, pharmaceutical compositions, uses and methods of treatment.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK2004/000594

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N9/64

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PERSSON EGON ET AL: "Substitution of aspartic acid for methionine-306 in factor VIIa abolishes the allosteric linkage between the active site and the binding interface with tissue factor" BIOCHEMISTRY, vol. 40, no. 11, 20 March 2001 (2001-03-20), pages 3251-3256, XP002311730 ISSN: 0006-2960 the whole document	

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 December 2004	14. 03. 2005

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Smalt, R

INTERNATIONAL SEARCH REPORT

Intern Application No
PCT/DK2004/000594

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DICKINSON C D ET AL: "Identification of surface residues mediating tissue factor binding and catalytic function of the serine protease factor VIIa" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, US, vol. 93, December 1996 (1996-12), pages 14379-14384, XP002267001 ISSN: 0027-8424 the whole document	

A	O'BRIEN D P ET AL: "Surface plasmon resonance studies of the interaction between factor VII and tissue factor. Demonstration of defective tissue factor binding in a variant FVII molecule (FVII-R79Q)" BIOCHEMISTRY, vol. 33, no. 47, 1994, pages 14162-14169, XP002311731 ISSN: 0006-2960 the whole document	

A	SRIDHARA S ET AL: "Activation of a recombinant human factor VII structural analogue alters its affinity of binding to tissue factor" AMERICAN JOURNAL OF HEMATOLOGY, NEW YORK, NY, US, vol. 53, 1996, pages 66-71, XP002273443 the whole document	

A	WO 02/38162 A (PETROVAN RAMONA J ; SCRIPPS RESEARCH INST (US); RUF WOLFRAM (US)) 16 May 2002 (2002-05-16) the whole document	

A	WO 01/58935 A (MAXYGEN APS) 16 August 2001 (2001-08-16) claims 6-8	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK2004/000594

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

5 (completely); 1-3, 30-44 (partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

Invention 1: claim 5 completely, and 1-3,30-44 partially

Factor VII with amino acid residue K18 substituted by E, D, A, or F, compositions, vectors, hosts, methods for producing, and use in manufacture of a medicament.

Inventions 2-23: claims 1-44, as far as applicable

Subject-matter essentially as defined above for invention 1, but relating instead to substitutions of residues R36, S43, K62, Q64, L65, I69, F71, L73, P74, E77, G78, R79, K85, Q88, N93, F275, R277, M306, T307, Q308, D309, or R379 (in that order, so invention 2 relates to R36, etc., and invention 23 to R379).

INTERNATIONAL SEARCH REPORT

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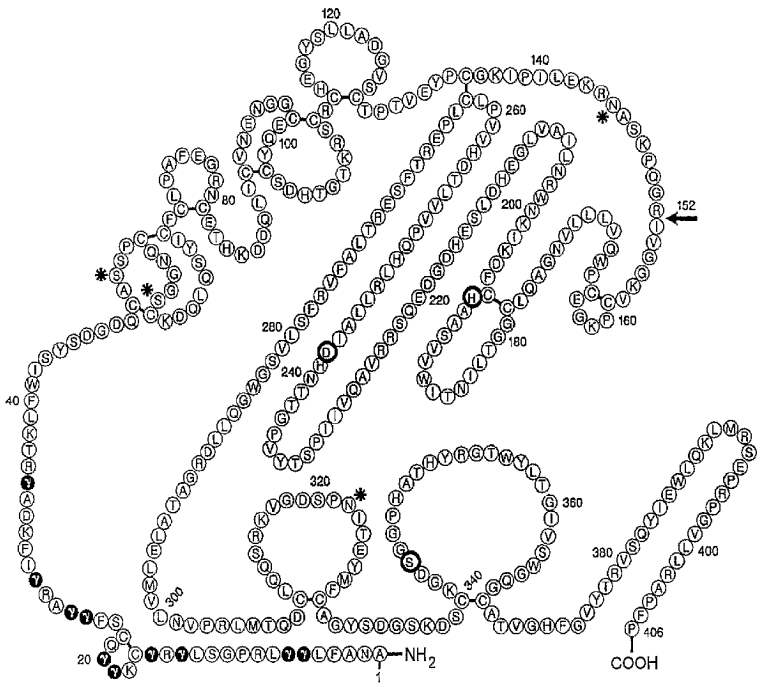
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(54) Title: COAGULATION FACTOR VII POLYPEPTIDES



(57) Abstract: The present invention relates to novel coagulation Factor VII polypeptides, polynucleotide constructs encoding such polypeptides, as well as vectors and host cells comprising and expressing the polynucleotide, pharmaceutical compositions, uses and methods of treatment.

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(54) Title: NOVEL COMPOUNDS

(57) Abstract: The present invention relates to novel coagulation factor FVIIa polypeptides.

NOVEL COMPOUNDS

FIELD OF THE INVENTION

The present invention relates to novel human coagulation Factor VII/VIIa proteins having coagulant potential/activity as well as pharmaceutical compositions comprising the polypeptides, uses and methods of treatment.

BACKGROUND OF THE INVENTION

Blood coagulation is a process consisting of a complex interaction of various blood components (or factors) that eventually gives rise to a fibrin clot. Generally, the blood components, which participate in what has been referred to as the coagulation "cascade", are enzymatically inactive proteins (proenzymes or zymogens) that are converted to proteolytic enzymes by the action of an activator (which itself is an activated clotting factor). Coagulation factors that have undergone such a conversion are generally referred to as "active factors", and are designated by the addition of the letter "a" to the name of the coagulation factor (e.g. Factor VIIa).

Initiation of the haemostatic process is mediated by the formation of a complex between tissue factor, exposed as a result of injury to the vessel wall, and Factor VIIa. This complex then converts Factors IX and X to their active forms. Factor Xa converts limited amounts of prothrombin to thrombin on the tissue factor-bearing cell. Thrombin activates platelets and Factors V and VIII into Factors Va and VIIIa, both cofactors in the further process leading to the full thrombin burst. This process includes generation of Factor Xa by Factor IXa (in complex with factor VIIIa) and occurs on the surface of activated platelets. Thrombin finally converts fibrinogen to fibrin resulting in formation of a fibrin clot. In recent years Factor VII and tissue factor have been found to be the main initiators of blood coagulation.

Factor VII is a trace plasma glycoprotein that circulates in blood as a single-chain zymogen. The zymogen is catalytically inactive. Single-chain Factor VII may be converted to two-chain Factor VIIa by Factor Xa, Factor XIIa, Factor IXa, Factor VIIa or thrombin in vitro. Factor Xa is believed to be the major physiological activator of Factor VII. Like several other plasma proteins involved in haemostasis, wild type Factor VII is, like a number of other coagulation proteins, dependent on Vitamin K for its activity, which is required for the gamma-carboxylation of multiple glutamic acid residues that are clustered close to the amino terminus of the protein. These gamma-carboxylated glutamic acids are required for the calcium ion-induced interaction of wild type Factor VII with phospholipids. The conversion of zymogen Factor VII into the activated two-chain molecule occurs by cleavage of an internal Arg152-Ile153 peptide bond.

In the presence of tissue factor, phospholipids and calcium ions, the two-chain Factor VIIa rapidly activates Factor X or Factor IX by limited proteolysis.

Thus, wild type Factor VII has a domain structure comprising a domain rich in gamma-carboxyglutamic acid residues (the "GLA domain"), a region containing sequences homologous to human epidermal growth factor, and a catalytic domain containing a serine protease catalytic triad. The catalytic domain is glycosylated in nature.

It is often desirable to stimulate or improve the coagulation cascade in a subject. Factor VIIa has been used to control bleeding disorders that have several causes such as clotting factor deficiencies (e.g. haemophilia A and B or deficiency of coagulation Factors XI or VII) or clotting factor inhibitors. Factor VIIa has also been used to control excessive bleeding occurring in subjects with a normally functioning blood clotting cascade (no clotting factor deficiencies or inhibitors against any of the coagulation factors). Such bleeding may, for example, be caused by a defective platelet function, thrombocytopenia or von Willebrand's disease. Bleeding is also a major problem in connection with surgery and other forms of tissue damage.

FVII can be prepared recombinantly, but the primary structure of wild type Factor VII renders production of the functional protein in prokaryotic host cells impossible, since bacteria do not have the capacity to introduce the vitamin K-dependent gamma-carboxylation essential to membrane binding of the protein. Traditionally, production of wild type FVII is restricted to expression in higher, mammalian cells. However, expression in mammalian cells is much more complicated and time-consuming than expression in prokaryotes, and the yields are as a rule more limited; in general production in mammalian cells is therefore more expensive than production using prokaryotic host cells.

Wild type FVIIa, in contrast to other, homologous serine proteases, possesses an active conformation that is energetically unfavourable. The consequence is a far from optimal enzymatic activity of free wild type human Factor VIIa, which is dramatically enhanced upon binding to the cognate, membrane-bound cofactor tissue factor (TF). In the natural environment, the zymogenicity of free wild type human Factor VIIa ensures timely triggering and appropriate location of FVIIa haemostatic activity upon vascular lesion and concomitant TF exposure.

European Patent No. 200,421 (ZymoGenetics) relates to the nucleotide sequence encoding human Factor VII and the recombinant expression of Factor VII in mammalian cells. Dickinson et al. (Proc. Natl. Acad. Sci. USA (1996) 93, 14379-14384) relates to a Factor VII variant wherein Leu305 has been replaced by Ala (FVII(Ala305)).

Iwanaga et al. (Thromb. Haemost. (supplement August 1999), 466, abstract 1474) relates to Factor VIIa variants wherein residues 316-320 are deleted or residues 311-322 are replaced with the corresponding residues from trypsin. Sakai et al. (J. Biol. Chem.

1990;265:1890-1894) and Nicolaisen et al. (FEBS Lett. 1992;306:157-160) relates to the GLA domain of FVIIa.

Published international patent applications WO 01/83725, WO 02/22776, WO 03/027147, WO 03/037932, and WO 04/029090 all relate to variants of Factor VIIa with preserved or increased activity. WO 02/077218 relates to derivatives of Factor VIIa with prolonged serum half-life.

SUMMARY OF THE INVENTION

It is an object of the invention to provide for less expensive, easier to produce, proteins having substantially the same or increased proteolytic activity compared to recombinant wild type human Factor VIIa.

The present invention relates in a broad aspect to a FVII polypeptide with substantially the same or increased proteolytic activity compared to recombinant wild type human Factor VIIa, wherein the FVII polypeptide is essentially free of a functional lipid membrane binding domain.

In a first aspect the present invention relates to a FVII polypeptide comprising one or more amino acid substitutions relative to the amino acid sequence of SEQ ID NO:1, wherein the amino acid substitutions are replacement with any other amino acid of one or more amino acids selected from the group consisting of K157, V158, E296, M298, L305, M306, D309, S314, D334, S336, K337, F374, and wherein the FVII polypeptide is essentially free of GLA residues.

In a second aspect the present invention relates to a polynucleotide construct encoding a Factor VII polypeptide comprising one or more amino acid substitutions relative to the amino acid sequence of SEQ ID NO:1, wherein the amino acid substitutions are replacement with any other amino acid of one or more amino acids selected from the group consisting of K157, V158, E296, M298, L305, M306, D309, S314, D334, S336, K337, F374, and wherein the FVII polypeptide is essentially free of GLA residues.

The term "construct" is intended to indicate a polynucleotide segment which may be based on a complete or partial naturally occurring nucleotide sequence encoding the polypeptide of interest. The construct may optionally contain other polynucleotide segments. In a similar way, the term "amino acids which can be encoded by polynucleotide constructs" covers amino acids which can be encoded by the polynucleotide constructs defined above, i.e. amino acids such as Ala, Val, Leu, Ile, Met, Phe, Trp, Pro, Gly, Ser, Thr, Cys, Tyr, Asn, Glu, Lys, Arg, His, Asp and Gln.

In a further aspect, the invention provides a recombinant vector comprising the polynucleotide construct encoding a Factor VII polypeptide of the invention.

The term "vector", as used herein, means any nucleic acid entity capable of the amplification in a host cell. Thus, the vector may be an autonomously replicating vector, i.e. a vector, which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated. The choice of vector will often depend on the host cell into which it is to be introduced. Vectors include, but are not limited to plasmid vectors, phage vectors, viruses or cosmid vectors. Vectors usually contains a replication origin and at least one selectable gene, i.e., a gene which encodes a product which is readily detectable or the presence of which is essential for cell growth.

In a further aspect, the invention provides a recombinant host cell comprising the polynucleotide construct or the vector. In one embodiment, the host cell is a eukaryotic cell.

In one embodiment, the host cell is of mammalian origin. In one embodiment, the host cell is an insect cell. In one embodiment, the host cell is selected from the group consisting of CHO cells , HEK cells and BHK cells. In one embodiment, the host cell is a microorganism. In one embodiment, the host cell is selected from the group consisting of Escherichia coli, Bacillus subtilis, Rhodococcus, Streptomycetes, Actinomycetes, Corynebacteria, Pseudomonas, Erwinia, Salmonella, Saccharomyces cerevisiae, Picchia, protozoans, Penicillium, Fusarium, Aspergillus, Podospora, and Neurospora.

The term "a host cell", as used herein, represent any cell, including hybrid cells, in which heterologous DNA can be expressed. Typical host cells includes, but are not limited to bacterial cells, insect cells, yeast cells, mammalian cells, including human cells, such as BHK, CHO, HEK, and COS cells.

In a further aspect, the invention provides a transgenic animal containing and expressing the polynucleotide construct.

In a further aspect, the invention provides a transgenic plant containing and expressing the polynucleotide construct.

In a further aspect the present invention relates to a method for producing a FVII polypeptide comprising one or more amino acid substitutions relative to the amino acid sequence of SEQ ID NO:1, wherein the amino acid substitutions are replacement with any other amino acid of one or more amino acids selected from the group consisting of K157, V158, E296, M298, L305, M306, D309, S314, D334, S336, K337, F374, and wherein the FVII polypeptide is essentially free of GLA residues, the method comprising cultivating a host cell comprising the polynucleotide construct encoding the Factor VII polypeptide in an appropriate growth medium under conditions allowing expression of the polynucleotide

construct and recovering the resulting polypeptide from the growth medium or the host cell. In one embodiment the resulting polypeptide is recovered for the host cell by cell lysis.

As used herein the term "appropriate growth medium" means a medium containing nutrients and other components required for the growth of cells and the expression of the nucleic acid sequence encoding the Factor VII polypeptide of the invention.

In a further aspect, the invention relates to a method for producing the Factor VII polypeptide, the method comprising recovering the polypeptide from milk produced by the transgenic animal.

In a further aspect, the invention relates to a method for producing the Factor VII polypeptide, the method comprising cultivating a host cell of a transgenic plant comprising the polynucleotide construct, and recovering the polypeptide from the resulting plant.

In a further aspect, the invention relates to a pharmaceutical composition comprising a FVII polypeptide, wherein the FVII polypeptide is essentially free of GLA residues; and, optionally, a pharmaceutically acceptable carrier. In one embodiment the FVII polypeptide is wild type human FVIIa.

In a further aspect the present invention relates to a pharmaceutical composition comprising a FVII polypeptide comprising one or more amino acid substitutions relative to the amino acid sequence of SEQ ID NO:1, wherein the amino acid substitutions are replacement with any other amino acid of one or more amino acids selected from the group consisting of K157, V158, E296, M298, L305, M306, D309, S314, D334, S336, K337, F374, and wherein the FVII polypeptide is essentially free of GLA residues; and, optionally, a pharmaceutically acceptable carrier.

In a further aspect the present invention relates to the use of a FVII polypeptide, wherein the FVII polypeptide is essentially free of GLA residues; for the preparation of a medicament for the treatment of bleeding disorders or bleeding episodes or for the enhancement of the normal haemostatic system. In one embodiment the FVII polypeptide is wild type human FVIIa.

In a further aspect the present invention relates to the use of a FVII polypeptide comprising one or more amino acid substitutions relative to the amino acid sequence of SEQ ID NO:1, wherein the amino acid substitutions are replacement with any other amino acid of one or more amino acids selected from the group consisting of K157, V158, E296, M298, L305, M306, D309, S314, D334, S336, K337, F374, and wherein the FVII polypeptide is essentially free of GLA residues; for the preparation of a medicament for the treatment of bleeding disorders or bleeding episodes or for the enhancement of the normal haemostatic system. In one embodiment the use is for the treatment of haemophilia A or B.

In the present context, the term "treatment" is means both prevention of an expected bleeding, such as in surgery, and regulation of an already occurring bleeding, such as in trauma, with the purpose of inhibiting or minimising the bleeding. Prophylactic administration of the Factor VIIa polypeptide according to the invention is thus included in the term "treatment".

The term "bleeding episodes" is meant to include uncontrolled and excessive bleeding. Bleeding episodes may be a major problem both in connection with surgery and other forms of tissue damage. Uncontrolled and excessive bleeding may occur in subjects having a normal coagulation system and subjects having coagulation or bleeding disorders. As used herein the term "bleeding disorder" reflects any defect, congenital, acquired or induced, of cellular or molecular origin that is manifested in bleedings. Examples are clotting factor deficiencies (e.g. haemophilia A and B or deficiency of coagulation Factors XI or VII), clotting factor inhibitors, defective platelet function, thrombocytopenia or von Willebrand's disease.

Excessive bleedings also occur in subjects with a normally functioning blood clotting cascade (no clotting factor deficiencies or -inhibitors against any of the coagulation factors) and may be caused by a defective platelet function, thrombocytopenia or von Willebrand's disease. In such cases, the bleedings may be likened to those bleedings caused by haemophilia because the haemostatic system, as in haemophilia, lacks or has abnormal essential clotting "compounds" (such as platelets or von Willebrand factor protein) that causes major bleedings. In subjects who experience extensive tissue damage in association with surgery or vast trauma, the normal haemostatic mechanism may be overwhelmed by the demand of immediate haemostasis and they may develop bleeding in spite of a normal haemostatic mechanism. Achieving satisfactory haemostasis also is a problem when bleedings occur in organs such as the brain, inner ear region and eyes with limited possibility for surgical haemostasis. The same problem may arise in the process of taking biopsies from various organs (liver, lung, tumour tissue, gastrointestinal tract) as well as in laparoscopic surgery. Common for all these situations is the difficulty to provide haemostasis by surgical techniques (sutures, clips, etc.) which also is the case when bleeding is diffuse (haemorrhagic gastritis and profuse uterine bleeding). Acute and profuse bleedings may also occur in subjects on anticoagulant therapy in whom a defective haemostasis has been induced by the therapy given. Such subjects may need surgical interventions in case the anticoagulant effect has to be counteracted rapidly. Radical retropubic prostatectomy is a commonly performed procedure for subjects with localized prostate cancer. The operation is frequently complicated by significant and sometimes massive blood loss. The considerable blood loss during prostatectomy is mainly related to the complicated anatomical situation,

with various densely vascularized sites that are not easily accessible for surgical haemostasis, and which may result in diffuse bleeding from a large area. Another situation that may cause problems in the case of unsatisfactory haemostasis is when subjects with a normal haemostatic mechanism are given anticoagulant therapy to prevent thromboembolic disease. Such therapy may include heparin, other forms of proteoglycans, warfarin or other forms of vitamin K-antagonists as well as aspirin and other platelet aggregation inhibitors. In one embodiment of the invention, the bleeding is associated with haemophilia. In another embodiment, the bleeding is associated with haemophilia with aquired inhibitors. In another embodiment, the bleeding is associated with thrombocytopenia. In another embodiment, the bleeding is associated with von Willebrand's disease. In another embodiment, the bleeding is associated with severe tissue damage. In another embodiment, the bleeding is associated with severe trauma. In another embodiment, the bleeding is associated with surgery. In another embodiment, the bleeding is associated with laparoscopic surgery. In another embodiment, the bleeding is associated with haemorrhagic gastritis. In another embodiment, the bleeding is profuse uterine bleeding. In another embodiment, the bleeding is occurring in organs with a limited possibility for mechanical haemostasis. In another embodiment, the bleeding is occurring in the brain, inner ear region or eyes. In another embodiment, the bleeding is associated with the process of taking biopsies. In another embodiment, the bleeding is associated with anticoagulant therapy.

The term "subject" as used herein is intended to mean any animal, in particular mammals, such as humans, and may, where appropriate, be used interchangeably with the term "patient".

The term "enhancement of the normal haemostatic system" means an enhancement of the ability to generate thrombin.

In a further aspect the present invention relates to a method for the treatment of bleeding disorders or bleeding episodes in a subject or for the enhancement of the normal haemostatic system, the method comprising administering a therapeutically or prophylactically effective amount of a FVII polypeptide, wherein said FVII polypeptide is essentially free of GLA residues; to a subject in need thereof. In one embodiment the FVII polypeptide is wild type human FVIIa.

In a further aspect the present invention relates to a method for the treatment of bleeding disorders or bleeding episodes in a subject or for the enhancement of the normal haemostatic system, the method comprising administering a therapeutically or prophylactically effective amount of a FVII polypeptide comprising one or more amino acid substitutions relative to the amino acid sequence of SEQ ID NO:1, wherein the amino acid substitutions are replacement with any other amino acid of one or more amino acids selected

from the group consisting of K157, V158, E296, M298, L305, M306, D309, S314, D334, S336, K337, F374, and wherein the FVII polypeptide is essentially free of GLA residues; to a subject in need thereof. In one embodiment the bleeding disorder is haemophilia A or B.

In a further aspect the present invention relates to a Factor VII polypeptide essentially free of GLA residues for use as a medicament.

In a further aspect the present invention relates to a FVII polypeptide comprising one or more amino acid substitutions relative to the amino acid sequence of SEQ ID NO:1, wherein the amino acid substitutions are replacement with any other amino acid of one or more amino acids selected from the group consisting of K157, V158, E296, M298, L305, M306, D309, S314, D334, S336, K337, F374, and wherein the FVII polypeptide is essentially free of GLA residues, for use as a medicament.

DETAILED DESCRIPTION OF THE INVENTION

The inventors of the present invention have realised that it will be feasible to prepare a Factor VII polypeptide having substantially the same or increased proteolytic activity compared to recombinant wild type human Factor VIIa, where the gamma-carboxyglutamic acid containing lipid membrane binding domain is lacking or is non-functional.

Recent advances in our understanding of the mechanisms regulating the activity of FVIIa have pinpointed side chains that function as zymogenicity determinants in the free enzyme. Replacements of these amino acid residues have resulted in FVIIa molecules with increased intrinsic (TF-independent) catalytic efficiency. The relatively high intrinsic activity of some of these FVIIa variants suggests that the zymogen-like conformation of free factor VIIa is dictated by a limited number of key amino acid residues. One of these superactive FVIIa variants, containing the mutations at positions 158, 296 and 298, exhibits several properties resembling TF-bound rather than free FVIIa. Apart from increased intrinsic enzymatic activity and inhibitor susceptibility as compared with wild-type FVIIa, these FVIIa variants have a diminished requirement for calcium ions and a more deeply buried protease domain N-terminus (Ile153) indicating salt bridge formation of this residue with Asp343.

As already suggested wild-type human FVIIa and FVIIa variants with increased proteolytic activity as compared to wild-type human FVIIa have different properties. Whereas free wild-type human FVIIa has a zymogen-like conformation, FVIIa variants with increased proteolytic activity as compared to wild-type human FVIIa exhibits several properties resembling TF-bound FVIIa.

According to the present invention, a FVII polypeptide which is "essentially free of GLA residues" is a FVII polypeptide where there is substantially no host-cell derived GLA residues, i.e. 4-carboxyglutamic acid (gamma-carboxyglutamic acid).

One aspect of the present invention relates to a FVII polypeptide essentially free of GLA residues. In one embodiment the FVII polypeptide is not a FVII polypeptide consisting of 39-406 relative to the amino acid sequence of SEQ ID NO:1 (des(1-38)- recombinant human FVIIa). In one embodiment the FVII polypeptide is not a FVII polypeptide consisting of 45-406 relative to the amino acid sequence of SEQ ID NO:1 (des(1-44)-recombinant human FVIIa).

In one embodiment the FVII polypeptide has no GLA residues at all. In one embodiment of the invention, the GLA residues at any position selected from the list consisting of 6, 7, 14, 16, 19, 20, 25, 26, 29, and 35 relative to the amino acid sequence of SEQ ID NO:1 has been replaced by any other amino acid. In one embodiment of the invention, the GLA residues at any position selected from the list consisting of 6, 7, 14, 16, 19, 20, 25, 26, 29, and 35 relative to the amino acid sequence of SEQ ID NO:1 has been replaced by an amino acid selected from the group consisting of Glu, Gln, Asp, and Asn. In one embodiment of the invention, the GLA residues at any position selected from 6, 7, 14, 16, 19, 20, 25, 26, 29, and 35 relative to the amino acid sequence of SEQ ID NO:1 has been replaced by a Glu. In one embodiment of the invention, the GLA residues at any position selected from 6, 7, 14, 16, 19, 20, 25, 26, 29, and 35 relative to the amino acid sequence of SEQ ID NO:1 has been replaced by a Gln. It is to be understood, that the replacement of GLA residues at any position selected from 6, 7, 14, 16, 19, 20, 25, 26, 29, and 35 relative to the amino acid sequence of SEQ ID NO:1 may be replaced by Glu by the removal of a functional propeptide from a nucleotide construct encoding the FVII polypeptide. Thus, in one embodiment of the invention, the polynucleotide construct encoding a Factor VII polypeptide according to the invention is devoid of a functional propeptide.

In one embodiment of the invention 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 GLA residues at any position selected from the list consisting of 6, 7, 14, 16, 19, 20, 25, 26, 29, and 35 relative to the amino acid sequence of SEQ ID NO:1 has been replaced by any other amino acid.

In another embodiment of the invention, the FVII polypeptide is essentially free of GLA residues by synthesis in a host cell, which is not able to gamma-carboxylate. In one embodiment, the host cell does not express a gamma-carboxylase.

In another embodiment of the invention, the FVII polypeptide is essentially free of GLA residues by synthesis in a host cell in the presence of a vitamin-K antagonist. In one

embodiment, the vitamin K antagonist is selected from the list consisting of warfarin, coumarin, and acenocoumarol.

According to the present invention, a FVII polypeptide may have a lipid membrane binding domain not normally present in human wild type FVII and/or derived from another polypeptide. Suitable lipid membrane binding domains are disclosed in 5,225,537, which is hereby incorporated by reference in its entirety.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein K157 is replaced with any other amino acid.

The term "any other amino acid" as used herein means one amino acid that are different from that amino acid naturally present at that position. This includes but is not limited to amino acids that can be encoded by a polynucleotide. Preferably the different amino acid is in natural L-form and can be encoded by a polynucleotide. A specific example being L-cysteine (Cys).

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein V158 is replaced with any other amino acid.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein E296 is replaced with any other amino acid.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein M298 is replaced with any other amino acid.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein L305 is replaced with any other amino acid.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein M306 is replaced with any other amino acid.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein D309 is replaced with any other amino acid.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein S314 is replaced with any other amino acid.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein D334 is replaced with any other amino acid.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein S336 is replaced with any other amino acid.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein K337 is replaced with any other amino acid.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein F374 is replaced with any other amino acid.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein at least one amino acid in the remaining positions in the protease domain has been replaced with any other amino acid.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein at the most 20 additional amino acids in the remaining positions in the protease domain have been replaced with any other amino acids.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein at least one amino acid corresponding to an amino acid at a position selected from 159-170 of SEQ ID NO:1 has been replaced with any other amino acid.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein at least one amino acid corresponding to an amino acid at a position selected from 290-304 of SEQ ID NO:1 has been replaced with any other amino acid.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein R304 has been replaced by an amino acid selected from the group consisting of Tyr, Phe, Leu, and Met.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein at least one amino acid corresponding to an amino acid at a position selected from 307-312 of SEQ ID NO:1 has been replaced with any other amino acid.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein at least one amino acid corresponding to an amino acid at a position selected from 330-339 of SEQ ID NO:1 has been replaced with any other amino acid.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein A274 has been replaced with any other amino acid.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein the A274 has been replaced by an amino acid selected from the group consisting of Met, Leu, Lys, and Arg.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein the K157 has been replaced by an amino acid selected from the group consisting of Gly, Val, Ser, Thr, Asn, Gln, Asp, and Glu.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein the V158 has been replaced by an amino acid selected from the group consisting of Ser, Thr, Asn, Gln, Asp, and Glu.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein the E296 has been replaced by an amino acid selected from the group consisting of Arg, Lys, Ile, Leu and Val.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein the M298 has been replaced by an amino acid selected from the group consisting of Lys, Arg, Gln, and Asn.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein the L305 has been replaced by an amino acid selected from the group consisting of Val, Tyr and Ile.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein M306 has been replaced by an amino acid selected from the group consisting of Asp, and Asn.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein D309 has been replaced by an amino acid selected from the group consisting of Ser, and Thr.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein the S314 has been replaced by an amino acid selected from the group consisting of Gly, Lys, Gln and Glu.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein the D334 has been replaced by an amino acid selected from the group consisting of Gly, and Glu.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein the S336 has been replaced by an amino acid selected from the group consisting of Gly, and Glu.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein the K337 has been replaced by an amino acid selected from the group consisting of Ala, Gly, Val, Ser, Thr, Asn, Gln, Asp, and Glu.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein the F374 has been replaced by an amino acid selected from the group consisting of Pro, and Tyr.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein the F374 has been replaced by Tyr.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein the Factor VII polypeptide does not contain the amino acid sequence 1-38 relative to the amino acid sequence of SEQ ID NO:1. In one embodiment, the amino acid sequence 1-38 relative to the amino acid sequence of SEQ ID NO:1 has been removed by a proteolytic enzyme after synthesis of the factor VII polypeptide. In one embodiment, the polynucleotide sequence encoding the amino acid sequence 1-38 relative to the amino acid sequence of

SEQ ID NO:1 has been removed from the polynucleotide construct encoding the factor VII polypeptide.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein the Factor VII polypeptide does not contain the amino acid sequence 1-44 relative to the amino acid sequence of SEQ ID NO:1. In one embodiment, the amino acid sequence 1-44 relative to the amino acid sequence of SEQ ID NO:1 has been removed by a proteolytic enzyme after synthesis of the factor VII polypeptide. In one embodiment, the polynucleotide sequence encoding the amino acid sequence 1-44 relative to the amino acid sequence of SEQ ID NO:1 has been removed from the polynucleotide construct encoding the factor VII polypeptide.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein the Factor VII polypeptide does not contain the amino acid sequence 1-85 relative to the amino acid sequence of SEQ ID NO:1.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein the Factor VII polypeptide does not contain the amino acid sequence 1-90 relative to the amino acid sequence of SEQ ID NO:1.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein the Factor VII polypeptide does not contain the amino acid sequence 1-152 relative to the amino acid sequence of SEQ ID NO:1.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein the Factor VII polypeptide at least consists of the amino acid sequence 39-406 relative to the amino acid sequence of SEQ ID NO:1.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein the Factor VII polypeptide at least consists of the amino acid sequence 45-406 relative to the amino acid sequence of SEQ ID NO:1.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein the Factor VII polypeptide at least consists of the amino acid sequence 86-406 relative to the amino acid sequence of SEQ ID NO:1.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein the Factor VII polypeptide at least consists of the amino acid sequence 91-406 relative to the amino acid sequence of SEQ ID NO:1.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein the Factor VII polypeptide at least consists of the amino acid sequence 153-406 relative to the amino acid sequence of SEQ ID NO:1.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein the amino acid has been replaced with any other amino acid which can be encoded by polynucleotide constructs.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein the Factor VII polypeptide is in activated form.

In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein the ratio between the proteolytic activity of the Factor VII polypeptide and the proteolytic activity of the native Factor VIIa polypeptide shown in SEQ ID NO:1 is at least about 1.25. In one embodiment of the invention, the factor VII polypeptide is a polypeptide, wherein the ratio is at least about 2.0, preferably at least about 4.0.

In a further embodiment of the invention, the Factor VII polypeptide has a proteolytic activity higher than wild type human FVIIa.

In a further embodiment of the invention, the Factor VII polypeptide is wild type human FVII, which does not contain the amino acid sequence 1-38 relative to the amino acid sequence of SEQ ID NO:1.

In a further embodiment of the invention, the Factor VII polypeptide is wild type human FVII, which does not contain the amino acid sequence 1-44 relative to the amino acid sequence of SEQ ID NO:1.

In a further embodiment of the invention, the Factor VII polypeptide is wild type human FVII, which does not contain the amino acid sequence 1-152 relative to the amino acid sequence of SEQ ID NO:1.

In a further embodiment of the invention, the Factor VII polypeptide comprises the amino acid sequence 39-406 of SEQ ID NO:1 of wild type human FVII.

In a further embodiment of the invention, the Factor VII polypeptide comprises the amino acid sequence 45-406 of SEQ ID NO:1 of wild type human FVII.

In a further embodiment of the invention, the Factor VII polypeptide comprises the amino acid sequence 153-406 of SEQ ID NO:1 of wild type human FVII.

In a further embodiment of the invention, the Factor VII polypeptide comprises amino acid substitutions relative to the amino acid sequence of SEQ ID NO:1 selected from the group consisting of: L305V, L305V/M306D/D309S, L305I, L305T, F374P, V158T/M298Q, V158D/E296V/M298Q, K337A, M298Q, V158D/M298Q, L305V/K337A, V158D/E296V/M298Q/L305V, V158D/E296V/M298Q/K337A, V158D/E296V/M298Q/L305V/K337A, K157A, E296V, E296V/M298Q, V158D/E296V, V158D/M298K, and S336G, L305V/K337A, L305V/V158D, L305V/E296V, L305V/M298Q, L305V/V158T, L305V/K337A/V158T, L305V/K337A/M298Q, L305V/K337A/E296V, L305V/K337A/V158D, L305V/V158D/M298Q, L305V/V158D/E296V, L305V/V158T/M298Q,

L305V/V158T/E296V, L305V/E296V/M298Q, L305V/V158D/E296V/M298Q, L305V/V158T/E296V/M298Q, L305V/V158T/K337A/M298Q, L305V/V158T/E296V/K337A, L305V/V158D/K337A/M298Q, L305V/V158D/E296V/K337A, L305V/V158D/E296V/M298Q/K337A, L305V/V158T/E296V/M298Q/K337A, S314E/K316H, S314E/K316Q, S314E/L305V, S314E/K337A, S314E/V158D, S314E/E296V, S314E/M298Q, S314E/V158T, K316H/L305V, K316H/K337A, K316H/V158D, K316H/E296V, K316H/M298Q, K316H/V158T, K316Q/L305V, K316Q/K337A, K316Q/V158D, K316Q/E296V, K316Q/M298Q, K316Q/V158T, S314E/L305V/K337A, S314E/L305V/V158D, S314E/L305V/E296V, S314E/L305V/M298Q, S314E/L305V/V158T, S314E/L305V/K337A/V158T, S314E/L305V/K337A/M298Q, S314E/L305V/K337A/E296V, S314E/L305V/K337A/V158D, S314E/L305V/V158D/M298Q, S314E/L305V/V158D/E296V, S314E/L305V/V158T/M298Q, S314E/L305V/V158T/E296V, S314E/L305V/E296V/M298Q, S314E/L305V/V158D/E296V/M298Q, S314E/L305V/V158T/E296V/M298Q, S314E/L305V/V158T/K337A/M298Q, S314E/L305V/V158T/E296V/K337A, S314E/L305V/V158D/K337A/M298Q, S314E/L305V/V158D/E296V/K337A, S314E/L305V/V158D/E296V/M298Q/K337A, S314E/L305V/V158T/E296V/M298Q/K337A, K316H/L305V/K337A, K316H/L305V/V158D, K316H/L305V/E296V, K316H/L305V/M298Q, K316H/L305V/V158T, K316H/L305V/K337A/V158T, K316H/L305V/K337A/M298Q, K316H/L305V/K337A/E296V, K316H/L305V/K337A/V158D, K316H/L305V/V158D/M298Q, K316H/L305V/V158D/E296V, K316H/L305V/V158T/M298Q, K316H/L305V/V158T/E296V, K316H/L305V/E296V/M298Q, K316H/L305V/V158D/E296V/M298Q, K316H/L305V/V158T/E296V/M298Q, K316H/L305V/V158T/K337A/M298Q, K316H/L305V/V158T/E296V/K337A, K316H/L305V/V158D/K337A/M298Q, K316H/L305V/V158D/E296V/K337A, K316H/L305V/V158D/E296V/M298Q/K337A, K316H/L305V/V158T/E296V/M298Q/K337A, K316Q/L305V/K337A, K316Q/L305V/V158D, K316Q/L305V/E296V, K316Q/L305V/M298Q, K316Q/L305V/V158T, K316Q/L305V/K337A/V158T, K316Q/L305V/K337A/M298Q, K316Q/L305V/K337A/E296V, K316Q/L305V/K337A/V158D, K316Q/L305V/V158D/M298Q, K316Q/L305V/V158D/E296V, K316Q/L305V/V158T/M298Q, K316Q/L305V/V158T/E296V, K316Q/L305V/E296V/M298Q, K316Q/L305V/V158D/E296V/M298Q, K316Q/L305V/V158T/E296V/M298Q, K316Q/L305V/V158T/K337A/M298Q, K316Q/L305V/V158T/E296V/K337A, K316Q/L305V/V158D/K337A/M298Q, K316Q/L305V/V158D/E296V/K337A, K316Q/L305V/V158D/E296V/M298Q/K337A, K316Q/L305V/V158T/E296V/M298Q/K337A, F374Y/K337A, F374Y/V158D, F374Y/E296V, F374Y/M298Q, F374Y/V158T, F374Y/S314E, F374Y/L305V, F374Y/L305V/K337A, F374Y/L305V/V158D, F374Y/L305V/E296V, F374Y/L305V/M298Q, F374Y/L305V/V158T, F374Y/L305V/S314E, F374Y/K337A/S314E,

F374Y/K337A/V158T, F374Y/K337A/M298Q, F374Y/K337A/E296V, F374Y/K337A/V158D, F374Y/V158D/S314E, F374Y/V158D/M298Q, F374Y/V158D/E296V, F374Y/V158T/S314E, F374Y/V158T/M298Q, F374Y/V158T/E296V, F374Y/E296V/S314E, F374Y/S314E/M298Q, F374Y/E296V/M298Q, F374Y/L305V/K337A/V158D, F374Y/L305V/K337A/E296V, F374Y/L305V/K337A/M298Q, F374Y/L305V/K337A/V158T, F374Y/L305V/K337A/S314E, F374Y/L305V/V158D/E296V, F374Y/L305V/V158D/M298Q, F374Y/L305V/V158D/S314E, F374Y/L305V/E296V/M298Q, F374Y/L305V/E296V/V158T, F374Y/L305V/E296V/S314E, F374Y/L305V/M298Q/V158T, F374Y/L305V/M298Q/S314E, F374Y/L305V/V158T/S314E, F374Y/K337A/S314E/V158T, F374Y/K337A/S314E/M298Q, F374Y/K337A/S314E/E296V, F374Y/K337A/S314E/V158D, F374Y/K337A/V158T/M298Q, F374Y/K337A/V158T/E296V, F374Y/K337A/M298Q/E296V, F374Y/K337A/M298Q/V158D, F374Y/K337A/E296V/V158D, F374Y/V158D/S314E/M298Q, F374Y/V158D/S314E/E296V, F374Y/V158D/M298Q/E296V, F374Y/V158T/S314E/E296V, F374Y/V158T/S314E/M298Q, F374Y/V158T/M298Q/E296V, F374Y/E296V/S314E/M298Q, F374Y/L305V/M298Q/K337A/S314E, F374Y/L305V/E296V/K337A/S314E, F374Y/E296V/M298Q/K337A/S314E, F374Y/L305V/E296V/M298Q/K337A, F374Y/L305V/E296V/M298Q/S314E, F374Y/V158D/E296V/M298Q/K337A, F374Y/V158D/E296V/M298Q/S314E, F374Y/L305V/V158D/K337A/S314E, F374Y/V158D/M298Q/K337A/S314E, F374Y/V158D/E296V/K337A/S314E, F374Y/L305V/V158D/E296V/M298Q, F374Y/L305V/V158D/M298Q/K337A, F374Y/L305V/V158D/E296V/K337A, F374Y/L305V/V158D/M298Q/S314E, F374Y/L305V/V158D/E296V/S314E, F374Y/V158T/E296V/M298Q/K337A, F374Y/V158T/E296V/M298Q/S314E, F374Y/L305V/V158T/K337A/S314E, F374Y/V158T/M298Q/K337A/S314E, F374Y/V158T/E296V/K337A/S314E, F374Y/L305V/V158T/E296V/M298Q, F374Y/L305V/V158T/M298Q/K337A, F374Y/L305V/V158T/E296V/K337A, F374Y/L305V/V158T/M298Q/S314E, F374Y/L305V/V158T/E296V/S314E, F374Y/E296V/M298Q/K337A/V158T/S314E, F374Y/V158D/E296V/M298Q/K337A/S314E, F374Y/L305V/V158D/E296V/M298Q/S314E, F374Y/L305V/E296V/M298Q/V158T/S314E, F374Y/L305V/E296V/M298Q/K337A/V158T, F374Y/L305V/E296V/K337A/V158T/S314E, F374Y/L305V/M298Q/K337A/V158T/S314E, F374Y/L305V/V158D/E296V/M298Q/K337A, F374Y/L305V/V158D/E296V/K337A/S314E, F374Y/L305V/V158D/M298Q/K337A/S314E, F374Y/L305V/E296V/M298Q/K337A/V158T/S314E, F374Y/L305V/V158D/E296V/M298Q/K337A/S314E, S52A-Factor VII, S60A-Factor VII; R152E-Factor VII, S344A-Factor VII, and P11Q/K33E, T106N, K143N/N145T, V253N, R290N/A292T, G291N, R315N/V317T, K143N/N145T/R315N/V317T; and FVII having substitutions, additions or deletions in the amino acid sequence from 233Thr to 240Asn, FVII

having substitutions, additions or deletions in the amino acid sequence from 304Arg to 329Cys.

In a further embodiment of the invention, the ratio between the proteolytic activity of the Factor VII polypeptide and the proteolytic activity of the wild type human Factor VIIa is at least about 1.25 when tested in assay 1 as described herein.

In a further embodiment of the invention, the ratio between the proteolytic activity of the Factor VII polypeptide and the proteolytic activity of the wild type human Factor VIIa is at least about 2.0, such as at least about 4.0, when tested in assay 1 as described herein.

In a further embodiment of the invention, the ratio between the proteolytic activity of the Factor VII polypeptide and the proteolytic activity of the wild type human Factor VIIa is at least about 1.25 when tested in assay 2 as described herein.

In a further embodiment of the invention, the ratio between the proteolytic activity of the Factor VII polypeptide and the proteolytic activity of the wild type human Factor VIIa is at least about 2.0, such as at least about 4.0, when tested in assay 2 as described herein.

As used herein, "wild type human FVIIa" is a polypeptide having the amino acid sequence disclosed in U.S. Patent No. 4,784,950 (figure 1).

As used herein, the terms "Factor VII polypeptide " or "FVII polypeptide" means any protein comprising the amino acid sequence 1-406 of native human Factor VII (SEQ ID NO: 1) or variants thereof. This includes but is not limited to human Factor VII, human Factor VIIa and variants thereof. This includes variants of Factor VII exhibiting substantially the same or increased proteolytic activity compared to recombinant wild type human Factor VIIa, as well as Factor VII derivatives and Factor VII conjugates. The term "Factor VII" is intended to encompass Factor VII polypeptides in their uncleaved (zymogen) form, as well as those that have been proteolytically processed to yield their respective bioactive forms, which may be designated Factor VIIa. Typically, Factor VII is cleaved between residues 152 and 153 to yield Factor VIIa. Such variants of Factor VII may exhibit different properties relative to human Factor VII, including stability, phospholipid binding, altered specific activity, and the like.

A "lipid membrane binding domain" is in the present context a protein domain that binds to cell membrane lipid domains. "Lipid membrane binding domains" typically include gamma-carboxyglutamic residues and will normally be derived from a vitamin K dependent protein such as factors VII, IX, and X; protein C, protein S; osteocalcin, matrix Gla protein, and proline-rich Gla protein 1. Other lipid membrane binding domains are disclosed in US 5,225,537, which US patent is hereby incorporated by reference.

The term "N-terminal Gla-domain" or just "Gla-domain" specifically means the amino acid sequence 1-37 of Factor VII set forth in SEQ ID NO: 1.

The three-letter indication "Gla" means 4-carboxyglutamic acid (gamma-carboxyglutamate).

The term "Factor VII derivative" as used herein, is intended to designate a FVII polypeptide exhibiting substantially the same or improved biological activity relative to wild-type Factor VII, in which one or more of the amino acids of the parent peptide have been genetically and/or chemically and/or enzymatically modified, e.g. by alkylation, glycosylation, PEGylation, acylation, ester formation or amide formation or the like. This includes but is not limited to PEGylated human Factor VIIa, cysteine-PEGylated human Factor VIIa and variants thereof. Non-limiting examples of Factor VII derivatives includes GlycoPegylated FVII derivatives as disclosed in WO 03/31464 and US Patent applications US 20040043446, US 20040063911, US 20040142856, US 20040137557, and US 20040132640 (Neose Technologies, Inc.); FVII conjugates as disclosed in WO 01/04287, US patent application 20030165996, WO 01/58935, WO 03/93465 (Maxygen ApS) and WO 02/02764, US patent application 20030211094 (University of Minnesota).

The term "PEGylated human Factor VIIa" means human Factor VIIa, having a PEG molecule conjugated to a Factor VIIa polypeptide. It is to be understood, that the PEG molecule may be attached to any part of the Factor VIIa polypeptide including any amino acid residue or carbohydrate moiety of the Factor VIIa polypeptide. The term "cysteine-PEGylated human Factor VIIa " means Factor VIIa having a PEG molecule conjugated to a sulfhydryl group of a cysteine introduced in human Factor VIIa.

The biological activity of Factor VIIa in blood clotting derives from its ability to (i) bind to tissue factor (TF) and (ii) catalyze the proteolytic cleavage of Factor IX or Factor X to produce activated Factor IX or X (Factor IXa or Xa, respectively). For purposes of the invention, Factor VIIa biological activity may be quantified by measuring the ability of a preparation to promote blood clotting using Factor VII-deficient plasma and thromboplastin, as described, e.g., in U.S. Patent No. 5,997,864. In this assay, biological activity is expressed as the reduction in clotting time relative to a control sample and is converted to "Factor VII units" by comparison with a pooled human serum standard containing 1 unit/ml Factor VII activity. Alternatively, Factor VIIa biological activity may be quantified by (i) measuring the ability of Factor VIIa to produce of Factor Xa in a system comprising TF embedded in a lipid membrane and Factor X. (Persson et al., J. Biol. Chem. 272:19919-19924, 1997); (ii) measuring Factor X hydrolysis in an aqueous system; (iii) measuring its physical binding to TF using an instrument based on surface plasmon resonance (Persson, FEBS Letts. 413:359-363, 1997) and (iv) measuring hydrolysis of a synthetic substrate.

Alternatively, Factor VIIa biological activity may be quantified by the generation of thrombin on platelets as described in Persson et al, PNAS 98, 13583-13588, 2001.

The term "substantially the same or increased proteolytic activity compared to recombinant wild type human Factor VIIa", as used herein, means an activity more than 50% of the activity of recombinant wild type human Factor VIIa, when tested in one or more of a clotting assay, or proteolysis assay, as described herein. In one embodiment the activity is more than 80% of the activity of recombinant wild type human Factor VIIa. In another embodiment the activity is more than 90% of the activity of recombinant wild type human Factor VIIa. In a further embodiment the activity is more than 100% of the activity of recombinant wild type human Factor VIIa. In a further embodiment the activity is more than 120% of the activity of recombinant wild type human Factor VIIa. In a further embodiment the activity is more than 200% of the activity of recombinant wild type human Factor VIIa. In a further embodiment the activity is more than 400% of the activity of recombinant wild type human Factor VIIa.

Variants of Factor VII, whether exhibiting substantially the same or increased proteolytic activity compared to recombinant wild type human Factor VIIa, include, without limitation, polypeptides having an amino acid sequence that differs from the sequence of wild-type Factor VII by insertion, deletion, or substitution of one or more amino acids.

The terms "variant" or "variants", as used herein, is intended to designate Factor VII having the sequence of wild type human FVII, wherein one or more amino acids of the parent protein have been substituted by another amino acid and/or wherein one or more amino acids of the parent protein have been deleted and/or wherein one or more amino acids have been inserted in protein and/or wherein one or more amino acids have been added to the parent protein. Such addition can take place either at the N-terminal end or at the C-terminal end of the parent protein or both. The "variant" or "variants" within this definition still have FVII activity in its activated form. In one embodiment the FVII polypeptide variant comprises at least the amino acid sequence 91-368 of native human Factor VII (SEQ ID NO: 1).

In one embodiment a variant is 70 % identical with the sequence of wild type human FVII. In one embodiment a variant is 80 % identical with the sequence of wild type human FVII. In another embodiment a variant is 90 % identical with the sequence of wild type human FVII. In a further embodiment a variant is 95 % identical with the sequence of wild type human FVII.

Non-limiting examples of Factor VII variants having substantially the same or increased proteolytic activity compared to recombinant wild type human Factor VIIa include S52A-FVIIa, S60A-FVIIa (Iino et al., Arch. Biochem. Biophys. 352: 182-192, 1998); FVIIa variants exhibiting increased proteolytic stability as disclosed in U.S. Patent No. 5,580,560; Factor VIIa that has been proteolytically cleaved between residues 290 and 291 or between residues 315 and 316 (Mollerup et al., Biotechnol. Bioeng. 48:501-505, 1995); oxidized forms

of Factor VIIa (Kornfelt et al., Arch. Biochem. Biophys. 363:43-54, 1999); FVII variants as disclosed in PCT/DK02/00189; and FVII variants exhibiting increased proteolytic stability as disclosed in WO 02/38162 (Scripps Research Institute); and FVII variants as disclosed in WO 01/58935 (Maxygen ApS) and WO 04/029091 (Maxygen ApS)..

Non-limiting examples of Factor VII variants having substantially the same or increased proteolytic activity compared to recombinant wild type human Factor VIIa include S52A-FVIIa, S60A-FVIIa (Lino et al., Arch. Biochem. Biophys. 352: 182-192, 1998); FVIIa variants exhibiting increased proteolytic stability as disclosed in U.S. Patent No. 5,580,560; Factor VIIa that has been proteolytically cleaved between residues 290 and 291 or between residues 315 and 316 (Mollerup et al., Biotechnol. Bioeng. 48:501-505, 1995); oxidized forms of Factor VIIa (Kornfelt et al., Arch. Biochem. Biophys. 363:43-54, 1999); FVII variants as disclosed in PCT/DK02/00189 (corresponding to WO 02/077218); and FVII variants exhibiting increased proteolytic stability as disclosed in WO 02/38162 (Scripps Research Institute); FVII variants having a modified Gla-domain and exhibiting an enhanced membrane binding as disclosed in WO 99/20767, US patents US 6017882 and US 6747003, US patent application 20030100506 (University of Minnesota) and WO 00/66753, US patent applications US 20010018414, US 2004220106, and US 200131005, US patents US 6762286 and US 6693075 (University of Minnesota); and FVII variants as disclosed in WO 01/58935, US patent US 6806063, US patent application 20030096338 (Maxygen ApS), WO 03/93465 (Maxygen ApS), WO 04/029091 (Maxygen ApS), WO 04/083361 (Maxygen ApS), and WO 04/111242 (Maxygen ApS), as well as in WO 04/108763 (Canadian Blood Services).

Non-limiting examples of FVII variants having increased biological activity compared to wild-type FVIIa include FVII variants as disclosed in WO 01/83725, WO 02/22776, WO 02/077218, PCT/DK02/00635 (corresponding to WO 03/027147), Danish patent application PA 2002 01423 (corresponding to WO 04/029090), Danish patent application PA 2001 01627 (corresponding to WO 03/027147); WO 02/38162 (Scripps Research Institute); and FVIIa variants with enhanced activity as disclosed in JP 2001061479 (Chemo-Sero-Therapeutic Res Inst.).

Examples of variants of factor VII include, without limitation, L305V-FVII, L305V/M306D/D309S-FVII, L305I-FVII, L305T-FVII, F374P-FVII, V158T/M298Q-FVII, V158D/E296V/M298Q-FVII, K337A-FVII, M298Q-FVII, V158D/M298Q-FVII, L305V/K337A-FVII, V158D/E296V/M298Q/L305V-FVII, V158D/E296V/M298Q/K337A-FVII, V158D/E296V/M298Q/L305V/K337A-FVII, K157A-FVII, E296V-FVII, E296V/M298Q-FVII, V158D/E296V-FVII, V158D/M298K-FVII, and S336G-FVII, L305V/K337A-FVII, L305V/V158D-FVII, L305V/E296V-FVII, L305V/M298Q-FVII, L305V/V158T-FVII,

L305V/K337A/V158T-FVII, L305V/K337A/M298Q-FVII, L305V/K337A/E296V-FVII, L305V/K337A/V158D-FVII, L305V/V158D/M298Q-FVII, L305V/V158D/E296V-FVII, L305V/V158T/M298Q-FVII, L305V/V158T/E296V-FVII, L305V/E296V/M298Q-FVII, L305V/V158D/E296V/M298Q-FVII, L305V/V158T/E296V/M298Q-FVII, L305V/V158T/K337A/M298Q-FVII, L305V/V158T/E296V/K337A-FVII, L305V/V158D/K337A/M298Q-FVII, L305V/V158D/E296V/K337A–FVII, L305V/V158D/E296V/M298Q/K337A-FVII, L305V/V158T/E296V/M298Q/K337A-FVII, S314E/K316H-FVII, S314E/K316Q-FVII, S314E/L305V-FVII, S314E/K337A-FVII, S314E/V158D-FVII, S314E/E296V-FVII, S314E/M298Q-FVII, S314E/V158T-FVII, K316H/L305V-FVII, K316H/K337A-FVII, K316H/V158D-FVII, K316H/E296V-FVII, K316H/M298Q-FVII, K316H/V158T-FVII, K316Q/L305V-FVII, K316Q/K337A-FVII, K316Q/V158D-FVII, K316Q/E296V-FVII, K316Q/M298Q-FVII, K316Q/V158T-FVII, S314E/L305V/K337A-FVII, S314E/L305V/V158D-FVII, S314E/L305V/E296V-FVII, S314E/L305V/M298Q-FVII, S314E/L305V/V158T-FVII, S314E/L305V/K337A/V158T-FVII, S314E/L305V/K337A/M298Q-FVII, S314E/L305V/K337A/E296V-FVII, S314E/L305V/K337A/V158D-FVII, S314E/L305V/V158D/M298Q-FVII, S314E/L305V/V158D/E296V-FVII, S314E/L305V/V158T/M298Q-FVII, S314E/L305V/V158T/E296V-FVII, S314E/L305V/E296V/M298Q-FVII, S314E/L305V/V158D/E296V/M298Q-FVII, S314E/L305V/V158T/E296V/M298Q-FVII, S314E/L305V/V158T/K337A/M298Q-FVII, S314E/L305V/V158T/E296V/K337A-FVII, S314E/L305V/V158D/K337A/M298Q-FVII, S314E/L305V/V158D/E296V/K337A-FVII, S314E/L305V/V158D/E296V/M298Q/K337A-FVII, S314E/L305V/V158T/E296V/M298Q/K337A-FVII, K316H/L305V/K337A-FVII, K316H/L305V/V158D-FVII, K316H/L305V/E296V-FVII, K316H/L305V/M298Q-FVII, K316H/L305V/V158T-FVII, K316H/L305V/K337A/V158T-FVII, K316H/L305V/K337A/M298Q-FVII, K316H/L305V/K337A/E296V-FVII, K316H/L305V/K337A/V158D-FVII, K316H/L305V/V158D/M298Q-FVII, K316H/L305V/V158D/E296V-FVII, K316H/L305V/V158T/M298Q-FVII, K316H/L305V/V158T/E296V-FVII, K316H/L305V/E296V/M298Q-FVII, K316H/L305V/V158D/E296V/M298Q-FVII, K316H/L305V/V158T/E296V/M298Q-FVII, K316H/L305V/V158T/K337A/M298Q-FVII, K316H/L305V/V158T/E296V/K337A-FVII, K316H/L305V/V158D/K337A/M298Q-FVII, K316H/L305V/V158D/E296V/K337A –FVII, K316H/L305V/V158D/E296V/M298Q/K337A-FVII, K316H/L305V/V158T/E296V/M298Q/K337A-FVII, K316Q/L305V/K337A-FVII, K316Q/L305V/V158D-FVII, K316Q/L305V/E296V-FVII, K316Q/L305V/M298Q-FVII, K316Q/L305V/V158T-FVII, K316Q/L305V/K337A/V158T-FVII, K316Q/L305V/K337A/M298Q-FVII, K316Q/L305V/K337A/E296V-FVII, K316Q/L305V/K337A/V158D-FVII,

K316Q/L305V/V158D/M298Q-FVII, K316Q/L305V/V158D/E296V-FVII, K316Q/L305V/V158T/M298Q-FVII, K316Q/L305V/V158T/E296V-FVII, K316Q/L305V/E296V/M298Q-FVII, K316Q/L305V/V158D/E296V/M298Q-FVII, K316Q/L305V/V158T/E296V/M298Q-FVII, K316Q/L305V/V158T/K337A/M298Q-FVII, K316Q/L305V/V158T/E296V/K337A-FVII, K316Q/L305V/V158D/K337A/M298Q-FVII, K316Q/L305V/V158D/E296V/K337A –FVII, K316Q/L305V/V158D/E296V/M298Q/K337A-FVII, K316Q/L305V/V158T/E296V/M298Q/K337A-FVII, F374Y/K337A-FVII, F374Y/V158D-FVII, F374Y/E296V-FVII, F374Y/M298Q-FVII, F374Y/V158T-FVII, F374Y/S314E-FVII, F374Y/L305V-FVII, F374Y/L305V/K337A-FVII, F374Y/L305V/V158D-FVII, F374Y/L305V/E296V-FVII, F374Y/L305V/M298Q-FVII, F374Y/L305V/V158T-FVII, F374Y/L305V/S314E-FVII, F374Y/K337A/S314E-FVII, F374Y/K337A/V158T-FVII, F374Y/K337A/M298Q-FVII, F374Y/K337A/E296V-FVII, F374Y/K337A/V158D-FVII, F374Y/V158D/S314E-FVII, F374Y/V158D/M298Q-FVII, F374Y/V158D/E296V-FVII, F374Y/V158T/S314E-FVII, F374Y/V158T/M298Q-FVII, F374Y/V158T/E296V-FVII, F374Y/E296V/S314E-FVII, F374Y/S314E/M298Q-FVII, F374Y/E296V/M298Q-FVII, F374Y/L305V/K337A/V158D-FVII, F374Y/L305V/K337A/E296V-FVII, F374Y/L305V/K337A/M298Q-FVII, F374Y/L305V/K337A/V158T-FVII, F374Y/L305V/K337A/S314E-FVII, F374Y/L305V/V158D/E296V-FVII, F374Y/L305V/V158D/M298Q-FVII, F374Y/L305V/V158D/S314E-FVII, F374Y/L305V/E296V/M298Q-FVII, F374Y/L305V/E296V/V158T-FVII, F374Y/L305V/E296V/S314E-FVII, F374Y/L305V/M298Q/V158T-FVII, F374Y/L305V/M298Q/S314E-FVII, F374Y/L305V/V158T/S314E-FVII, F374Y/K337A/S314E/V158T-FVII, F374Y/K337A/S314E/M298Q-FVII, F374Y/K337A/S314E/E296V-FVII, F374Y/K337A/S314E/V158D-FVII, F374Y/K337A/V158T/M298Q-FVII, F374Y/K337A/V158T/E296V-FVII, F374Y/K337A/M298Q/E296V-FVII, F374Y/K337A/M298Q/V158D-FVII, F374Y/K337A/E296V/V158D-FVII, F374Y/V158D/S314E/M298Q-FVII, F374Y/V158D/S314E/E296V-FVII, F374Y/V158D/M298Q/E296V-FVII, F374Y/V158T/S314E/E296V-FVII, F374Y/V158T/S314E/M298Q-FVII, F374Y/V158T/M298Q/E296V-FVII, F374Y/E296V/S314E/M298Q-FVII, F374Y/L305V/M298Q/K337A/S314E-FVII, F374Y/L305V/E296V/K337A/S314E-FVII, F374Y/E296V/M298Q/K337A/S314E-FVII, F374Y/L305V/E296V/M298Q/K337A –FVII, F374Y/L305V/E296V/M298Q/S314E-FVII, F374Y/V158D/E296V/M298Q/K337A-FVII, F374Y/V158D/E296V/M298Q/S314E-FVII, F374Y/L305V/V158D/K337A/S314E-FVII, F374Y/V158D/M298Q/K337A/S314E-FVII, F374Y/V158D/E296V/K337A/S314E-FVII, F374Y/L305V/V158D/E296V/M298Q-FVII, F374Y/L305V/V158D/M298Q/K337A-FVII,

F374Y/L305V/V158D/E296V/K337A-FVII, F374Y/L305V/V158D/M298Q/S314E-FVII, F374Y/L305V/V158D/E296V/S314E-FVII, F374Y/V158T/E296V/M298Q/K337A-FVII, F374Y/V158T/E296V/M298Q/S314E-FVII, F374Y/L305V/V158T/K337A/S314E-FVII, F374Y/V158T/M298Q/K337A/S314E-FVII, F374Y/V158T/E296V/K337A/S314E-FVII, F374Y/L305V/V158T/E296V/M298Q-FVII, F374Y/L305V/V158T/M298Q/K337A-FVII, F374Y/L305V/V158T/E296V/K337A-FVII, F374Y/L305V/V158T/M298Q/S314E-FVII, F374Y/L305V/V158T/E296V/S314E-FVII, F374Y/E296V/M298Q/K337A/V158T/S314E-FVII, F374Y/V158D/E296V/M298Q/K337A/S314E-FVII, F374Y/L305V/V158D/E296V/M298Q/S314E-FVII, F374Y/L305V/E296V/M298Q/V158T/S314E-FVII, F374Y/L305V/E296V/M298Q/K337A/V158T-FVII, F374Y/L305V/E296V/K337A/V158T/S314E-FVII, F374Y/L305V/M298Q/K337A/V158T/S314E-FVII, F374Y/L305V/V158D/E296V/M298Q/K337A-FVII, F374Y/L305V/V158D/E296V/K337A/S314E-FVII, F374Y/L305V/V158D/M298Q/K337A/S314E-FVII, F374Y/L305V/E296V/M298Q/K337A/V158T/S314E-FVII, F374Y/L305V/V158D/E296V/M298Q/K337A/S314E-FVII, S52A-Factor VII, S60A-Factor VII; R152E-Factor VII, S344A-Factor VII, T106N-FVII, K143N/N145T-FVII, V253N-FVII, R290N/A292T-FVII, G291N-FVII, R315N/V317T-FVII, K143N/N145T/R315N/V317T-FVII; and FVII having substitutions, additions or deletions in the amino acid sequence from 233Thr to 240Asn; FVII having substitutions, additions or deletions in the amino acid sequence from 304Arg to 329Cys; and FVII having substitutions, additions or deletions in the amino acid sequence from 153Ile to 223Arg.

The terminology for amino acid substitutions used is as follows. The first letter represents the amino acid naturally present at a position of human wild type FVII. The following number represents the position in human wild type FVII. The second letter represent the different amino acid substituting for (replacing) the natural amino acid. An example is M298Q, where a methionine at position 298 of human wild type FVII is replaced by a glutamine. In another example, V158T/M298Q, the valine in position 158 of human wild type FVII is replaced by a threonine and the methionine in position 298 of human wild type FVII is replaced by a Glutamine in the same Factor VII polypeptide.

In a further embodiment of the invention, the Factor VII polypeptide polypeptide is a polypeptide, wherein the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at least about 1.25. In one embodiment the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human

Factor VIIa is at least about 2.0. In a further embodiment the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at least about 4.0.

In a further embodiment of the invention, the Factor VII polypeptide is a polypeptide, wherein the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at least about 1.25 when tested in a Factor VIIa activity assay. In one embodiment the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at least about 2.0 when tested in a Factor VIIa activity assay. In a further embodiment the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at least about 4.0 when tested in a Factor VIIa activity assay. The Factor VIIa activity may be measured by the assays described under "assays".

In a further embodiment of the invention, the Factor VII polypeptide is a polypeptide, wherein the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at least about 1.25 when tested in the "*In Vitro* Hydrolysis Assay". In one embodiment the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at least about 2.0 when tested in the "*In Vitro* Hydrolysis Assay". In a further embodiment the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at least about 4.0 when tested in the "*In Vitro* Hydrolysis Assay".

In a further embodiment of the invention, the Factor VII polypeptide is a polypeptide, wherein the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at least about 1.25 when tested in the "*In Vitro* Proteolysis Assay". In one embodiment the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at least about 2.0 when tested in the "*In Vitro* Proteolysis Assay". In a further embodiment the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at least about 4.0 when tested in the "*In Vitro* Proteolysis Assay". In a further embodiment the ratio between the activity of the Factor VII polypeptide and the activity of the wild type human Factor VIIa is at least about 8.0 when tested in the "*In Vitro* Proteolysis Assay".

The present invention is suitable for Factor VII/VIIa variants with increased activity compared to wild type human Factor VIIa. Factor VII/VIIa variants with increased activity may be found by testing in suitable assays described in the following. These assays can be performed as a simple preliminary in vitro test. Thus, the section "assays" discloses a simple test (entitled "In Vitro Hydrolysis Assay") for the activity of Factor VIIa variants of the invention. Based thereon, Factor VIIa variants which are of particular interest are such

variants where the ratio between the activity of the variant and the activity of wild type human Factor VII is above 1.0, e.g. at least about 1.25, preferably at least about 2.0, such as at least about 3.0 or, even more preferred, at least about 4.0 when tested in the "In Vitro Hydrolysis Assay".

The activity of the variants can also be measured using a physiological substrate such as factor X ("In Vitro Proteolysis Assay") (see under "assays"), suitably at a concentration of 100-1000 nM, where the factor Xa generated is measured after the addition of a suitable chromogenic substrate (e.g. S-2765). In addition, the activity assay may be run at physiological temperature.

The ability of the Factor VIIa variants to generate thrombin can also be measured in an assay comprising all relevant coagulation factors and inhibitors at physiological concentrations (minus factor VIII when mimicking hemophilia A conditions) and activated platelets (as described on p. 543 in Monroe et al. (1997) Brit. J. Haematol. 99, 542-547 which is hereby incorporated as reference).

The term "identity" as known in the art, refers to a relationship between the sequences of two or more polypeptide molecules or two or more nucleic acid molecules, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between nucleic acid molecules or between polypeptides, as the case may be, as determined by the number of matches between strings of two or more nucleotide residues or two or more amino acid residues. "Identity" measures the percent of identical matches between the smaller of two or more sequences with gap alignments (if any) addressed by a particular mathematical model or computer program (i.e., "algorithms").

The term "similarity" is a related concept, but in contrast to "identity", refers to a sequence relationship that includes both identical matches and conservative substitution matches. If two polypeptide sequences have, for example, (fraction (10/20)) identical amino acids, and the remainder are all non-conservative substitutions, then the percent identity and similarity would both be 50%. If, in the same example, there are 5 more positions where there are conservative substitutions, then the percent identity remains 50%, but the percent similarity would be 75% ((fraction (15/20))). Therefore, in cases where there are conservative substitutions, the degree of similarity between two polypeptides will be higher than the percent identity between those two polypeptides.

The term "isolated polypeptide" refers to a polypeptide of the present invention that (1) has been separated from at least about 50 percent of polynucleotides, lipids, carbohydrates or other materials (i.e., contaminants) with which it is naturally associated, (2) is not covalently linked to all or a portion of a polypeptide to which the "isolated polypeptide" is linked in nature, (3) is operably linked covalently to a polypeptide to which it is not

covalently linked in nature, or (4) does not occur in nature. Preferably, the isolated polypeptide is substantially free from any other contaminating polypeptides or other contaminants that are found in its natural environment which would interfere with its therapeutic, diagnostic, prophylactic or research use.

Conservative modifications to the amino acid sequence of wild type human FVII (and the corresponding modifications to the encoding nucleotides) will produce FVII polypeptides having functional and chemical characteristics similar to those of naturally occurring FVII polypeptide. In contrast, substantial modifications in the functional and/or chemical characteristics of FVII polypeptides may be accomplished by selecting substitutions in the amino acid sequence of wild type human FVII that differ significantly in their effect on maintaining (a) the structure of the molecular backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain.

For example, a "conservative amino acid substitution" may involve a substitution of a native amino acid residue with a nonnative residue such that there is little or no effect on the polarity or charge of the amino acid residue at that position. Furthermore, any native residue in the polypeptide may also be substituted with alanine, as has been previously described for "alanine scanning mutagenesis" (see, for example, MacLennan et al., 1998, Acta Physiol. Scand. Suppl. 643:55-67; Sasaki et al., 1998, Adv. Biophys. 35:1-24, which discuss alanine scanning mutagenesis).

Identity and similarity of related polypeptides can be readily calculated by known methods. Such methods include, but are not limited to, those described in Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part 1, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M. Stockton Press, New York, 1991; and Carillo et al., SIAM J. Applied Math., 48:1073 (1988).

Preferred methods to determine identity and/or similarity are designed to give the largest match between the sequences tested. Methods to determine identity and similarity are described in publicly available computer programs. Preferred computer program methods to determine identity and similarity between two sequences include, but are not limited to, the GCG program package, including GAP (Devereux et al., Nucl. Acid. Res., 12:387 (1984); Genetics Computer Group, University of Wisconsin, Madison, Wis.), BLASTP, BLASTN, and FASTA (Altschul et al., J. Mol. Biol., 215:403-410 (1990)). The BLASTX program is publicly available from the National Center for Biotechnology Information (NCBI) and other sources

(BLAST Manual, Altschul et al. NCB/NLM/NIH Bethesda, Md. 20894; Altschul et al., supra). The well known Smith Waterman algorithm may also be used to determine identity.

Certain alignment schemes for aligning two amino acid sequences may result in the matching of only a short region of the two sequences, and this small aligned region may have very high sequence identity even though there is no significant relationship between the two full length sequences. Accordingly, in a preferred embodiment, the selected alignment method (GAP program) will result in an alignment that spans at least 50 contiguous amino acids of the target polypeptide.

For example, using the computer algorithm GAP (Genetics Computer Group, University of Wisconsin, Madison, Wis.), two polypeptides for which the percent sequence identity is to be determined are aligned for optimal matching of their respective amino acids (the "matched span", as determined by the algorithm). A gap opening penalty (which is calculated as 3.times. the average diagonal; the "average diagonal" is the average of the diagonal of the comparison matrix being used; the "diagonal" is the score or number assigned to each perfect amino acid match by the particular comparison matrix) and a gap extension penalty (which is usually (fraction (1/10)) times the gap opening penalty), as well as a comparison matrix such as PAM 250 or BLOSUM 62 are used in conjunction with the algorithm. A standard comparison matrix (see Dayhoff et al., Atlas of Protein Sequence and Structure, vol. 5, supp.3 (1978) for the PAM 250 comparison matrix; Henikoff et al., Proc. Natl. Acad. Sci USA, 89:10915-10919 (1992) for the BLOSUM 62 comparison matrix) is also used by the algorithm.

Preferred parameters for a polypeptide sequence comparison include the following: Algorithm: Needleman et al., J. Mol. Biol, 48:443-453 (1970); Comparison matrix: BLOSUM 62 from Henikoff et al., Proc. Natl. Acad. Sci. USA, 89:10915-10919 (1992); Gap Penalty: 12, Gap Length Penalty: 4, Threshold of Similarity: 0.

The GAP program is useful with the above parameters. The aforementioned parameters are the default parameters for polypeptide comparisons (along with no penalty for end gaps) using the GAP algorithm.

Preferred parameters for nucleic acid molecule sequence comparisons include the following: Algorithm: Needleman et al., J. Mol Biol., 48:443-453 (1970); Comparison matrix: matches=+10, mismatch=0, Gap Penalty: 50, Gap Length Penalty: 3.

The GAP program is also useful with the above parameters. The aforementioned parameters are the default parameters for nucleic acid molecule comparisons.

Other exemplary algorithms, gap opening penalties, gap extension penalties, comparison matrices, thresholds of similarity, etc. may be used,, including those set forth in the Program Manual, Wisconsin Package, Version 9, September, 1997. The particular

choices to be made will be apparent to those of skill in the art and will depend on the specific comparison to be made, such as DNA to DNA, protein to protein, protein to DNA; and additionally, whether the comparison is between given pairs of sequences (in which case GAP or BestFit are generally preferred) or between one sequence and a large database of sequences (in which case FASTA or BLASTA are preferred).

Factor VII variants having a substantially modified biological activity relative to wild-type Factor VII include, without limitation, Factor VII variants that exhibit TF-independent Factor X proteolytic activity.

As described above the present invention further relates to a method for the treatment of bleeding episodes in a subject or for the enhancement of the normal haemostatic system, the method comprising administering to a subject in need thereof a therapeutically or prophylactically effective amount of a composition comprising a Factor VII polypeptide.

It is to be understood, that the subject is treated for the same bleeding or series of bleedings.

The term "enhancement of the normal haemostatic system" means an enhancement of the ability to generate thrombin.

As used herein the term "bleeding disorder" reflects any defect, congenital, acquired or induced, of cellular or molecular origin that is manifested in bleedings. Examples are clotting factor deficiencies (e.g. haemophilia A and B or deficiency of coagulation Factors XI or VII), clotting factor inhibitors, defective platelet function, thrombocytopenia or von Willebrand's disease.

The term "bleeding episodes" is meant to include uncontrolled and excessive bleeding which is a major problem both in connection with surgery and other forms of tissue damage. Uncontrolled and excessive bleeding may occur in subjects having a normal coagulation system and subjects having coagulation or bleeding disorders. Clotting factor deficiencies (haemophilia A and B, deficiency of coagulation factors XI or VII) or clotting factor inhibitors may be the cause of bleeding disorders. Excessive bleedings also occur in subjects with a normally functioning blood clotting cascade (no clotting factor deficiencies or -inhibitors against any of the coagulation factors) and may be caused by a defective platelet function, thrombocytopenia or von Willebrand's disease. In such cases, the bleedings may be likened to those bleedings caused by haemophilia because the haemostatic system, as in haemophilia, lacks or has abnormal essential clotting "compounds" (such as platelets or von Willebrand factor protein) that causes major bleedings. In subjects who experience extensive tissue damage in association with surgery or vast trauma, the normal haemostatic mechanism may be overwhelmed by the demand of immediate haemostasis and they may

develop bleeding in spite of a normal haemostatic mechanism. Achieving satisfactory haemostasis also is a problem when bleedings occur in organs such as the brain, inner ear region and eyes with limited possibility for surgical haemostasis. The same problem may arise in the process of taking biopsies from various organs (liver, lung, tumour tissue, gastrointestinal tract) as well as in laparoscopic surgery. Common for all these situations is the difficulty to provide haemostasis by surgical techniques (sutures, clips, etc.) which also is the case when bleeding is diffuse (haemorrhagic gastritis and profuse uterine bleeding). Acute and profuse bleedings may also occur in subjects on anticoagulant therapy in whom a defective haemostasis has been induced by the therapy given. Such subjects may need surgical interventions in case the anticoagulant effect has to be counteracted rapidly. Radical retropubic prostatectomy is a commonly performed procedure for subjects with localized prostate cancer. The operation is frequently complicated by significant and sometimes massive blood loss. The considerable blood loss during prostatectomy is mainly related to the complicated anatomical situation, with various densely vascularized sites that are not easily accessible for surgical haemostasis, and which may result in diffuse bleeding from a large area. Another situation that may cause problems in the case of unsatisfactory haemostasis is when subjects with a normal haemostatic mechanism are given anticoagulant therapy to prevent thromboembolic disease. Such therapy may include heparin, other forms of proteoglycans, warfarin or other forms of vitamin K-antagonists as well as aspirin and other platelet aggregation inhibitors.

In one embodiment of the invention, the bleeding is associated with haemophilia. In another embodiment, the bleeding is associated with haemophilia with acquired inhibitors. In another embodiment, the bleeding is associated with thrombocytopenia. In another embodiment, the bleeding is associated with von Willebrand's disease. In another embodiment, the bleeding is associated with severe tissue damage. In another embodiment, the bleeding is associated with severe trauma. In another embodiment, the bleeding is associated with surgery. In another embodiment, the bleeding is associated with laparoscopic surgery. In another embodiment, the bleeding is associated with haemorrhagic gastritis. In another embodiment, the bleeding is profuse uterine bleeding. In another embodiment, the bleeding is occurring in organs with a limited possibility for mechanical haemostasis. In another embodiment, the bleeding is occurring in the brain, inner ear region or eyes. In another embodiment, the bleeding is associated with the process of taking biopsies. In another embodiment, the bleeding is associated with anticoagulant therapy.

The term "subject" as used herein is intended to mean any animal, in particular mammals, such as humans, and may, where appropriate, be used interchangeably with the term "patient".

In the present specification and claims, the term "amino acid" denotes any molecule having the formula COOH-CR-NH_3, i.e. the term includes within its scope both naturally occurring and non-naturally occurring L- and D-amino acids. In most cases, amino acid manipulation discussed herein will involve use of naturally occurring L-amino acids:

Amino acid	Tree-letter code	One-letter code
Glycine	Gly	G
Proline	Pro	P
Alanine	Ala	A
Valine	Val	V
Leucine	Leu	L
Isoleucine	Ile	I
Methionine	Met	M
Cysteine	Cys	C
Phenylalanine	Phe	F
Tyrosine	Tyr	Y
Tryptophan	Trp	W
Histidine	His	H
Lysine	Lys	K
Arginine	Arg	R
Glutamine	Gln	Q
Asparagine	Asn	N
Glutamic Acid	Glu	E
Aspartic Acid	Asp	D
Serine	Ser	S
Threonine	Thr	T

The Factor VII polypeptides described herein may be produced by means of recombinant nucleic acid techniques. In general, a cloned wild-type Factor VII nucleic acid sequence is modified to encode the desired protein. This modified sequence is then inserted into an expression vector, which is in turn transformed or transfected into host cells. In one embodiment, higher eukaryotic cells, in particular cultured mammalian cells, are used as host cells. The complete nucleotide and amino acid sequences for human Factor VII are known (see U.S. 4,784,950, where the cloning and expression of recombinant human Factor VII is described). The bovine Factor VII sequence is described in Takeya et al., J. Biol. Chem. 263:14868-14872 (1988)).

The amino acid sequence alterations may be accomplished by a variety of techniques. Modification of the nucleic acid sequence may be by site-specific mutagenesis. Techniques for site-specific mutagenesis are well known in the art and are described in, for example, Zoller and Smith (DNA 3:479-488, 1984) or "Splicing by extension overlap", Horton et al., Gene 77, 1989, pp. 61-68. Thus, using the nucleotide and amino acid sequences of Factor VII, one may introduce the alteration(s) of choice. Likewise, procedures for preparing a DNA construct using polymerase chain reaction using specific primers are well known to

persons skilled in the art (cf. PCR Protocols, 1990, Academic Press, San Diego, California, USA).

The nucleic acid construct encoding the Factor VII polypeptide of the invention may suitably be of genomic or cDNA origin, for instance obtained by preparing a genomic or cDNA library and screening for DNA sequences coding for all or part of the polypeptide by hybridization using synthetic oligonucleotide probes in accordance with standard techniques (cf. Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd. Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989).

The nucleic acid construct encoding the Factor VII polypeptide may also be prepared synthetically by established standard methods, e.g. the phosphoamidite method described by Beaucage and Caruthers, Tetrahedron Letters 22 (1981), 1859 - 1869, or the method described by Matthes et al., EMBO Journal 3 (1984), 801 - 805. According to the phosphoamidite method, oligonucleotides are synthesised, e.g. in an automatic DNA synthesiser, purified, annealed, ligated and cloned in suitable vectors.

Furthermore, the nucleic acid construct may be of mixed synthetic and genomic, mixed synthetic and cDNA or mixed genomic and cDNA origin prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate), the fragments corresponding to various parts of the entire nucleic acid construct, in accordance with standard techniques.

The nucleic acid construct is preferably a DNA construct. DNA sequences for use in producing Factor VII polypeptides according to the present invention will typically encode a pre-pro polypeptide at the amino-terminus of Factor VII to obtain proper posttranslational processing (e.g. gamma-carboxylation of glutamic acid residues) and secretion from the host cell. The pre-pro polypeptide may be that of Factor VII or another vitamin K-dependent plasma protein, such as Factor IX, Factor X, prothrombin, protein C or protein S. As will be appreciated by those skilled in the art, additional modifications can be made in the amino acid sequence of the Factor VII polypeptides where those modifications do not significantly impair the ability of the protein to act as a coagulant. For example, the Factor VII polypeptides can also be modified in the activation cleavage site to inhibit the conversion of zymogen Factor VII into its activated two-chain form, as generally described in U.S. 5,288,629.

Expression vectors for use in expressing Factor VIIa variants will comprise a promoter capable of directing the transcription of a cloned gene or cDNA. Preferred promoters for use in cultured mammalian cells include viral promoters and cellular promoters. Viral promoters include the SV40 promoter (Subramani et al., Mol. Cell. Biol. 1:854-864, 1981) and the CMV promoter (Boshart et al., Cell 41:521-530, 1985). A

particularly preferred viral promoter is the major late promoter from adenovirus 2 (Kaufman and Sharp, Mol. Cell. Biol. 2:1304-1319, 1982). Cellular promoters include the mouse kappa gene promoter (Bergman et al., Proc. Natl. Acad. Sci. USA 81:7041-7045, 1983) and the mouse VH promoter (Loh et al., Cell 33:85-93, 1983). A particularly preferred cellular promoter is the mouse metallothionein-I promoter (Palmiter et al., Science 222:809-814, 1983). Expression vectors may also contain a set of RNA splice sites located downstream from the promoter and upstream from the insertion site for the Factor VII sequence itself. Preferred RNA splice sites may be obtained from adenovirus and/or immunoglobulin genes. Also contained in the expression vectors is a polyadenylation signal located downstream of the insertion site. Particularly preferred polyadenylation signals include the early or late polyadenylation signal from SV40 (Kaufman and Sharp, ibid.), the polyadenylation signal from the adenovirus 5 Elb region, the human growth hormone gene terminator (DeNoto et al. Nucl. Acids Res. 9:3719-3730, 1981) or the polyadenylation signal from the human Factor VII gene or the bovine Factor VII gene. The expression vectors may also include a noncoding viral leader sequence, such as the adenovirus 2 tripartite leader, located between the promoter and the RNA splice sites; and enhancer sequences, such as the SV40 enhancer.

Cloned DNA sequences are introduced into cultured mammalian cells by, for example, calcium phosphate-mediated transfection (Wigler et al., Cell 14:725-732, 1978; Corsaro and Pearson, Somatic Cell Genetics 7:603-616, 1981; Graham and Van der Eb, Virology 52d:456-467, 1973) or electroporation (Neumann et al., EMBO J. 1:841-845, 1982). To identify and select cells that express the exogenous DNA, a gene that confers a selectable phenotype (a selectable marker) is generally introduced into cells along with the gene or cDNA of interest. Preferred selectable markers include genes that confer resistance to drugs such as neomycin, hygromycin, and methotrexate. The selectable marker may be an amplifiable selectable marker. A preferred amplifiable selectable marker is a dihydrofolate reductase (DHFR) sequence. Selectable markers are reviewed by Thilly (Mammalian Cell Technology, Butterworth Publishers, Stoneham, MA, incorporated herein by reference). The person skilled in the art will easily be able to choose suitable selectable markers.

Selectable markers may be introduced into the cell on a separate plasmid at the same time as the gene of interest, or they may be introduced on the same plasmid. If, on the same plasmid, the selectable marker and the gene of interest may be under the control of different promoters or the same promoter, the latter arrangement producing a dicistronic message. Constructs of this type are known in the art (for example, Levinson and Simonsen, U.S. 4,713,339). It may also be advantageous to add additional DNA, known as "carrier DNA," to the mixture that is introduced into the cells. After the cells have taken up the DNA, they are grown in an appropriate growth medium, typically for 1-2 days, to begin expressing

the gene of interest. The medium used to culture the cells may be any conventional medium suitable for growing the host cells, such as minimal or complex media containing appropriate supplements. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. in catalogues of the American Type Culture Collection). The media are prepared using procedures known in the art (see, e.g., references for bacteria and yeast; Bennett, J.W. and LaSure, L., editors, More Gene Manipulations in Fungi, Academic Press, CA, 1991). Growth media generally include a carbon source, a nitrogen source, essential amino acids, essential sugars, vitamins, salts, phospholipids, proteins and growth factors. For production of gamma-carboxylated Factor VII polypeptides, the medium will contain vitamin K, preferably at a concentration of about 0.1 mg/ml to about 5 mg/ml. Drug selection is then applied to select for the growth of cells that are expressing the selectable marker in a stable fashion. For cells that have been transfected with an amplifiable selectable marker the drug concentration may be increased to select for an increased copy number of the cloned sequences, thereby increasing expression levels. Clones of stably transfected cells are then screened for expression of the desired Factor VII polypeptide.

Preferred mammalian cell lines include the CHO (ATCC CCL 61), COS-1 (ATCC CRL 1650), baby hamster kidney (BHK) and HEK293 (e.g., ATCC CRL 1573; Graham et al., J. Gen. Virol. 36:59-72, 1977) cell lines. A preferred BHK cell line is the tk- ts13 BHK cell line (Waechter and Baserga, Proc. Natl. Acad. Sci. USA 79:1106-1110, 1982), hereinafter referred to as BHK 570 cells. The BHK 570 cell line is available from the American Type Culture Collection, 12301 Parklawn Dr., Rockville, MD 20852, under ATCC accession number CRL 10314. A tk- ts13 BHK cell line is also available from the ATCC under accession number CRL 1632. In addition, a number of other cell lines may be used, including Rat Hep I (Rat hepatoma; ATCC CRL 1600), Rat Hep II (Rat hepatoma; ATCC CRL 1548), TCMK (ATCC CCL 139), Human lung (ATCC HB 8065), NCTC 1469 (ATCC CCL 9.1) and DUKX cells (Urlaub and Chasin, Proc. Natl. Acad. Sci. USA 77:4216-4220, 1980).

Transgenic animal technology may be employed to produce the Factor VII polypeptides of the invention. It is preferred to produce the proteins within the mammary glands of a host female mammal. Expression in the mammary gland and subsequent secretion of the protein of interest into the milk overcomes many difficulties encountered in isolating proteins from other sources. Milk is readily collected, available in large quantities, and biochemically well characterized. Furthermore, the major milk proteins are present in milk at high concentrations (typically from about 1 to 15 g/l).

From a commercial point of view, it is clearly preferable to use as the host a species that has a large milk yield. While smaller animals such as mice and rats can be used (and are preferred at the proof of principle stage), it is preferred to use livestock mammals

including, but not limited to, pigs, goats, sheep and cattle. Sheep are particularly preferred due to such factors as the previous history of transgenesis in this species, milk yield, cost and the ready availability of equipment for collecting sheep milk (see, for example, WO 88/00239 for a comparison of factors influencing the choice of host species). It is generally desirable to select a breed of host animal that has been bred for dairy use, such as East Friesland sheep, or to introduce dairy stock by breeding of the transgenic line at a later date. In any event, animals of known, good health status should be used.

To obtain expression in the mammary gland, a transcription promoter from a milk protein gene is used. Milk protein genes include those genes encoding caseins (see U.S. 5,304,489), beta lactoglobulin, a lactalbumin, and whey acidic protein. The beta lactoglobulin (BLG) promoter is preferred. In the case of the ovine beta lactoglobulin gene, a region of at least the proximal 406 bp of 5' flanking sequence of the gene will generally be used, although larger portions of the 5' flanking sequence, up to about 5 kbp, are preferred, such as a ~4.25 kbp DNA segment encompassing the 5' flanking promoter and non coding portion of the beta lactoglobulin gene (see Whitelaw et al., Biochem. J. 286: 31 39 (1992)). Similar fragments of promoter DNA from other species are also suitable.

Other regions of the beta lactoglobulin gene may also be incorporated in constructs, as may genomic regions of the gene to be expressed. It is generally accepted in the art that constructs lacking introns, for example, express poorly in comparison with those that contain such DNA sequences (see Brinster et al., Proc. Natl. Acad. Sci. USA 85: 836 840 (1988); Palmiter et al., Proc. Natl. Acad. Sci. USA 88: 478 482 (1991); Whitelaw et al., Transgenic Res. 1: 3 13 (1991); WO 89/01343; and WO 91/02318, each of which is incorporated herein by reference). In this regard, it is generally preferred, where possible, to use genomic sequences containing all or some of the native introns of a gene encoding the protein or polypeptide of interest, thus the further inclusion of at least some introns from, e.g, the beta lactoglobulin gene, is preferred. One such region is a DNA segment that provides for intron splicing and RNA polyadenylation from the 3' non coding region of the ovine beta lactoglobulin gene. When substituted for the natural 3' non coding sequences of a gene, this ovine beta lactoglobulin segment can both enhance and stabilize expression levels of the protein or polypeptide of interest. Within other embodiments, the region surrounding the initiation ATG of the variant Factor VII sequence is replaced with corresponding sequences from a milk specific protein gene. Such replacement provides a putative tissue specific initiation environment to enhance expression. It is convenient to replace the entire variant Factor VII pre pro and 5' non coding sequences with those of, for example, the BLG gene, although smaller regions may be replaced.

For expression of Factor VII polypeptides in transgenic animals, a DNA segment encoding variant Factor VII is operably linked to additional DNA segments required for its expression to produce expression units. Such additional segments include the above mentioned promoter, as well as sequences that provide for termination of transcription and polyadenylation of mRNA. The expression units will further include a DNA segment encoding a secretory signal sequence operably linked to the segment encoding modified Factor VII. The secretory signal sequence may be a native Factor VII secretory signal sequence or may be that of another protein, such as a milk protein (see, for example, von Heijne, Nucl. Acids Res. 14: 4683 4690 (1986); and Meade et al., U.S. 4,873,316, which are incorporated herein by reference).

Construction of expression units for use in transgenic animals is conveniently carried out by inserting a variant Factor VII sequence into a plasmid or phage vector containing the additional DNA segments, although the expression unit may be constructed by essentially any sequence of ligations. It is particularly convenient to provide a vector containing a DNA segment encoding a milk protein and to replace the coding sequence for the milk protein with that of a variant Factor VII polypeptide; thereby creating a gene fusion that includes the expression control sequences of the milk protein gene. In any event, cloning of the expression units in plasmids or other vectors facilitates the amplification of the variant Factor VII sequence. Amplification is conveniently carried out in bacterial (e.g. E. coli) host cells, thus the vectors will typically include an origin of replication and a selectable marker functional in bacterial host cells. The expression unit is then introduced into fertilized eggs (including early stage embryos) of the chosen host species. Introduction of heterologous DNA can be accomplished by one of several routes, including microinjection (e.g. U.S. Patent No. 4,873,191), retroviral infection (Jaenisch, Science 240: 1468 1474 (1988)) or site directed integration using embryonic stem (ES) cells (reviewed by Bradley et al., Bio/Technology 10: 534 539 (1992)). The eggs are then implanted into the oviducts or uteri of pseudopregnant females and allowed to develop to term. Offspring carrying the introduced DNA in their germ line can pass the DNA on to their progeny in the normal, Mendelian fashion, allowing the development of transgenic herds. General procedures for producing transgenic animals are known in the art (see, for example, Hogan et al., Manipulating the Mouse Embryo: A Laboratory Manual, Cold Spring Harbor Laboratory, 1986; Simons et al., Bio/Technology 6: 179 183 (1988); Wall et al., Biol. Reprod. 32: 645 651 (1985); Buhler et al., Bio/Technology 8: 140 143 (1990); Ebert et al., Bio/Technology 9: 835 838 (1991); Krimpenfort et al., Bio/Technology 9: 844 847 (1991); Wall et al., J. Cell. Biochem. 49: 113 120 (1992); U.S. 4,873,191; U.S. 4,873,316; WO 88/00239, WO 90/05188, WO 92/11757; and GB 87/00458). Techniques for introducing foreign DNA sequences into mammals and

their germ cells were originally developed in the mouse (see, e.g., Gordon et al., Proc. Natl. Acad. Sci. USA 77: 7380 7384 (1980); Gordon and Ruddle, Science 214: 1244 1246 (1981); Palmiter and Brinster, Cell 41: 343 345 (1985); Brinster et al., Proc. Natl. Acad. Sci. USA 82: 4438 4442 (1985); and Hogan et al. (ibid.)). These techniques were subsequently adapted for use with larger animals, including livestock species (see, e.g., WO 88/00239, WO 90/05188, and WO 92/11757; and Simons et al., Bio/Technology 6: 179 183 (1988)). To summarise, in the most efficient route used to date in the generation of transgenic mice or livestock, several hundred linear molecules of the DNA of interest are injected into one of the pro nuclei of a fertilized egg according to established techniques. Injection of DNA into the cytoplasm of a zygote can also be employed.

Production in transgenic plants may also be employed. Expression may be generalised or directed to a particular organ, such as a tuber (see, Hiatt, Nature 344:469 479 (1990); Edelbaum et al., J. Interferon Res. 12:449 453 (1992); Sijmons et al., Bio/Technology 8:217 221 (1990); and EP 0 255 378).

The Factor VII polypeptides of the invention are recovered from cell culture medium or milk. The Factor VII polypeptides of the present invention may be purified by a variety of procedures known in the art including, but not limited to, chromatography (e.g., ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (e.g., preparative isoelectric focusing (IEF), differential solubility (e.g., ammonium sulfate precipitation), or extraction (see, e.g., Protein Purification, J.-C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989). Preferably, they may be purified by affinity chromatography on an anti-Factor VII antibody column. The use of calcium-dependent monoclonal antibodies is described by Wakabayashi et al., J. Biol. Chem. 261:11097-11108, (1986) and Thim et al., Biochemistry 27: 7785-7793, (1988). Additional purification may be achieved by conventional chemical purification means, such as high performance liquid chromatography. Other methods of purification, including barium citrate precipitation, are known in the art, and may be applied to the purification of the novel Factor VII polypeptides described herein (see, for example, Scopes, R., Protein Purification, Springer-Verlag, N.Y., 1982).

For the purpose of recombinant expression of Factor VII polypeptides in prokaryotic cells, the nucleic acid fragments encoding the Factor VII polypeptides will normally be inserted in suitable vectors to form cloning or expression vectors carrying the nucleic acid fragments of the invention. Details concerning the construction of these vectors of the invention will be discussed in context of transformed cells and microorganisms below. The vectors can be in the form of plasmids, phages, cosmids, or mini-chromosomes. Preferred

cloning and expression vectors used in the invention are capable of autonomous replication, thereby enabling high copy-numbers for the purposes of high-level expression or high-level replication for subsequent cloning.

The general outline of a vector for use in the of the invention comprises the following features in the 5'-3' direction and in operable linkage: a promoter for driving expression of the nucleic acid fragment encoding the Factor VII polypeptides, optionally a nucleic acid sequence encoding a leader peptide enabling secretion (to the extracellular phase or, where applicable, into the periplasm) of or integration into the membrane of the Factor VII polypeptides, the nucleic acid fragment of the invention, and optionally a nucleic acid sequence encoding a terminator. When operating with expression vectors in producer strains or cell-lines it is for the purposes of genetic stability of the transformed cell preferred that the vector when introduced into a host cell is integrated in the host cell genome.

The vectors of the invention are used to transform host cells to produce the Factor VII polypeptides. Such transformed cells can be cultured cells or cell lines used for propagation of the nucleic acid fragments and vectors encoding the second polyamino acod or used for recombinant production of Factor VII polypeptides.

In one embodiment, the transformed cells of the invention are microorganisms such as bacteria (such as the species Escherichia [e.g. E. coli], Bacillus [e.g. Bacillus subtilis], Rhodococcus, Streptomycetes, Actinomycetes, Corynebacteria, Pseudomonas, Erwinia or Salmonella; yeasts (such as Saccharomyces cerevisiae and Picchia); insect cells; and protozoans. Alternatively, the transformed cells are derived from a multicellular organism such as a fungi (Penicillium, Fusarium, Aspergillus, Podospora, Neurospora) or a plant cell.

In one embodiment of the invention, the Factor VII polypeptide is non-glycosylated.

Prokaryotic host cells, including strains of the bacteria Escherichia coli, Bacillus and other genera are the most useful host cells within the present invention. Techniques for transforming these hosts and expressing foreign DNA sequences cloned therein are well known in the art (see, e.g., Sambrook et al., ibid.). When expressing DNA encoding a Factor VII polypeptides in bacteria such as E. coli, the polypeptide may be retained in the cytoplasm, typically as insoluble granules, or may be directed to the periplasmic space by a bacterial secretion sequence. In the former case, the cells are lysed, and the granules are recovered and denatured using, for example, guanidine isothiocyanate or urea. The denatured polypeptide can then be refolded by diluting the denaturant, such as by dialysis against a solution of urea and a combination of reduced and oxidized glutathione, followed by dialysis against a buffered saline solution. In the latter case, the polypeptide can be recovered from the periplasmic space in a soluble and functional form by disrupting the cells (by, for example, sonication or osmotic shock) to release the contents of the periplasmic

space and recovering the polypeptide, thereby obviating the need for denaturation and refolding. Methods for producing heterologous disulfide bond-containing polypeptides in bacterial cells are disclosed by Georgiou et al., U.S. Pat. No. 6,083,715.

For the purposes of cloning and/or optimized expression it is preferred that the transformed cell is capable of replicating the nucleic acid fragment encoding the Factor VII polypeptides. To ultimately produce the Factor VII polypeptides, transformed cells must express the nucleic acid fragment encoding the first polyamino acid. It is convenient, although far from essential, that the expression product is either exported out into the culture medium or carried on the surface of the transformed cell.

When an effective producer cell has been identified it is preferred, on the basis thereof, to establish a stable cell line which carries the which expresses the nucleic acid fragment encoding the Factor VII polypeptides. Preferably, this stable cell line secretes or carries on its surfaces the Factor VII polypeptides, thereby facilitating purification thereof. In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with the hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, E. coli is typically transformed using pBR322, a plasmid derived from an E. coli species. The pBR322 plasmid contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR plasmid, or other microbial plasmid or phage must also contain, or be modified to contain, promoters which can be used by the prokaryotic microorganism for expression.

Those promoters most commonly used in prokaryotic recombinant DNA construction include the B-lactamase (penicillinase) and lactose promoter systems (Chang et al., 1978; Itakura et al., 1977; Goeddel et al., 1979) and a tryptophan (trp) promoter system (Goeddel et al., 1979; EP-A-0 036 776). While these are the most commonly used, other microbial promoters have been discovered and utilized, and details concerning their nucleotide sequences have been published, enabling a skilled worker to ligate them functionally with plasmid vectors (Siebwenlist et al., 1980). Certain genes may be expressed efficiently in E. coli from their own promoter sequences, precluding the need for addition of another promoter by artificial means.

In addition to prokaryotes, eukaryotic microbes, such as yeast cultures may also be used, and here the promoter should be capable of driving expression. Saccharomyces cerevisiase, or common baker's yeast is the most commonly used among eukaryotic microorganisms, although a number of other strains are commonly available. For expression in Saccharomyces, the plasmid YRp7, for example, is commonly used. This plasmid already

contains the trpl gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan for example ATCC No. 44076 or PEP4-1. The presence of the trpl lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoting sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase or other glycolytic enzymes, such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. In constructing suitable expression plasmids, the termination sequences associated with these genes are also ligated into the expression vector 3' of the sequence desired to be expressed to provide polyadenylation of the mRNA and termination.

Other promoters, which have the additional advantage of transcription controlled by growth conditions are the promoter region for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Any plasmid vector containing a yeast-compatible promoter, origin of replication and termination sequences is suitable.

For therapeutic purposes it is preferred that the Factor VII polypeptides of the invention are substantially pure. Thus, in a preferred embodiment of the invention the Factor VII polypeptides of the invention is purified to at least about 90 to 95% homogeneity, preferably to at least about 98% homogeneity. Purity may be assessed by e.g. gel electrophoresis and amino-terminal amino acid sequencing.

The Factor VII polypeptide is cleaved at its activation site in order to convert it to its two-chain form. Activation may be carried out according to procedures known in the art, such as those disclosed by Osterud, et al., Biochemistry 11:2853-2857 (1972); Thomas, U.S. Patent No. 4,456,591; Hedner and Kisiel, J. Clin. Invest. 71:1836-1841 (1983); or Kisiel and Fujikawa, Behring Inst. Mitt. 73:29-42 (1983). Alternatively, as described by Bjoern et al. (Research Disclosure, 269 September 1986, pp. 564-565), Factor VII may be activated by passing it through an ion-exchange chromatography column, such as Mono Q (Pharmacia fine Chemicals) or the like. The resulting activated Factor VII polypeptide may then be formulated and administered as described below.

Assays

Assay 1:

In Vitro Hydrolysis Assay

Wild type (native) Factor VIIa and Factor VIIa variant (both hereafter referred to as "Factor VIIa") are assayed in parallel to directly compare their specific activities. The assay is carried out in a microtiter plate (MaxiSorp, Nunc, Denmark). The chromogenic substrate D-Ile-Pro-Arg-p-nitroanilide (S-2288, Chromogenix, Sweden), final concentration 1 mM, is added to Factor VIIa (final concentration 100 nM) in 50 mM Hepes, pH 7.4, containing 0.1 M NaCl, 5 mM $CaCl_2$ and 1 mg/ml bovine serum albumin. The absorbance at 405 nm is meas-ured continuously in a SpectraMax® 340 plate reader (Molecular Devices, USA). The absorbance developed during a 20-minute incubation, after subtraction of the absorbance in a blank well containing no enzyme, is used to calculate the ratio between the activities of vari-ant and wild-type Factor VIIa:

Ratio = (A405 nm Factor VIIa variant)/(A405 nm Factor VIIa wild-type).

Assay 2:

In Vitro Proteolysis Assay

Wild type (native) Factor VIIa and Factor VIIa variant (both hereafter referred to as "Factor VIIa") are assayed in parallel to directly compare their specific activities. The assay is carried out in a microtiter plate (MaxiSorp, Nunc, Denmark). Factor VIIa (10 nM) and Factor X (0.8 microM) in 100 microL 50 mM Hepes, pH 7.4, containing 0.1 M NaCl, 5 mM $CaCl_2$ and 1 mg/ml bovine serum albumin, are incubated for 15 min. Factor X cleavage is then stopped by the addition of 50 microL 50 mM Hepes, pH 7.4, containing 0.1 M NaCl, 20 mM EDTA and 1 mg/ml bovine serum albumin. The amount of Factor Xa generated is measured by addition of the chromogenic substrate Z-D-Arg-Gly-Arg-p-nitroanilide (S-2765, Chromogenix, Sweden), final concentration 0.5 mM. The absorbance at 405 nm is measured continuously in a SpectraMax® 340 plate reader (Molecular Devices, USA). The absorbance developed dur-ing 10 minutes, after subtraction of the absorbance in a blank well containing no FVIIa, is used to calculate the ratio between the proteolytic activities of variant and wild-type Factor VIIa:

Ratio = (A405 nm Factor VIIa variant)/(A405 nm Factor VIIa wild-type).

The invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed, since these embodiments are intended as illustrations of several aspects of the invention. Any equivalent embodiments are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the

foregoing description. Such modifications are also intended to fall within the scope of the appended claims. In the case of conflict, the present disclosure including definitions will control.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. The amino acid sequence of wild type human coagulation Factor VII.

EXAMPLES

Example 1

Production of FVII polypeptides according to the invention by Cathepsin G cleavage for removal of amino acid residues 1-44 relative to the amino acid sequence of SEQ ID NO:1.

DNA constructs encoding FVII polypeptides comprising one or more amino acid substitutions relative to the amino acid sequence of SEQ ID NO:1 may be prepared by site-directed mutagenesis using a supercoiled, double stranded DNA vector with an insert of interest and two synthetic primers containing the desired mutation as described in Published international patent applications WO 01/83725, WO 02/22776, WO 03/027147, WO 02/077218, and WO 03/037932 and Danish patent application PA 2002 01423. Briefly oligonucleotide primers, each complementary to opposite strands of the vector, are extended during temperature cycling by means of Pfu DNA polymerase. On incorporation of the primers, a mutated plasmid containing staggered nicks is generated. Following temperature cycling, the product is treated with DpnI which is specific for methylated and hemi-methylated DNA to digest the parental DNA template and to select for mutation-containing synthesized DNA.

Procedures for preparing a DNA construct using polymerase chain reaction using specific primers are well known to persons skilled in the art (cf. PCR Protocols, 1990, Academic Press, San Diego, California, USA).

FVII polypeptides comprising one or more amino acid substitutions relative to the amino acid sequence of SEQ ID NO:1 may be prepared by transfection in BHK cells essentially as previously described (Thim et al. (1988) Biochemistry 27, 7785-7793; Persson and Nielsen (1996) FEBS Lett. 385, 241-243) to obtain expression of the FVII polypeptide variant.

The FVII polypeptides comprising one or more amino acid substitutions relative to the amino acid sequence of SEQ ID NO:1 may be purified as follows:
Conditioned medium is loaded onto a 25-ml column of Q Sepharose Fast Flow (Pharmacia Biotech) after addition of 5 mM EDTA, 0.1% Triton X-100 and 10 mM Tris, adjustment of pH to 8.0 and adjustment of the conductivity to 10-11 mS/cm by adding water.
Elution of the protein is accomplished by stepping from 10 mM Tris, 50 mM NaCl, 0.1% Triton X-100, pH 8.0 to 10 mM Tris, 50 mM NaCl, 25 mM CaCl2, 0.1% Triton X-100, pH 8.0. The fractions containing the FVII polypeptide variant are pooled and applied to a 25-ml column containing monoclonal antibody F1A2 (Novo Nordisk, Bagsværd, Denmark) coupled to CNBr-activated Sepharose 4B (Pharmacia Biotech).
The column is equilibrated with 50 mM Hepes, pH 7.5, containing 10 mM CaCl2,100 mM NaCl and 0.02% Triton X-100. After washing with equilibration buffer and equilibration buffer containing 2 M NaCl, bound material is eluted with equilibration buffer containing 10 mM EDTA instead of CaCl2. Before use or storage, excess CaCl2 over EDTA is added or the FVII polypeptide variant is transferred to a Ca2+-containing buffer. The yield of each step is followed by factor VII ELISA measurements and the purified protein is analysed by SDS-PAGE.

Following purification of the the FVII polypeptides comprising one or more amino acid substitutions relative to the amino acid sequence of SEQ ID NO:1 the polypeptide is treated with Cathepsin G cleavage for removal of amino acid residues 1-44 relative to the amino acid sequence of SEQ ID NO:1 as described in Nicolaisen et al. (1992) FEBS Lett. 306, 157-160.

Example 2

Production of FVII polypeptides according to the invention by removal of DNA sequence encoding functional lipid membrane binding domain amino acid residues from DNA constructs encoding FVII polypeptides.

Construction of DNA encoding truncated versions of FVII polypeptides as examplified by FVII polypeptides encompassing residues 42-406 (FVII-(42-406)) and 83-406 (FVII-(83-406)):
DNA constructs encoding N-terminally truncated versions of FVII polypeptides were prepared by site-directed mutagenesis using two synthetic primers bridging the desired deletion and a supercoiled, double stranded DNA vector with insert of unprocessed human FVII, i.e. comprising the FVII leader peptide (as described in US Patent 4,784,950) followed by the mature FVII sequence (Figure 1, SEQ ID NO:1). Each primer was designed to simultaneously anneal to the two regions of the DNA vector flanking the desired deletion. Since deletions

encompassed the N-terminal part of mature FVII, one flanking region (underlined) was located in the leader-peptide sequence.

For FVII-(42-406):

5'-GGCGTCCTGCACCGGCGCCGGCGCATTTCTTACAGTGATGGGGACCAGTGTGCCTC-3'
(SEQ ID NO:2)

5'-GAGGCACACTGGTCCCCATCACTGTAAGAAATGCGCCGGCGCCGGTGCAGGACGCC-3'
(SEQ ID NO:3)

For FVII-(83-406):

5'- GGCGTCCTGCACCGGCGCCGGCGCACGCACAAGGATGACCAGCTGATCTGTGTG-3'
(SEQ ID NO:4)

5'- CACACAGATCAGCTGGTCATCCTTGTGCGTGCGCCGGCGCCGGTGCAGGACGCC-3'
(SEQ ID NO:5)

The oligonucleotide primers, each complementary to opposite strands of the vector insert, were extended during temperature cycling by means of Pfu DNA polymerase. On incorporation of the primers, a mutated plasmid containing staggered nicks was generated. Following temperature cycling, the product was treated with DpnI which is specific for methylated and hemimethylated DNA to digest the parental DNA template and to select for mutation-containing synthesized DNA.

Procedures for preparing a DNA construct using polymerase chain reaction using specific primers are well known to persons skilled in the art (cf. *PCR Protocols*, 1990, Academic Press, San Diego, California, USA).

Example 3

Demonstration of the procoagulant activity of a FVII polypeptide lacking residues 1-44.

Anti-FVIII antibody (final concentration 10 human Bethesda units/ml) was added to citrated human blood from a normal donor to induce a haemophilia A-like condition. Full-length or des(1-44)-FVIIa was added to this blood to a final concentration of 25 or 100 nM. Innovin (relipidated recombinant human tissue factor, final dilution 1:50000) and t-PA (final concentration 1.8 nM) was also added. Blood clotting was initiated by the addition of buffer containing $CaCl_2$, and the development of a fibrin clot was monitored using thrombelastography. The procoagulant activity of des(1-44)-FVIIa was similar to that of FVIIa. For instance, the presence of 100 nM des(1-44)-

FVIIa gave clotting times of 490 and 455 seconds in two samples, whereas 100 nM FVIIa gave clotting times of 390 and 410 seconds. All four samples were based on the same donor blood. The rate by which the clot grew, represented by the so-called angle value, was also similar in the two des(1-44)-FVIIa samples (59 and 62°) and in the two FVIIa samples (57 and 61°). The maximal clot strength, represented by the maximal amplitude value, was between 51 and 54 mm in the four samples, i.e. similar for FVIIa and des(1-44)-FVIIa. The FVIIa variant V158D/E296V/M298Q-FVIIa also displayed similar results in its full-length and des(1-44) forms when tested at 1 nM.

CLAIMS

1. A FVII polypeptide comprising one or more amino acid substitutions relative to the amino acid sequence of SEQ ID NO:1, wherein said amino acid substitutions are replacement with any other amino acid of one or more amino acids selected from the group consisting of K157, V158, E296, M298, L305, M306, D309, S314, D334, S336, K337, F374, and wherein said FVII polypeptide is essentially free of GLA residues.

2. The Factor VII polypeptide according to claim 1, wherein K157 is replaced with any other amino acid.

3. The Factor VII polypeptide according to claims 1 or 2, wherein V158 is replaced with any other amino acid.

4. The Factor VII polypeptide according to any of claims 1-3, wherein E296 is replaced with any other amino acid.

5. The Factor VII polypeptide according to any of claims 1-4, wherein M298 is replaced with any other amino acid.

6. The Factor VII polypeptide according to any of claims 1-5, wherein L305 is replaced with any other amino acid.

7. The Factor VII polypeptide according to any of claims 1-6, wherein M306 is replaced with any other amino acid.

8. The Factor VII polypeptide according to any of claims 1-7, wherein D309 is replaced with any other amino acid.

9. The Factor VII polypeptide according to any of claims 1-8, wherein S314 is replaced with any other amino acid.

10. The Factor VII polypeptide according to any of claims 1-9, wherein D334 is replaced with any other amino acid.

11. The Factor VII polypeptide according to any of claims 1-10, wherein S336 is replaced with any other amino acid.

12. The Factor VII polypeptide according to any of claims 1-11, wherein K337 is replaced with any other amino acid.

13. The Factor VII polypeptide according to any of claims 1-12, wherein F374 is replaced with any other amino acid.

14. The Factor VII polypeptide according to any of claims 1-13, wherein at least one amino acid in the remaining positions in the protease domain has been replaced with any other amino acid.

15. The Factor VII polypeptide according to claims 14, wherein at the most 20 additional amino acids in the remaining positions in the protease domain have been replaced with any other amino acids.

16. The Factor VII polypeptide according to any of claims 14-15, wherein at least one amino acid corresponding to an amino acid at a position selected from 159-170 of SEQ ID NO:1 has been replaced with any other amino acid.

17. The Factor VII polypeptide according to any of claims 14-16, wherein at least one amino acid corresponding to an amino acid at a position selected from 290-304 of SEQ ID NO:1 has been replaced with any other amino acid.

18. The Factor VII polypeptide according to claim 17, wherein R304 has been replaced by an amino acid selected from the group consisting of Tyr, Phe, Leu, and Met.

19. The Factor VII polypeptide according to any of claims 14-18, wherein at least one amino acid corresponding to an amino acid at a position selected from 307-312 of SEQ ID NO:1 has been replaced with any other amino acid.

20. The Factor VII polypeptide according to any of claims 14-19, wherein at least one amino acid corresponding to an amino acid at a position selected from 330-339 of SEQ ID NO:1 has been replaced with any other amino acid.

21. The Factor VII polypeptide according to any of claims 14-20, wherein A274 has been replaced with any other amino acid.

22. The Factor VII polypeptide according to claim 21, wherein said A274 has been replaced by an amino acid selected from the group consisting of Met, Leu, Lys, and Arg.

23. The Factor VII polypeptide according to any of claims 1-22, wherein said K157 has been replaced by an amino acid selected from the group consisting of Gly, Val, Ser, Thr, Asn, Gln, Asp, and Glu.

24. The Factor VII polypeptide according to any of claims 1-23, wherein said V158 has been replaced by an amino acid selected from the group consisting of Ser, Thr, Asn, Gln, Asp, and Glu.

25. The Factor VII polypeptide according to any of claims 1-24, wherein said E296 has been replaced by an amino acid selected from the group consisting of Arg, Lys, Ile, Leu and Val.

26. The Factor VII polypeptide according to any of claims 1-25, wherein said M298 has been replaced by an amino acid selected from the group consisting of Lys, Arg, Gln, and Asn.

27. The Factor VII polypeptide according to any of claims 1-26, wherein said L305 has been replaced by an amino acid selected from the group consisting of Val, Tyr and Ile.

28. The Factor VII polypeptide according to any of claims 1-27, wherein M306 has been replaced by an amino acid selected from the group consisting of Asp, and Asn.

29. The Factor VII polypeptide according to any of claims 1-28, wherein D309 has been replaced by an amino acid selected from the group consisting of Ser, and Thr.

30. The Factor VII polypeptide according to any of claims 1-29, wherein said S314 has been replaced by an amino acid selected from the group consisting of Gly, Lys, Gln and Glu.

31. The Factor VII polypeptide according to any of claims 1-30, wherein said D334 has been replaced by an amino acid selected from the group consisting of Gly, and Glu.

32. The Factor VII polypeptide according to any of claims 1-31, wherein said S336 has been replaced by an amino acid selected from the group consisting of Gly, and Glu.

33. The Factor VII polypeptide according to any of claims 1-32, wherein said K337 has been replaced by an amino acid selected from the group consisting of Ala, Gly, Val, Ser, Thr, Asn, Gln, Asp, and Glu.

34. The Factor VII polypeptide according to any of claims 1-33, wherein said F374 has been replaced by an amino acid selected from the group consisting of Pro, and Tyr.

35. The Factor VII polypeptide according to claim 34, wherein said F374 has been replaced by Tyr.

36. The Factor VII polypeptide according to any of claims 1-35, wherein said Factor VII polypeptide does not contain the amino acid sequence 1-38 relative to the amino acid sequence of SEQ ID NO:1.

37. The Factor VII polypeptide according to any of claims 1-35, wherein said Factor VII polypeptide does not contain the amino acid sequence 1-44 relative to the amino acid sequence of SEQ ID NO:1.

38. The Factor VII polypeptide according to any of claims 1-35, wherein said Factor VII polypeptide does not contain the amino acid sequence 1-152 relative to the amino acid sequence of SEQ ID NO:1.

39. The Factor VII polypeptide according to any of claims 1-36, wherein said Factor VII polypeptide at least consists of the amino acid sequence 39-406 relative to the amino acid sequence of SEQ ID NO:1.

40. The Factor VII polypeptide according to any of claims 1-37, wherein said Factor VII polypeptide at least consists of the amino acid sequence 45-406 relative to the amino acid sequence of SEQ ID NO:1.

41. The Factor VII polypeptide according to any of claims 1-38, wherein said Factor VII polypeptide at least consists of the amino acid sequence 153-406 relative to the amino acid sequence of SEQ ID NO:1.

42. The Factor VII polypeptide according to any of claims 1-41, wherein the amino acid has been replaced with any other amino acid which can be encoded by polynucleotide constructs.

43. The Factor VII polypeptide according to any of claims 1-42, wherein said Factor VII polypeptide is in activated form.

44. The Factor VII polypeptide according to any of claims 1-43, wherein the ratio between the proteolytic activity of said Factor VII polypeptide and the proteolytic activity of the native Factor VIIa polypeptide shown in SEQ ID NO:1 is at least about 1.25.

45. The Factor VII polypeptide according to claim 44, wherein said ratio is at least about 2.0, preferably at least about 4.0.

46. A polynucleotide construct encoding a Factor VII polypeptide according to any of claims 1-45.

47. The polynucleotide construct according to claim 46, which is a vector.

48. A host cell comprising the polynucleotide construct according to any one of the claims 46-47.

49. The host cell according to claim 48, which is a eukaryotic cell.

50. The host cell according to claim 49, which is of mammalian origin.

51. The host cell according to claim 49, which is an insect cell.

52. The host cell according to claim 50, wherein the cell is selected from the group consisting of CHO cells , HEK cells and BHK cells.

53. The host cell according to claim 48, which is a microorganism.

54. The host cell according to claim 53, wherein the microorganism is selected from the group consisting of Escherichia coli, Bacillus subtilis, Rhodococcus, Streptomycetes, Actinomycetes, Corynebacteria, Pseudomonas, Erwinia, Salmonella, Saccharomyces

cerevisiae, Picchia, protozoans, Penicillium, Fusarium, Aspergillus, Podospora, and Neurospora.

55. A transgenic animal containing and expressing the polynucleotide construct as defined in claim 46.

56. A transgenic plant containing and expressing the polynucleotide construct as defined in claim 46.

57. A method for producing the Factor VII polypeptide defined in any of claims 1-45, the method comprising cultivating a host cell as defined in any one of claims 48-54 in an appropriate growth medium under conditions allowing expression of the polynucleotide construct and recovering the resulting polypeptide from said growth medium or said host cell.

58. A method for producing the Factor VII polypeptide defined in any of claims 1-45, the method comprising recovering the Factor VII polypeptide from milk produced by the transgenic animal defined in claim 55.

59. A method for producing the Factor VII polypeptide defined in any of claims 1-45, the method comprising cultivating a host cell of a transgenic plant as defined in claim 56, and recovering the Factor VII polypeptide from the plant.

60. A pharmaceutical composition comprising a FVII polypeptide, wherein said FVII polypeptide is essentially free of GLA residues; and, optionally, a pharmaceutically acceptable carrier.

61. A pharmaceutical composition comprising a FVII polypeptide comprising one or more amino acid substitutions relative to the amino acid sequence of SEQ ID NO:1, wherein said amino acid substitutions are replacement with any other amino acid of one or more amino acids selected from the group consisting of K157, V158, E296, M298, L305, M306, D309, S314, D334, S336, K337, F374, and wherein said FVII polypeptide is essentially free of GLA residues; and, optionally, a pharmaceutically acceptable carrier.

62. The pharmaceutical composition according to claim 60, wherein said Factor VII polypeptide is wild type human FVII essentially free of GLA residues; and, optionally, a pharmaceutically acceptable carrier.

63. The pharmaceutical composition according to any of claims 60-62, wherein said Factor VII polypeptide does not contain the amino acid sequence 1-38 relative to the amino acid sequence of SEQ ID NO:1.

64. The pharmaceutical composition according to any of claims 60-63, wherein said Factor VII polypeptide does not contain the amino acid sequence 1-44 relative to the amino acid sequence of SEQ ID NO:1.

65. The pharmaceutical composition according to any of claims 60-64, wherein said Factor VII polypeptide does not contain the amino acid sequence 1-152 relative to the amino acid sequence of SEQ ID NO:1.

66. The pharmaceutical composition according to any of claims 60, 62-65, wherein said Factor VII polypeptide comprises the amino acid sequence 39-406 of SEQ ID NO:1.

67. The pharmaceutical composition according to any of claims 60, 62-66, wherein said Factor VII polypeptide comprises the amino acid sequence 45-406 of SEQ ID NO:1.

68. The pharmaceutical composition according to any of claims 60, 62-67, wherein said Factor VII polypeptide comprises the amino acid sequence 153-406 of SEQ ID NO:1.

69. The pharmaceutical composition according to any of claims 60-68, wherein the amino acid has been replaced with any other amino acid which can be encoded by polynucleotide constructs.

70. The pharmaceutical composition according to any of claims 60-69, wherein said Factor VII polypeptide is in activated form.

71. A pharmaceutical composition comprising a Factor VII polypeptide as defined in any of claims 1-45, and, optionally, a pharmaceutically acceptable carrier.

72. Use of a FVII polypeptide, wherein said FVII polypeptide is essentially free of GLA residues; for the preparation of a medicament for the treatment of bleeding disorders or bleeding episodes or for the enhancement of the normal haemostatic system.

73. Use of a FVII polypeptide comprising one or more amino acid substitutions relative to the amino acid sequence of SEQ ID NO:1, wherein said amino acid substitutions are replacement with any other amino acid of one or more amino acids selected from the group consisting of K157, V158, E296, M298, L305, M306, D309, S314, D334, S336, K337, F374, and wherein said FVII polypeptide is essentially free of GLA residues; for the preparation of a medicament for the treatment of bleeding disorders or bleeding episodes or for the enhancement of the normal haemostatic system.

74. Use according to claim 72, wherein said Factor VII polypeptide is wild type human FVII essentially free of GLA residues; and, optionally, a pharmaceutically acceptable carrier.

75. Use according to any of claims 72-74, wherein said Factor VII polypeptide does not contain the amino acid sequence 1-38 relative to the amino acid sequence of SEQ ID NO:1.

76. Use according to any of claims 72-75, wherein said Factor VII polypeptide does not contain the amino acid sequence 1-44 relative to the amino acid sequence of SEQ ID NO:1.

77. Use according to any of claims 72-76, wherein said Factor VII polypeptide does not contain the amino acid sequence 1-152 relative to the amino acid sequence of SEQ ID NO:1.

78. Use according to any of claims 72, 74-77, wherein said Factor VII polypeptide comprises the amino acid sequence 39-406 of SEQ ID NO:1.

79. Use according to any of claims 72, 74-78, wherein said Factor VII polypeptide comprises the amino acid sequence 45-406 of SEQ ID NO:1.

80. Use according to any of claims 72, 74-79, wherein said Factor VII polypeptide comprises the amino acid sequence 153-406 of SEQ ID NO:1.

81. Use according to any of claims 72-80, wherein the amino acid has been replaced with any other amino acid which can be encoded by polynucleotide constructs.

82. Use according to any of claims 72-81, wherein said Factor VII polypeptide is in activated form.

83. Use of a Factor VII polypeptide as defined in any of claims 1-45 for the preparation of a

medicament for the treatment of bleeding disorders or bleeding episodes or for the enhancement of the normal haemostatic system.

84. Use according to any of claims 72-83 for the treatment of haemophilia A or B.

85. A method for the treatment of bleeding disorders or bleeding episodes in a subject or for the enhancement of the normal haemostatic system, the method comprising administering a therapeutically or prophylactically effective amount of a FVII polypeptide, wherein said FVII polypeptide is essentially free of GLA residues; to a subject in need thereof.

86. A method for the treatment of bleeding disorders or bleeding episodes in a subject or for the enhancement of the normal haemostatic system, the method comprising administering a therapeutically or prophylactically effective amount of a FVII polypeptide comprising one or more amino acid substitutions relative to the amino acid sequence of SEQ ID NO:1, wherein said amino acid substitutions are replacement with any other amino acid of one or more amino acids selected from the group consisting of K157, V158, E296, M298, L305, M306, D309, S314, D334, S336, K337, F374, and wherein said FVII polypeptide is essentially free of GLA residues; to a subject in need thereof.

87. The method according to claim 85, wherein said Factor VII polypeptide is wild type human FVII essentially free of GLA residues; and, optionally, a pharmaceutically acceptable carrier.

88. The method according to any of claims 85-87, wherein said Factor VII polypeptide does not contain the amino acid sequence 1-38 relative to the amino acid sequence of SEQ ID NO:1.

89. The method according to any of claims 85-88, wherein said Factor VII polypeptide does not contain the amino acid sequence 1-44 relative to the amino acid sequence of SEQ ID NO:1.

90. The method according to any of claims 85-89, wherein said Factor VII polypeptide does not contain the amino acid sequence 1-152 relative to the amino acid sequence of SEQ ID NO:1.

91. The method according to any of claims 85, 87-90, wherein said Factor VII polypeptide comprises the amino acid sequence 39-406 of SEQ ID NO:1.

92. The method according to any of claims 85, 87-91, wherein said Factor VII polypeptide comprises the amino acid sequence 45-406 of SEQ ID NO:1.

93. The method according to any of claims 85, 87-92, wherein said Factor VII polypeptide comprises the amino acid sequence 153-406 of SEQ ID NO:1.

94. The method according to any of claims 85-93, wherein the amino acid has been replaced with any other amino acid which can be encoded by polynucleotide constructs.

95. The method according to any of claims 85-94, wherein said Factor VII polypeptide is in activated form.

96. A method for the treatment of bleeding disorders or bleeding episodes in a subject or for the enhancement of the normal haemostatic system, the method comprising administering a therapeutically or prophylactically effective amount of a Factor VII polypeptide as defined in any of claims 1-45 to a subject in need thereof.

97. A Factor VII polypeptide essentially free of GLA residues for use as a medicament.

98. The Factor VII polypeptide according to claim 97, wherein said Factor VII polypeptide is wild type human FVII essentially free of GLA residues; and, optionally, a pharmaceutically acceptable carrier.

99. The Factor VII polypeptide according to any of claims 97-98, wherein said Factor VII polypeptide does not contain the amino acid sequence 1-38 relative to the amino acid sequence of SEQ ID NO:1.

100. The Factor VII polypeptide according to any of claims 97-99, wherein said Factor VII polypeptide does not contain the amino acid sequence 1-44 relative to the amino acid sequence of SEQ ID NO:1.

101. The Factor VII polypeptide according to any of claims 97-100, wherein said Factor VII polypeptide does not contain the amino acid sequence 1-152 relative to the amino acid sequence of SEQ ID NO:1.

102. The Factor VII polypeptide according to any of claims 97-101, wherein said Factor VII polypeptide comprises the amino acid sequence 39-406 of SEQ ID NO:1.

103. The Factor VII polypeptide according to any of claims 97-102, wherein said Factor VII polypeptide comprises the amino acid sequence 45-406 of SEQ ID NO:1.

104. The Factor VII polypeptide according to any of claims 97-103, wherein said Factor VII polypeptide comprises the amino acid sequence 153-406 of SEQ ID NO:1.

105. The Factor VII polypeptide according to any of claims 97-104, wherein the amino acid has been replaced with any other amino acid which can be encoded by polynucleotide constructs.

106. The Factor VII polypeptide according to any of claims 97-105, wherein said Factor VII polypeptide is in activated form.

107. A Factor VII polypeptide as defined in any of claims 1-45 for use as a medicament.

Fig. 1. Sequence listing:

SEQ ID NO:1: The amino acid sequence of wild type human coagulation Factor VII. The three-letter indication "GLA" means 4-carboxyglutamic acid (γ-carboxyglutamate)

```
Ala-Asn-Ala-Phe-Leu-GLA-GLA-Leu-Arg-Pro-Gly-Ser-Leu-GLA-Arg-GLA-Cys-Lys-
                  5                  10                  15

GLA-GLA-Gln-Cys-Ser-Phe-GLA-GLA-Ala-Arg-GLA-Ile-Phe-Lys-Asp-Ala-GLA-Arg-
     20                  25                  30                  35

Thr-Lys-Leu-Phe-Trp-Ile-Ser-Tyr-Ser-Asp-Gly-Asp-Gln-Cys-Ala-Ser-Ser-Pro-
             40                  45                  50

Cys-Gln-Asn-Gly-Gly-Ser-Cys-Lys-Asp-Gln-Leu-Gln-Ser-Tyr-Ile-Cys-Phe-Cys-
 55                  60                  65                  70

Leu-Pro-Ala-Phe-Glu-Gly-Arg-Asn-Cys-Glu-Thr-His-Lys-Asp-Asp-Gln-Leu-Ile-
         75                  80                  85                  90

Cys-Val-Asn-Glu-Asn-Gly-Gly-Cys-Glu-Gln-Tyr-Cys-Ser-Asp-His-Thr-Gly-Thr-
                 95                 100                 105

Lys-Arg-Ser-Cys-Arg-Cys-His-Glu-Gly-Tyr-Ser-Leu-Leu-Ala-Asp-Gly-Val-Ser-
    110                 115                 120                 125

Cys-Thr-Pro-Thr-Val-Glu-Tyr-Pro-Cys-Gly-Lys-Ile-Pro-Ile-Leu-Glu-Lys-Arg-
            130                 135                 140

Asn-Ala-Ser-Lys-Pro-Gln-Gly-Arg-Ile-Val-Gly-Gly-Lys-Val-Cys-Pro-Lys-Gly-
145                 150                 155                 160

Glu-Cys-Pro-Trp-Gln-Val-Leu-Leu-Leu-Val-Asn-Gly-Ala-Gln-Leu-Cys-Gly-Gly-
        165                 170                 175                 180

Thr-Leu-Ile-Asn-Thr-Ile-Trp-Val-Val-Ser-Ala-Ala-His-Cys-Phe-Asp-Lys-Ile-
                185                 190                 195
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Lys-Asn-Trp-Arg-Asn-Leu-Ile-Ala-Val-Leu-Gly-Glu-His-Asp-Leu-Ser-Glu-His-
200                 205                 210                 215

Asp-Gly-Asp-Glu-Gln-Ser-Arg-Arg-Val-Ala-Gln-Val-Ile-Ile-Pro-Ser-Thr-Tyr-
            220                 225                 230

Val-Pro-Gly-Thr-Thr-Asn-His-Asp-Ile-Ala-Leu-Leu-Arg-Leu-His-Gln-Pro-Val-
235                 240                 245                 250

Val-Leu-Thr-Asp-His-Val-Val-Pro-Leu-Cys-Leu-Pro-Glu-Arg-Thr-Phe-Ser-Glu-
        255                 260                 265                 270

Arg-Thr-Leu-Ala-Phe-Val-Arg-Phe-Ser-Leu-Val-Ser-Gly-Trp-Gly-Gln-Leu-Leu-
                275                 280                 285

Asp-Arg-Gly-Ala-Thr-Ala-Leu-Glu-Leu-Met-Val-Leu-Asn-Val-Pro-Arg-Leu-Met-
    290                 295                 300                 305 306

Thr-Gln-Asp-Cys-Leu-Gln-Gln-Ser-Arg-Lys-Val-Gly-Asp-Ser-Pro-Asn-Ile-Thr-
            310                 315                 320

Glu-Tyr-Met-Phe-Cys-Ala-Gly-Tyr-Ser-Asp-Gly-Ser-Lys-Asp-Ser-Cys-Lys-Gly-
325                 330                 335                 340

Asp-Ser-Gly-Gly-Pro-His-Ala-Thr-His-Tyr-Arg-Gly-Thr-Trp-Tyr-Leu-Thr-Gly-
        345                 350                 355                 360

Ile-Val-Ser-Trp-Gly-Gln-Gly-Cys-Ala-Thr-Val-Gly-His-Phe-Gly-Val-Tyr-Thr-
                365                 370                 375

Arg-Val-Ser-Gln-Tyr-Ile-Glu-Trp-Leu-Gln-Lys-Leu-Met-Arg-Ser-Glu-Pro-Arg-
    380                 385                 390                 395

Pro-Gly-Val-Leu-Leu-Arg-Ala-Pro-Phe-Pro
            400                 405 406
```

Fig. 1 (continued)

SEQ ID NO:2:
5'-GGCGTCCTGCACCGGCGCCGGCGCATTTCTTACAGTGATGGGGACCAGTGTGCCTC-3'

SEQ ID NO:3:
5'-GAGGCACACTGGTCCCCATCACTGTAAGAAATGCGCCGGCGCCGGTGCAGGACGCC-3'

SEQ ID NO:4:
5'- GGCGTCCTGCACCGGCGCCGGCGCACGCACAAGGATGACCAGCTGATCTGTGTG-3'

SEQ ID NO:5:
5'- CACACAGATCAGCTGGTCATCCTTGTGCGTGCGCCGGCGCCGGTGCAGGACGCC-3'

Fig. 1 (continued)

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(54) Title: COAGULATION FACTOR VII/VIIA VARIANTS LACKING A FUNCTIONAL LIPID MEMBRANE BINDING DOMAIN

(57) Abstract: The present invention relates to novel coagulation factor FVIIa polypeptides.

WO 2005/075635 A3

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2005/050474

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N9/64 A61K38/48

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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NICOLAISEN E M: "GENERATION OF GLA-DOMAINLESS FVIIA BY CATHEPSIN G-MEDIATED CLEAVAGE" FEBS LETTERS, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL, vol. 306, no. 2 / 3, 20 July 1992 (1992-07-20), pages 157-160, XP000286918 ISSN: 0014-5793 cited in the application the whole document	

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Smalt, R

Form PCT/ISA/210 (second sheet) (January 2004)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NEUENSCHWANDER PIERRE F ET AL: "Roles of the membrane-interactive regions of factor VIIa and tissue factor: The factor VIIa Gla domain is dispensable for binding to tissue factor but important for activation of factor X" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 11, 1994, pages 8007-8013, XP002338319 ISSN: 0021-9258 the whole document	
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2005/050474

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 00/66753 A (REGENTS OF THE UNIVERSITY OF MINNESOTA; NELSESTUEN, GARY, L) 9 November 2000 (2000-11-09) the whole document	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2005/050474

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 85-96 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2005/050474

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(74) Common Representative: **NOVO NORDISK A/S**; Corporate Patents, Novo Allé, DK-2880 Bagsværd (DK).

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(54) Title: DIMERIC AND MULTIMERIC FVIIA COMPOUNDS

(57) Abstract: The present invention relates to dimeric or multimeric FVIIa compounds comprising at least two FVIIa polypeptides covalently connected such as to retain the intrinsic catalytic activity of the FVIIa polypeptides.

DIMERIC AND MULTIMERIC FVIIa COMPOUNDS

FIELD OF THE INVENTION

The present invention relates to novel FVIIa compounds, methods for their synthesis, pharmaceutical compositions comprising the novel compounds as well as their use in treatment of coagulation disorders.

BACKGROUND OF THE INVENTION

Coagulation factor VIIa (FVIIa) is the key initiator of haemostasis. It is a 50-kDa plasma protein with a functional circulatory half life around 1-3 hours. The zymogen, FVII, is a single-chain protein being catalytically inactive and may be converted into the catalytically active two-chain FVIIa by cleavage of an internal Arg_{152}-Ile_{153} peptide bond. FVIIa is widely used as therapeutic protein for the treatment of various coagulation disorders that may be caused by clotting factor deficiencies or clotting factor inhibitors. FVIIa has also been used to control excessive bleeding occurring in subjects with a normally functioning blood clotting cascade. Such bleeding may for example be caused by a defective platelet function, thrombocytopenia, von Willebrands disease as well as during surgery and other forms of tissue damage.

For many of the therapeutic applications of FVIIa it is desirable to perform the treatment using FVIIa variants with improved properties relative to native FVIIa. One area where FVIIa variants with enhanced biological activity are desirable is the treatment of uncontrolled bleedings that are partially or completely refractory to conventional rFVIIa therapy due to suboptimal potency of FVIIa. Presently these so-called superactive FVIIa variants can be divided into two classes according to their mode of action. One group encompasses variants with improved proteolytic activity resulting from a shift in the conformational equilibrium of the protein towards the catalytically competent state, see e.g. Persson et al. (2001a), (2001b), (2002), (2004) and Soejima et al. (2002). The other group is mainly variants with optimized GLA domains exhibiting increased affinity for anionic phospholipids membranes (Shah et al. (1998), Nelsestuen et al. (2001) and Harvey et al. (2003)). It is believed that improved phospholipid-binding serves to localize and concentrate FVIIa onto the activated platelet membrane at the site of vascular injury thereby promoting haemostasis.

Covalent modification, e.g. by PEGylation or lipid attachment, has been successfully applied on several protein-based pharmaceutics to improve their pharmacokinetic and pharmacodynamic profiles. Conjugation via native or engineered cysteines provides an

attractive means of site-specific modification due to the rarity of this amino acid on the surface of proteins, particularly those secreted by the cell, as well as the high selectivity of the thiol-coupling chemistry. The lack of free thiols in native Factor VIIa has led to the proposal that prolongation of the circulatory half life might be achieved by modification, e.g. PEGylation, of engineered solvent-exposed cysteines, see, e.g. WO 02/077218 A1 and WO 01/58935 A2.

Hence, WO 01/58935 A2 discloses FVIIa conjugates with non-polypeptide moieties and their preparation. It is, i.a., suggested that the non-polypeptide moiety is conjugated to the FVIIa polypeptide via a cysteine. Similarly, WO 02/077218 A1 discloses FVIIa polypeptide conjugates with chemical groups and their preparation. It is, i.a., suggested that the chemical group is conjugated to the FVIIa polypeptide via a cysteine.

In practice, however, this approach does not always warrant FVIIa compounds with the optimal match of pharmacokinetic and pharmacodynamic profiles, i.e. high activity, prolonged circulatory half-life etc.

Thus, there is a need for improved FVIIa compounds which exhibit high specific activity, prolonged plasma half-life as well as acceptable physico-chemical properties in relation to manufacturing, handling and formulation.

WO 03/076461 discloses dimeric Tissue Factor antagonists and their use in the treatment of e.g. vascular diseases and inflammatory diseases.

SE 9501285A discloses a process for the *in vitro* production of appropriately folded, biologically active disulfide-crosslinked proteins using a mixture of a protein disulfide oxidoreductase (e.g. protein disulfide isomerase (PDI)), a glutaredoxin and a redox buffer. The reference is focused on cysteines involved in intramolecular disulfide bonds.

SUMMARY OF THE INVENTION

In order to overcome the above-mentioned limitations of the known FVIIa compounds, the present invention now provides dimeric and multimeric FVIIa compounds comprising at least two FVIIa polypeptides covalently connected such as to retain the intrinsic catalytic activity of the FVIIa polypeptides. Such FVIIa compounds have surprisingly shown to be super-active and they are believed to exhibit enhanced membrane affinity resulting from the avidity effect arising when two or more FVIIa polypeptides are covalently linked.

In one aspect the present invention provides a FVIIa compound wherein two FVIIa polypeptides are covalently connected to form a dimeric FVIIa compound. In one embodiment the FVIIa polypeptides are covalently connected via engineered cysteines in said FVIIa polypeptides. In another embodiment two identical FVIIa polypeptides are covalently connected to form a dimeric FVIIa compound.

In another aspect the present invention provides a dimeric or multimeric FVIIa compound comprising a linker between the constituent FVIIa polypeptides.

The term "retain the intrinsic catalytic activity" refers to the property that the dimeric or multimeric FVIIa polypeptides have substantially the same peptidolytic activity as that of the constituent FVIIa polypeptides, e.g. an active site concentration which is at least 70%, preferably at least 80%, as measured by the assay disclosed herein.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Identification of low-molecular weight thiols engaged in mixed disulfides with FVIIa 407C. Protein was incubated in the presence of TCEP and SBD-f to liberate and derivatize thiols attached to the engineered Cys407 in the preparation of FVIIa 407C. SBD-derivatized thiols were separated by reversed-phase HPLC and detected by fluorescence (excitation and emission wavelengths of 386 and 516 nm, respectively). HPLC traces of wild-type FVIIa and a series of low-molecular weight thiol compounds (10 pmol of each; denoted standard) are shown for comparison. Peaks marked with an asterisk arise from impurities in commercially available cysteamine. The two asterisks indicate a peak of unknown identity in the HPLC trace of FVIIa 407C. Percentages above each peak indicate the amount of a given thiol species (identified from its retention time) relative to the amount of FVIIa 407C analyzed. Based on retention times, it can be concluded that major thiols conjugated to FVIIa 407C are glutathione, cysteine, and homocysteine. Abbreviations: GSH (glutathione), γ-GC (γ-glutamylcysteine), CG (cysteinylglycine), Cys (cysteine), Hcy (homocysteine) and Cya (cysteamine).

Figure 2: Residual amidolytic activity of FVIIa incubated at pH 7 in the presence of varying concentrations of reduced and oxidized glutathione (given by the $[GSH]^2/[GSSG]$ ratio), 1 μM yeast glutaredoxin 1 (yGrx1p), and either no (open circles) or 25 mM *p*-aminobenzamidine (open squares). All samples were allowed to equilibrate for 3.5 hrs at 30°C before the amidolytic activity was measured. Amidolytic activities are normalized to 1 for fully active FVIIa.

Figure 3: Redox titration of the mixed-disulfide between FVIIa Cys407 and glutathione. FVIIa 407C was allowed to equilibrate in pH 7 buffer containing varying ratios of reduced and oxidized glutathione (given by the [GSH]/[GSSG] ratio) and 10 µM E.coli glutaredoxin 2 (Grx2). Following equilibration for 5 hrs at 30°C, free FVIIa 407C was detected and quantified by HPLC after alkylation with PEG5k-maleimide. Peak areas are normalized to 1 for fully 5k-PEGylated FVIIa.

Figure 4: Residual amidolytic activity (stippled lines) and fraction of FVIIa 407C with a free Cys407 thiol group (solid line) at equilibrium in a redox buffer consisting of 0.5 mM GSH, varying concentrations of GSSG, and either no (-PABA) or 25 mM *p*-aminobenzamidine (+PABA). Curves were drawn using Eq. 2 and 4, K_{ox} values of 93 (-PABA) and 166 mM (+PABA), respectively, and a K_{scox} value of 1.02. The shaded area indicates the concentration range of GSSG resulting in >90% residual activity and >90% free 407C thiol.

Figure 5: As Figure 4 with 0.25 mM GSH instead. The shaded area indicates the concentration range of GSSG resulting in >90% residual activity and >90% free FVIIa 407C thiol.

Figure 6: As Figure 4 with 1.0 mM GSH instead. The shaded area indicates the concentration range of GSSG resulting in >90% residual activity and >90% free FVIIa 407C thiol.

Figure 7: HPLC analysis of FVIIa 407C before (dotted line) and after selective reduction (dashed-dotted line), and after modification with PEG20k-maleimide (solid line). 407C, 407C-SR, and 407C-PEG20k indicate peaks representing free, low-molecular weight thiol-conjugated, and 20k-PEGylated FVIIa 407C, respectively. Asterisks indicate peaks of unknown identity probably representing hyper-PEGylated species. Peak integration yielded 89% free FVIIa 407C at the end of the reduction step versus 11% in the untreated material. After thiol alkylation, 85% of FVIIa 407C was converted into the mono-PEGylated species.

Figure 8: Reducing (right panel) and non-reducing (left panel) SDS-PAGE analysis of FVIIa 407C (lane A), FVIIa 407C-PEG5k (lane B), FVIIa 407C-PEG20k (lane C), FVIIa 407C-PEG40k (lane D), and FVIIa 407C-PEG3.4k-FVIIa 407C (lane E).

Figure 9: Reducing (right panel) and non-reducing (left panel) SDS-PAGE analysis of FVIIa 407C (lane A), FVIIa 407C-PEG3.4k-FVIIa 407C (lane B), FVIIa 407C-PEG20k-FVIIa 407C (lane C).

Figure 10: (A) Reducing SDS-PAGE analysis of FVIIa R396C treated with 2.5 (lane A) or 5.0 mM (lane B) triphenylphosphine-3,3',3'' trisulfonic acid (PPh_3S_3) for 16.3 hrs at room

temperature and then labelled with PEG20k-maleimide. Lane C contains untreated FVIIa as a reference. (B) Relative amidolytic activities of FVIIa R396C before (100% activity) and after 16.3 hrs incubation with PPh_3S_3.

DETAILED DESCRIPTION OF THE INVENTION

As mentioned above, the present invention provides dimeric and multimeric FVIIa compounds comprising at least two FVIIa polypeptides covalently connected such as to retain the intrinsic catalytic activity of the FVIIa polypeptides. In one embodiment the dimeric or multimeric FVIIa compound has an active site concentration which is at least 70%, preferably at least 80%, of the active site concentration of the constituent FVIIa polypeptides as measured by the assay disclosed herein (cf. Example 1). In another embodiment the dimeric or multimeric FVIIa compound has an increased biological activity as compared to the biological activity of the constituent FVIIa polypeptides.

In a preferred aspect of the invention, two FVIIa polypeptides are covalently connected to form a dimeric FVIIa compound. The resulting dimeric FVIIa compound thus has a molecular weight which is usually larger than approx. 100 kDa.

In another aspect the invention provides multimeric FVIIa compounds comprising at least three FVIIa polypeptides covalently connected such as to retain the intrinsic catalytic activity of the FVIIa polypeptides.

FVIIa polypeptides

A number of different FVIIa polypeptides may be covalently connected to form the dimeric or multimeric FVIIa compounds according to the present invention.

The term "Factor VII polypeptide" or "FVII polypeptide" as used herein means the inactive one-chain zymogen Factor VII molecule as well as variants thereof. The one chain zymogen Factor VII is a polypeptide comprising 406 amino acid residues, 10 of which are γ-carboxylated glutamic acid residues, N-glycosylated asparagines residues (no 145 and no 322), and O-glycosylated serine residues in position 52 and 60. The variant forms of FVII encompasses i.a. molecules wherein one or more amino acid residues have been substituted, added or deleted, molecules with different number of GLA residues, molecules with a modified or uncomplete glycosylation pattern. Non-limiting examples of modifications of amino acid residues are amidation, alkylation, acylation and PEGylation.

The term "Factor VIIa polypeptide" or "FVIIa polypeptide" as used herein means the active two-chain Factor VIIa molecule as well as variants thereof. The two-chain Factor VIIa is a polypeptide produced from FVII by hydrolysis of the Arg_{152}-Ile_{153} peptide bond of FVII. FVIIa also comprises 406 amino acid residues, 10 of which are γ-carboxylated glutamic acid residues, N-glycosylated asparagines residues (no 145 and no 322), and O-glycosylated serine residues in position 52 and 60. The variant forms of FVIIa encompasses i.a. molecules wherein one or more amino acid residues have been substituted, added or deleted, molecules with different number of GLA residues, molecules with a modified or uncomplete glycosylation pattern.

The term "GLA" as used herein means 4-carboxyglutamic acid (γ-carboxyglutamate).

The term "polypeptide" as used herein means a compound comprising at least five constituent amino acid residues covalently connected by peptide bonds. The constituent amino acids may be from the group of the amino acids encoded by the genetic code and they may be natural amino acids which are not encoded by the genetic code, as well as synthetic amino acids. Natural amino acids which are not encoded by the genetic code are e.g. hydroxyproline, γ-carboxy-glutamic acid, ornithine, phophoserine, D-alanine, D-glutamic acid. Synthetic amino acids comprise amino acids manufactured by organic synthesis, e.g. D-isomers of the amino acids encoded by the genetic code and Aib (α-aminoisobutyric acid), Abu (α-aminobutyric acid), Tle (tert-butylglycine), and β-alanine. A polypeptide may comprise a single peptide chain or it may comprise more than one polypeptide chain, such as FVII being a single chain and FVIIa being two chains connected by disulphide bonds.

The term "Factor VIIa derivative" or "FVIIa derivative" as used herein, is intended to designate a FVIIa polypeptide exhibiting substantially the same or improved biological activity relative to wild-type Factor VIIa, in which one or more of the amino acids of the parent peptide have been genetically and/or chemically and/or enzymatically modified, e.g. by alkylation, glycosylation, deglycosylation, PEGylation, acylation, ester formation or amide formation or the like. This includes but is not limited to PEGylated Factor VIIa, cysteine-PEGylated human Factor VIIa and variants thereof.

The term "PEGylated Factor VIIa" (and the like) means a Factor VIIa polypeptide conjugated with a PEG molecule. It is to be understood, that the PEG molecule may be attached to any part of the Factor VIIa polypeptide including any amino acid residue or carbohydrate moiety of the Factor VIIa polypeptide. The term "cysteine-PEGylated Factor VIIa" means Factor VIIa polypeptide having a PEG molecule conjugated to a sulfhydryl group of a non-native cysteine of the Factor VIIa polypeptide.

Non-limiting examples of Factor VIIa derivatives includes GlycoPegylated FVIIa derivatives as disclosed in WO 03/31464 and US Patent applications US 20040043446, US 20040063911, US 20040142856, US 20040137557, and US 20040132640 (Neose Technologies, Inc.); FVIIa conjugates as disclosed in WO 01/04287, US patent application 20030165996, WO 01/58935, WO 03/93465 (Maxygen ApS) and WO 02/02764, US patent application 20030211094 (University of Minnesota).

The term "improved biological activity" refers to FVIIa polypeptides with i) substantially the same or increased proteolytic activity compared to recombinant wild type human Factor VIIa or ii) to FVIIa polypeptides with substantially the same or increased TF binding activity compared to recombinant wild type human Factor VIIa or iii) to FVIIa polypeptides with substantially the same or increased half life in blood plasma compared to recombinant wild type human Factor VIIa.

The term "PEGylated human Factor VIIa" means human Factor VIIa, having a PEG molecule conjugated to a human Factor VIIa polypeptide. It is to be understood, that the PEG molecule may be attached to any part of the Factor VIIa polypeptide including any amino acid residue or carbohydrate moiety of the Factor VIIa polypeptide. The term "cysteine-PEGylated human Factor VIIa" means Factor VIIa having a PEG molecule conjugated to a sulfhydryl group of a cysteine introduced in human Factor VIIa.

Non-limiting examples of Factor VIIa variants having substantially the same or increased proteolytic activity compared to recombinant wild type human Factor VIIa include S52A-FVIIa, S60A-FVIIa (Lino et al., Arch. Biochem. Biophys. 352: 182-192, 1998); FVIIa variants exhibiting increased proteolytic stability as disclosed in U.S. Patent No. 5,580,560; Factor VIIa that has been proteolytically cleaved between residues 290 and 291 or between residues 315 and 316 (Mollerup et al., Biotechnol. Bioeng. 48:501-505, 1995); oxidized forms of Factor VIIa (Kornfelt et al., Arch. Biochem. Biophys. 363:43-54, 1999); FVIIa variants as disclosed in PCT/DK02/00189 (corresponding to WO 02/077218); and FVIIa variants exhibiting increased proteolytic stability as disclosed in WO 02/38162 (Scripps Research Institute); FVIIa variants having a modified Gla-domain and exhibiting an enhanced membrane binding as disclosed in WO 99/20767, US patents US 6017882 and US 6747003, US patent application 20030100506 (University of Minnesota) and WO 00/66753, US patent applications US 20010018414, US 2004220106, and US 200131005, US patents US 6762286 and US 6693075 (University of Minnesota); and FVIIa variants as disclosed in WO 01/58935, US patent US 6806063, US patent application 20030096338 (Maxygen ApS), WO 03/93465 (Maxygen ApS), WO 04/029091 (Maxygen ApS), WO 04/083361 (Maxygen ApS), and WO 04/111242 (Maxygen ApS), as well as in WO 04/108763 (Canadian Blood Services).

Non-limiting examples of FVIIa variants having increased biological activity compared to wild-type FVIIa include FVIIa variants as disclosed in WO 01/83725, WO 02/22776, WO 02/077218, PCT/DK02/00635 (corresponding to WO 03/027147), Danish patent application PA 2002 01423 (corresponding to WO 04/029090), Danish patent application PA 2001 01627 (corresponding to WO 03/027147); WO 02/38162 (Scripps Research Institute); and FVIIa variants with enhanced activity as disclosed in JP 2001061479 (Chemo-Sero-Therapeutic Res Inst.).

Specific examples of interesting "engineered" Factors VIIa polypeptides are those disclosed in WO 02/077218 A1 (Novo Nordisk A/S) and WO 01/58935 A2 (Maxygen ApS) pages 21-24.

Particularly interesting are FVIIa polypeptides wherein a cysteine residue has been introduced. Examples of positions, wherein cysteine residues may be introduced include, but is not limited to, positions at or in the vicinity of the proteolytic degradation sites. Thus, in an interesting embodiment of the invention the cysteine residue(s) to be introduced, preferably by substitution, is selected from the group consisting of I30C, K32C, D33C, A34C, T37C, K38C, W41C, Y44C, S45C, D46C, L141C, E142C, K143C, R144C, L288C, D289C, R290C, G291C, A292C, S314C, R315C, K316C, V317C, L390C, M391C, R392C, S393C, E394C, P395C, R396C, P397C, G398C, V399C, L401C, R402C, A403C, P404C and combinations thereof, in particular selected from the group consisting of K32C, Y44C, K143C, R290C, R315C, K341C, R392C, R396C, R402C and combinations thereof. In a further interesting embodiment of the invention the cysteine residue(s) is/are introduced into a position that in wildtype hFVII is occupied by a threonine or serine residue having at least 25% of its side chain exposed to the surface. For instance, in the Factor VII polypeptide a cysteine residue is introduced, preferably by substitution, into at least one position selected from the group consisting of S12, S23, S43, S45, S52, S53, S60, S67, T83, S103, T106, T108, S111, S119, S126, T128, T130, S147, T185, S214, S222, S232, T233, T238, T239, T255, T267, T293, T307, S320, T324, S333, S336, T370 and S393. Even more preferable, the cysteine residue is introduced into at least one position of hFVII containing an S residue, the position being selected from the group consisting of S12, S23, S43, S45, S52, S53, S60, S67, S103, S111, S119, S126, S147, S214, S222, S232, S320, S333, S336 and S393. In a further embodiment the cysteine residue(s) is/are introduced into a position that in wildtype hFVII is occupied by a threonine or serine residue having at least 50% of its side chain exposed to the surface. For instance, in the Factor VII polypeptide a cysteine residue is introduced, preferably by substitution, into at least one position selected from the group consisting of S23, S43, S52, S53, S60, S67, T106, T108, S111, S119, S147, S214, T238, T267 and T293, even more preferable, a position selected from the group consisting of S23, S43, S52, S53, S60, S67, S111, S119, S147 and S214. In a still further embodiment a cysteine residue is introduced into at least one position selected from any of the above-mentioned positions, which is not

located in an active site region. Preferably, the position is one occupied by a T or an S residue. As an example, the Factor VII polypeptide comprises a cysteine residue introduced into at least one position selected from the group consisting of S12, S23, S43, S45, S52, S53, S60, S67, T83, S103, T106, T108, S111, S119, S126, T128, T130, S147, T185, S214, S222, T255, T267, T307, S320, S333, S336, T370 and S393 (having more than 25% of its side chain exposed to the surface), in particular selected from the group consisting of S12, S23, S43, S45, S52, S53, S60, S67, S103, S111, S1 19, S 126, S147, S214, S222, S320, S333, S336 and S393 (occupied by S residue), and, more preferable, from the group consisting of S23, S43, S52, S53, S60, S67, T106, T108, S111, S119, S147, S214 and T267 (having more than 50% of its side chain exposed to the surface), in particular from the group consisting of S23, S43, S52, S53, S60, S67, S111, S119, S147 and S214 (occupied by an S residue). In an even further embodiment a cysteine residue is introduced into at least one position selected from any of the above lists, which is not located in a tissue factor binding site region. Preferably, the position is one occupied by a T or an S residue. As an example, the Factor VII polypeptide comprises a cysteine residue introduced into at least one position selected from the group consisting of S12, S23, S45, S52, S53, S67, T83, S103, T106, T108, S111, S119, S126, T128, T130, S147, T185, S214, S222, S232, T233, T238, T239, T255, T267, T293, S320, T324, S333, S336, T370 and S393 (having more than 25% of its side chain exposed to the surface), in particular selected from the group consisting of S12, S23, S45, S52, S53, S67, S103, S111, S119, S126, S147, S214, S222, S232, S320, S333, S336 and S393 (occupied by S residue), and, more preferable, from the group consisting of S23, S52, S53, S67, T106, T108, S111, S119, S147, S214, T238, T267 and T293 (having more than 50% of its side chain exposed to the surface), in particular from the group consisting of S23, S52, S53, S67, S111, S119, S147 and S214 (occupied by an S residue). In a still further embodiment a cysteine residue is introduced into at least one position selected from any of the above lists, which is neither located in a tissue factor binding site region nor in an active site region. Preferably, the position is one occupied by a T or an S residue. As an example, the Factor VII polypeptide comprises a cysteine residue introduced into at least one position selected from the group consisting of S12, S23, S45, S52, S53, S67, T83, S103, T106, T108, S111, S119, S126, T128, T130, S147, T185, S214, S222, T255, T267, S320, S333, S336, T370 and S393 (having more than 25% of its side chain exposed to the surface), in particular selected from the group consisting of S12, S23, S45, S52, S53, S67, S103, S111, S119, S126, S147, S214, S222, S320, S333, S336 and S393 (occupied by S residue), and, more preferable, from the group consisting of S23, S52, S53, S67, T106, T108, S111, S119, S147, S214 and T267 (having more than 50% of its side chain exposed to the surface), in particular from the group consisting of S23, S52, S53, S67, S111, S119, S147 and S214 (occupied by an S residue).

Other useful examples of Factor VIIa polypeptide include those where an amino acid at a position selected from 247-260, 393-405 or 406, in particular R396, Q250 or P406, or K157,

V158, M298, L305, D334, S336, K337 or F374 has been substituted with a cysteine, or where a cysteine has been introduced in the terminal, e.g. Factor VIIa 407C.

In a preferred series of embodiments the FVIIa polypeptide is FVIIa 407C, a FVIIa 407C variant, FVIIa P406C, a FVIIa P406C variant, FVIIa R396C, a FVIIa R396C variant, FVIIa Q250C or a FVIIa Q250C variant.

In another embodiment the FVIIa polypeptides are FVIIa variants having optimized GLA domains, e.g. combinations of Y4 (insertion), P10Q, K32E, D33F, D33E, and A34E.

In yet another embodiment the FVIIa polypeptides are FVIIa variants having increased intrinsic proteolytic activity, e.g. comprising substitutions selected from the group consisting of M298Q, V158D/E296V/M298Q, V158D/E296V/M298Q/K337A, F374Y/L305V, F374Y/L305V/S314E/K337A, F374Y/L305V/S314E, F374Y/L305V/K337A, L305V/K337A, V158D/E296V/M298Q/L305V, V158D/E296V/M298Q/L305V/K337A, V158T/M298Q, E296V/M298Q, V158D/E296V, V158D/M298Q and the 407C variants thereof.

In yet another embodiment the FVIIa polypeptide is a FVIIa variant comprising substitutions selected from the group consisting of L305V, L305V/M306D/D309S, L305I, L305T, F374P, V158T/M298Q, V158D/E296V/M298Q, K337A, M298Q, V158D/M298Q, L305V/K337A, V158D/E296V/M298Q/L305V, V158D/E296V/M298Q/K337A, V158D/E296V/M298Q/L305V/K337A, K157A, E296V, E296V/M298Q, V158D/E296V, V158D/M298K, S336G, L305V/K337A, L305V/V158D, L305V/E296V, L305V/M298Q, L305V/V158T, L305V/K337A/V158T, L305V/K337A/M298Q, L305V/K337A/E296V, L305V/K337A/V158D, L305V/V158D/M298Q, L305V/V158D/E296V, L305V/V158T/M298Q, L305V/V158T/E296V, L305V/E296V/M298Q, L305V/V158D/E296V/M298Q, L305V/V158T/E296V/M298Q, L305V/V158T/K337A/M298Q, L305V/V158T/E296V/K337A, L305V/V158D/K337A/M298Q, L305V/V158D/E296V/K337A, L305V/V158D/E296V/M298Q/K337A, L305V/V158T/E296V/M298Q/K337A, S314E/K316H, S314E/K316Q, S314E/L305V, S314E/K337A, S314E/V158D, S314E/E296V, S314E/M298Q, S314E/V158T, K316H/L305V, K316H/K337A, K316H/V158D, K316H/E296V, K316H/M298Q, K316H/V158T, K316Q/L305V, K316Q/K337A, K316Q/V158D, K316Q/E296V, K316Q/M298Q, K316Q/V158T, S314E/L305V/K337A, S314E/L305V/V158D, S314E/L305V/E296V, S314E/L305V/M298Q, S314E/L305V/V158T, S314E/L305V/K337A/V158T, S314E/L305V/K337A/M298Q, S314E/L305V/K337A/E296V, S314E/L305V/K337A/V158D, S314E/L305V/V158D/M298Q, S314E/L305V/V158D/E296V, S314E/L305V/V158T/M298Q, S314E/L305V/V158T/E296V, S314E/L305V/E296V/M298Q, S314E/L305V/V158D/E296V/M298Q, S314E/L305V/V158T/E296V/M298Q, S314E/L305V/V158T/K337A/M298Q, S314E/L305V/V158T/E296V/K337A,

S314E/L305V/V158D/K337A/M298Q, S314E/L305V/V158D/E296V/K337A, S314E/L305V/V158D/E296V/M298Q/K337A, S314E/L305V/V158T/E296V/M298Q/K337A, K316H/L305V/K337A, K316H/L305V/V158D, K316H/L305V/E296V, K316H/L305V/M298Q, K316H/L305V/V158T, K316H/L305V/K337A/V158T, K316H/L305V/K337A/M298Q, K316H/L305V/K337A/E296V, K316H/L305V/K337A/V158D, K316H/L305V/V158D/M298Q, K316H/L305V/V158D/E296V, K316H/L305V/V158T/M298Q, K316H/L305V/V158T/E296V, K316H/L305V/E296V/M298Q, K316H/L305V/V158D/E296V/M298Q, K316H/L305V/V158T/E296V/M298Q, K316H/L305V/V158T/K337A/M298Q, K316H/L305V/V158T/E296V/K337A, K316H/L305V/V158D/K337A/M298Q, K316H/L305V/V158D/E296V/K337A, K316H/L305V/V158D/E296V/M298Q/K337A, K316H/L305V/V158T/E296V/M298Q/K337A, K316Q/L305V/K337A, K316Q/L305V/V158D, K316Q/L305V/E296V, K316Q/L305V/M298Q, K316Q/L305V/V158T, K316Q/L305V/K337A/V158T, K316Q/L305V/K337A/M298Q, K316Q/L305V/K337A/E296V, K316Q/L305V/K337A/V158D, K316Q/L305V/V158D/M298Q, K316Q/L305V/V158D/E296V, K316Q/L305V/V158T/M298Q, K316Q/L305V/V158T/E296V, K316Q/L305V/E296V/M298Q, K316Q/L305V/V158D/E296V/M298Q, K316Q/L305V/V158T/E296V/M298Q, K316Q/L305V/V158T/K337A/M298Q, K316Q/L305V/V158T/E296V/K337A, K316Q/L305V/V158D/K337A/M298Q, K316Q/L305V/V158D/E296V/K337A, K316Q/L305V/V158D/E296V/M298Q/K337A, K316Q/L305V/V158T/E296V/M298Q/K337A, and the 407C variants thereof.

In yet another embodiment the FVIIa polypeptides are PEGylated. It is to be understood that the PEGylation can be performed prior to the formation of the dimeric or multimeric FVIIa compound, or it can be performed after the formation of the dimeric or multimeric FVIIa compound.

In yet another embodiment the FVIIa compound is a dimer containing two FVIIa polypeptides. In yet another embodiment the FVIIa compound is a trimer containing three FVIIa polypeptides. In yet another embodiment the FVIIa compound is a tetramer containing four FVIIa polypeptides.

Dimeric and multimeric FVIIa compounds

A range of coupling chemistries may be used for producing the dimeric or multimeric FVIIa compounds which may optionally encompass a linker moiety.

In a preferred embodiment the FVIIa polypeptides covalently connected to form the dimeric or multimeric FVIIa compounds are identical FVIIa polypeptides. In another embodiment the

FVIIa polypeptides covalently connected to form the dimeric or multimeric FVIIa compounds are two different FVIIa polypeptides.

In a particularly preferred embodiment the dimeric or multimeric FVIIa compounds comprise at least two FVIIa polypeptides which are covalently connected via engineered cysteines in said FVIIa polypeptides. For the synthesis of such dimeric or multimeric FVIIa compounds comprising FVIIa polypeptides being connected via cysteines, the method disclosed below for producing selectively reduced FVIIa polypeptides is useful. In another embodiment the dimeric or multimeric FVIIa compounds according to the invention comprise a linker between the constituent FVIIa polypeptides. One class of such linkers is linkers having the structure that results from a reaction involving a bivalent cysteine reactive PEG. One such preferred linker has the structure that results from a reaction involving maleimide-PEG-maleimide, e.g. maleimide-PEG2kD-maleimide, maleimide-PEG3.4kD-maleimide, maleimide-PEG5kD-maleimide, maleimide-PEG10kD-maleimide, and maleimide-PEG20kD-maleimide. In another embodiment said linker comprises an amino acid sequence.

In yet another embodiment the dimeric or multimeric FVIIa compounds according to the invention comprises FVIIa polypeptides that are covalently connected via free amines.

In yet another embodiment the dimeric or multimeric FVIIa compounds according to the invention comprises FVIIa polypeptides that are covalently connected via natural or engineered glycans attached to said FVIIa polypeptides.

In yet another embodiment the dimeric or multimeric FVIIa compounds according to the invention comprises at least two FVIIa polypeptides that are covalently connected via a chemical structure resulting from an enzyme catalyzed coupling reaction, e.g. by transglutaminase or sortase.

In the situation where the dimeric or multimeric FVIIa compounds comprise FVIIa polypeptides covalently connected via engineered cysteines, preferred FVIIa polypeptides are selected from FVIIa 407C, a FVIIa 407C variant, FVIIa P406C, a FVIIa P406C variant, FVIIa R396C, a FVIIa R396C variant, FVIIa Q250C and a FVIIa Q250C variant.

Also within the scope of the present invention is conjugates between the dimeric or multimeric FVIIa compounds and a protractor group, such as human serum albumin or a variant thereof, a PEG moiety, a lipophilic moiety etc.

In general it is preferred to prepare a dimeric or multimeric FVIIa compound according to the invention by a method comprising the steps :

a) synthesis and purification of said FVIIa polypeptides;
b) synthesis of said compound by coupling said FVIIa polypeptides;
c) isolation of said dimeric or multimeric FVIIa compound.

When employing cysteine coupling chemistry it is preferred to prepare a dimeric or multimeric FVIIa compound according to the invention by a method comprising the steps :
a) synthesis and purification of said FVIIa polypeptides;
b) selective reduction of the cysteine residue on the FVIIa polypeptides which are to be linked;
c) synthesis of said dimeric or multimeric FVIIa compound by coupling said FVIIa polypeptides with reduced cysteine residues in the presence of an activated linker;
d) isolation of said dimeric or multimeric FVIIa compound.

In a preferred embodiment of the method, step b) comprises selective reduction of the cysteine residue on the FVIIa polypeptide by reaction with a redox buffer or a triarylphosphine reducing agent (cf section below on selective reduction).

Selective reduction of non-native cysteine of FVIIa monomer.

As mentioned above, when the dimeric or multimeric FVIIa compound is to be synthesized from the constituent FVIIa molecules or FVII molecules via non-native cysteines it may be necessary to selectively reduce the non-native cysteines in the FVIIa or FVII polypeptides prior to coupling. The FVIIa and FVII polypeptides typically comprises one or more cysteine moieties conjugated through a disulfide bridge to a low-molecular weight thiol (RS-Cys), said moiety/moieties not being involved in intramolecular S-S bridges (Cys-S-S-Cys) when the FVIIa polypeptide is in its active form.

Selective reduction using redox buffer.

One method for the selective reduction comprises the step of allowing the low-molecular weight thiol-conjugated FVIIa polypeptide to react with a mixture comprising a redox buffer.

When used herein, the term "redox buffer" is intended to mean a thiol/disulfide redox pair in a ratio that is sufficiently reducing to disrupt the FVIIa or FVII polypeptide-low-molecular weight thiol mixed disulfide(s) (RS-Cys) and at the same time sufficiently oxidizing to preserve the integrity of the native disulfide bonds in the FVIIa polypeptide.

Preferably, the redox buffer comprises a low molecular weight thiol/disulfide redox pair. By the term "low molecular weight" is meant that the thiol-form of the redox pair has a

molecular weight of at the most 500 g/mol. Illustrative examples of such redox pairs are the ones selected from (i) reduced and oxidized glutathione and (ii) reduced and oxidized γ-glutamylcysteine, (iii) reduced and oxidized cysteinylglycine, (iv) reduced and oxidized cysteine, (v) reduced and oxidized N-acetylcysteine, (vi) cysteamine, and (vii) dihydrolipoamide/lipoamide, preferably from (i) reduced and oxidized glutathione.

The optimal redox conditions can be determined by performing a redox titration of the protein as known to the person skilled in the art and as demonstrated in Figures 2, 3, and 4; see also Gilbert (1995).

In one embodiment, the redox buffer is a redox pair of reduced and oxidized glutathione, and the concentration of the reduced glutathione is in the range of 0-100 mM, and the ratio between reduced glutathione and oxidized glutathione is in the range of 2-200.

In another embodiment, the redox buffer is a redox pair of reduced and oxidized glutathione, and the concentration of the reduced glutathione is in the range of 0-100 mM, e.g. 0.01-50 mM, and the concentration of the oxidized glutathione is in the range of 0-5 mM, e.g. 0.001-5 mM. For Factor VII polypeptides, the concentration of the reduced glutathione is preferably in the range of 0-5 mM, e.g. 0.01-2 mM, and the concentration of the oxidized glutathione is in the range of 0.001-2 mM, e.g. 0.001-0.200 mM.

Since glutathione and other low molecular-weight thiols are generally poor reductants/-oxidants in terms of reaction kinetics, a thiol/disulfide redox catalyst is most preferably included in the mixture in conjunction with the redox buffer in order to enhance the rate of the reaction.

Suitable thiol/disulfide redox catalysts to be included in the mixture include dithiol-type and monothiol-type glutaredoxins. Glutaredoxins and their functions are generally described in Fernandes et al. (2004). Useful examples of glutaredoxins are those selected from Grx1, Grx2 or Grx3 from *Escherichia coli* (Holmgren et al., 1995), Grx1p, Grx2p, Grx3p, Grx4p, and Grx5p from *Saccharomyces cerevisiae* (Luikenhuis et al. 1998; Rodriguez-Manzaneque et al., 1999; Grant, 2001), Grx1 and Grx2 from *Homo sapiens* (Padilla et al. 1995; Lundberg et al., 2001), and variants hereof. Variants include, but is not restricted to, dithiol-type glutaredoxins in which the C-terminal cysteine in the CXXC motif has been replaced by another amino acid typically serine (see Yang et al., 1998).

The redox catalyst (in particular a glutaredoxin) is preferably used in a concentration of 0.001-20 μM.

It is preferred that the mixture does not comprise a protein disulfide isomerase (PDI).

The redox buffer may further comprise other components such as salts, pH buffers, etc., and the method of the invention may be conducted at any temperature which is suitable for the FVIIa protein in question, e.g. a temperature in the range of from -5°C to 50°C, such as in the range of from 0°C to 25°C, of course dependent on the stability of the protein under the given conditions.

Selective reduction using a triarylphosphine reducing agent.

Another method for the selective reduction comprises the step of allowing the low-molecular weight thiol-conjugated FVIIa protein to react with a triarylphosphine reducing agent.

The term "triarylphosphine reducing agent" is intended to mean a triarylphosphine optionally substituted with one or more substituents.

The aryl groups of the triarylphosphine reducing agent are preferably selected from phenyl, naphthyl, 1,2,3,4-tetrahydronaphthyl, anthracyl, phenanthracyl, pyrenyl, benzopyrenyl, fluorenyl and xanthenyl, in particular phenyl, and in currently selected embodiments, the aryl groups are preferably identical. In the currently most interesting embodiment, all three aryl groups are phenyl. Examples of substituents, which may be present in the aryl groups, in particular phenyl groups, are typically those selected from sulfonic acid, carboxylic acid, C_{1-6}-alkyl, C_{1-6}-alkoxy, and C_{1-6}-alkoxy-C_{1-6}-alkyl, or C_{3-6}-alkylene (representing a ring with two neighboring aryl carbon atoms) or C_{2-6}-alkyleneoxy (representing a ring with two neighboring aryl carbon atoms) or C_{1-4}-alkylene-oxy-C_{1-4}-alkylen (representing a ring with two neighboring aryl carbon atoms).

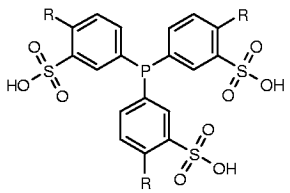
In one embodiment, at least one aryl (e.g. phenyl) has at least one substituent selected from sulfonic acid and carboxylic acid, in particular sulfonic acid; such substituent preferably being arranged in the meta position relative to the bond to the phosphor atom.

Preferably, all three aryl groups have a sulfonic acid substituent, e.g. all three aryl groups have a sulfonic acid substituent and at least one further substituent, in particular at least a substituent in the para-position relative to the bond to the phosphor atom, in particular an oxygen substituent in this para-position.

It is currently believed that the aryl groups of preferred reducing agents do not have any substituents in the ortho-position relative to the bond to the phosphor atom.

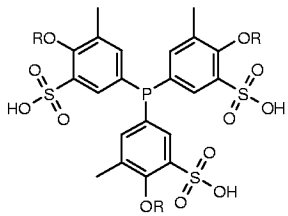
The term "C_{1-6}-alkyl" is intended to encompass linear or branched saturated hydrocarbon residues which have 1-6 carbon atoms. Particular examples are methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl, tert-butyl, n-pentyl, isopentyl, n-hexyl, etc. Similarly, the term "C_{1-4}-alkyl" encompasses linear or branched saturated hydrocarbon residues which have 1-4 carbon atoms. The terms "C_{1-6}-alkylene", "C_{2-6}-alkylene", etc. represent the biradicals corresponding to "C_{1-6}-alkyl", "C_{2-6}-alkyl", respectively.

Suitable triarylphosphine reducing agents are those having a useful balance between reduction potential and steric hinderance. The chemical nature of the triarylphosphine reducing agent is dictated by its ability to cleave the protein-low-molecular weight thiol mixed disulfide (RS-Cys) while preserving the integrity of the native disulfide bonds in the protein. Currently very interesting compounds are triarylphosphine trisulfonic acids, such as triphenylphosphine-3,3',3''-trisulfonic acid and analogues hereof. Illustrative examples hereof are triarylphosphine reducing agents selected from triphenylphosphine-3,3',3''-trisulfonic acid and analogues thereof, e.g. one of those selected from the compounds 9-11 below:



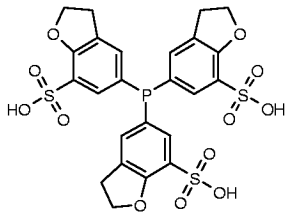
R = H, Me, Et, Pr, 2-Pr, Bu, MeO, EtO, PrO, 2-PrO, BuO.

9



R = Me, Et, Pr, 2-Pr, Bu.

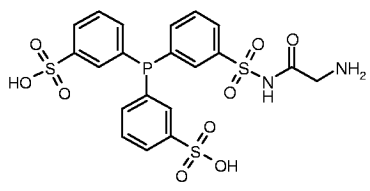
10



11

The triarylphosphine reducing agent is preferably used in a concentration of 0.001-100 mM, such as 0.01-50 mM or 0.1-25 mM.

In one interesting embodiment, the triarylphosphine reducing agent is immobilized to a solid support. This will facilitate the easy separation of the reducing agent from the protein. In general, triarylphosphine reducing agent, such as compounds **9**-**11,** may be immobilized by means known to the person skilled in the art, e.g. by introducing a linker group in one of the aryl groups. The triarylphosphine reagent **12** is an example of a linkable variant of **1**.



12

The reaction is typically conducted at a temperature in the range of 0-40°C, such as at ambient temperature, for a period of from 5 seconds to several days, as the case may be. The reaction may be followed by HPLC in order to confirm the conversion. The solvent is preferably an aqueous buffer, optionally including co-solvents such as DMSO or DMF. The solvent may also comprise salts, e.g. calcium salts.

In one variant, the triarylphosphine reducing agent is used in combination with an inhibitor for the protein, e.g. an active site S_1 pocket inhibitor, see further below.

In one currently preferred embodiment, the method for selective reduction of a FVIIa or FVII polypeptide comprising one or more cysteine moieties conjugated through a disulfide bridge to a low-molecular weight thiol (RS-Cys), said moiety/moieties not being involved in intramolecular S-S bridges (Cys-S-S-Cys) when the FVIIa polypeptide is in its active form, the method comprising the step of allowing the low-molecular weight thiol-conjugated FVIIa or FVII polypeptide to react with a mixture comprising reduced and oxidized glutathione and a glutaredoxin, and the simultaneous and/or subsequent step of conjugating at least one of the selectively reduced cysteine (HS-Cys) moieties with a chemical group.

In another currently preferred embodiment, the method for selective reduction of a FVIIa or FVII polypeptide comprising one or more cysteine moieties conjugated through a disulfide bridge to a low-molecular weight thiol (RS-Cys), said moiety/moieties not being involved in intramolecular S-S bridges (Cys-S-S-Cys) when the FVIIa polypeptide is in its active form, the method comprising the step of allowing the low-molecular weight thiol-conjugated FVIIa polypeptide to react with a mixture comprising a triarylphosphine-3,3',3''-trisulfonic acid compound and an active site S_1 pocket inhibitor, and the simultaneous and/or subsequent step of conjugating at least one of the selectively reduced cysteine (HS-Cys) moieties with a chemical group.

The FVIIa polypeptide

The selective reduction strategy using a redox buffer as described above is believed to be generally applicable for engineered FVIIa or FVII polypeptides comprising at least one non-native cysteine, in particular such proteins having engineered cysteines not being involved in intramolecular S-S bridges (Cys-S-S-Cys) when the protein is in its active form, and therefore potentially being in low-molecular weight thiol-conjugated (RS-Cys) form.

The term "low-molecular weight thiol-conjugated (RS-Cys) form" and similar terms are intended to mean that a thiol group of a cysteine of the FVIIa polypeptide in question is conjugated with a compound having a thiol group, wherein said compound has a molecular

weight of less than 500 Da. Examples of such compounds are glutathione, gamma-glutamylcysteine, cysteinylglycine, cysteine, homocysteine, N-acetylcysteine, cysteamine, etc.

The term "active form" refers to the form (or forms) of the protein wherein it is capable of performing a desirable action, e.g. as a catalyst (enzyme), zymogen, or as a co-factor, etc. The "active form" is sometimes referred to as the "correctly folded form".

In one interesting embodiment, a substantial portion of the FVIIa polypeptide (i.e. at least 50%) is in its active form when the selective reduction reaction is conducted.

The FVIIa polypeptide is generally an "engineered" polypeptide which compared to native FVIIa includes at least one non-native cysteine. Such "engineered" polypeptides are preferably prepared by recombinant techniques as will be apparent for the person skilled in the art; see also WO 02/077218 A1 and WO 01/58935 A2.

Protein inhibitor.

In one interesting embodiment, the mixture further comprises an inhibitor of the FVIIa polypeptide. By including an inhibitor in the mixture, it is believed that the conformation of the protein is somewhat stabilized whereby the intramolecular disulfide bonds have a lower tendency to become reduced by the redox buffer. Preferably, the inhibitor of the protein is an active-site inhibitor.

In the case of a FVIIa polypeptide, the presence of an active-site inhibitor extending into the S_1 binding pocket might be required during the selective reduction reaction to protect internal disulfide bonds in the active-site region from reduction. Inhibitors useful for this purpose include benzamidines, such as 4-aminobenzamidine, arginines, and other more potent analogues, see, e.g., those disclosed in WO 05/016365 A3 and those disclosed by Aventis in EP 1 162 194 A1, cf. in particular those defined in claims 1-6 and in sections [0009]-[0052], and in EP 1 270 551 A1, cf. in particular claims 1 and 2 and sections [0010]-[0032].

Conjugates

One important purpose of the selective reduction methods described above is to liberate a cysteine group which can be used for attachment (conjugation) of a chemical group, e.g. a non-polypeptide moiety.

Hence, in one important embodiment, the method further comprises the simultaneous and/or subsequent step of conjugating at least one of the selectively reduced cysteine (HS-Cys) moiety/moieties with a chemical group.

It should be understood that the conjugation of the at least one selectively reduced cysteine moieties with a chemical group may be conducted simultaneously, i.e. by addition of one or more reagents leading to the conjugation to the mixture comprising the redox buffer, or in a subsequent step, e.g. after purification and/or isolation of the selectively reduced protein.

It is also to be understood that conjugation may be conducted on a FVIIa polypeptide before synthesis of the dimeric or polymeric FVIIa compound, or it may be conducted after the dimeric or polymeric FVIIa compound has been synthesized.

In one embodiment, the chemical group is a protractor group, i.e. a group which upon conjugation to the FVIIa polypeptide increases the circulation half-life of said FVIIa polypeptide, when compared to the un-modified polypeptide. The specific principle behind the protractive effect may be caused by increased size, shielding of peptide sequences that can be recognized by peptidases or antibodies, or masking of glycanes in such way that they are not recognized by glycan specific receptores present in e.g. the liver or on macrophages, preventing or decreasing clearance. The protractive effect of the protractor group can e.g. also be caused by binding to blood components sush as albumin, or unspecific adhesion to vascular tissue. The conjugated glycoprotein should substantially preserve its biological activity.

In one embodiment of the invention the protractor group is selected from the group consisting of:

(a) A low molecular organic charged radical (15-1,000 Da), which may contain one or more carboxylic acids, amines, sulfonic acids, tetrazoles, acylsulfonamides, phosphonic acids, or combination thereof.

(b) A low molecular (15-1,000 Da) neutral hydrophilic molecule, such as cyclodextrin, or a polyethylene chain which may optionally branched.

(c) A low molecular (15-1,000 Da) hydrophobic molecule such as a fatty acid or cholic acid or derivatives thereof.

(d) Polyethyleneglycol with an average molecular weight of 2,000-60,000 Da.

(e) A well defined precision polymer such as a dendrimer with an exact molecular mass ranging from 700 to 20,000 Da, or more preferable between 700-10,000 Da.

(f) A substantially non-immunogenic polypeptide such as albumin or an antibody or part of an antibody optionally containing an Fc-domain.

(g) A high molecular weight organic polymer such as dextran.

In another embodiment of the invention the protractor group is selected from the group consisting of dendrimers, polyalkylene oxide (PAO), including polyalkylene glycol (PAG), such as polyethylene glycol (PEG) and polypropylene glycol (PPG), branched PEGs, polyvinyl alcohol (PVA), polycarboxylate, poly-vinylpyrolidone, polyethylene-co-maleic acid anhydride, polystyrene-co-maleic acid anhydride, and dextran, including carboxymethyl-dextran. In one particularly interesting embodiment of the invention, the protractor group is a PEG group.

The term "branched polymer", or interchangebly "dendritic polymer", "dendrimer" or "dendritic structure" means an organic polymer assembled from a selection of monomer building blocks of which, some contains branches.

In one embodiment of the invention the protractor group is a selected from the goup consisting of serum protein binding-ligands, such as compounds which bind to albumin, like fatty acids, C5-C24 fatty acid, aliphatic diacid (e.g. C5-C24). Other examples of protractor groups includes small organic molecules containing moieties that under physiological conditions alters charge properties, such as carboxylic acids or amines, or neutral substituents that prevent glycan specific recognition such as smaller alkyl substituents (e.g., C1-C5 alkyl). In one embodiment of the invention the protractor group is albumin.

In one embodiment, the chemical group is a non-polypeptide.

In one interesting embodiment, the chemical group is a polyethyleneglycol (PEG), in particular one having an average molecular weight of in the range of 500-100,000, such as 1,000-75,000, or 2,000-60,000.

Conjugation can be conducted as disclosed in WO 02/077218 A1 and WO 01/58935 A2.

Particularly interesting is the use of PEG as a chemical group for conjugation with the protein. The term "polyethylene glycol" or "PEG" means a polyethylene glycol compound or a derivative thereof, with or without coupling agents, coupling or activating moeities (e.g., with thiol, triflate, tresylate, azirdine, oxirane, pyridyldithio, vinyl sulfone, or preferably with a

maleimide moiety). Compounds such as maleimido monomethoxy PEG are exemplary of activated PEG compounds of the invention.

PEG is a suitable polymer molecule, since it has only few reactive groups capable of cross-linking compared to polysaccharides such as dextran. In particular, monofunctional PEG, e.g. methoxypolyethylene glycol (mPEG), is of interest since its coupling chemistry is relatively simple (only one reactive group is available for conjugating with attachment groups on the FVIIa polypeptide). Consequently, the risk of cross-linking is eliminated, the resulting FVIIa polypeptide conjugates are more homogeneous and the reaction of the polymer molecules with the FVIIa polypeptide is easier to control.

To effect covalent attachment of the polymer molecule(s) to the FVIIa polypeptide, the hydroxyl end groups of the polymer molecule are provided in activated form, i.e. with reactive functional groups. Suitable activated polymer molecules are commercially available, e.g. from Shearwater Corp., Huntsville, Ala., USA, or from PolyMASC Pharmaceuticals plc, UK. Alternatively, the polymer molecules can be activated by conventional methods known in the art, e.g. as disclosed in WO 90/13540. Specific examples of activated linear or branched polymer molecules for use in the present invention are described in the Shearwater Corp. 1997 and 2000 Catalogs (Functionalized Biocompatible Polymers for Research and pharmaceuticals, Polyethylene Glycol and Derivatives, incorporated herein by reference). Specific examples of activated PEG polymers include the following linear PEGs: NHS-PEG (e.g. SPA-PEG, SSPA-PEG, SBA-PEG, SS-PEG, SSA-PEG, SC-PEG, SG-PEG, and SCM-PEG), and NOR-PEG), BTC-PEG, EPOX-PEG, NCO-PEG, NPC-PEG, CDI-PEG, ALD-PEG, TRES-PEG, VS-PEG, IODO-PEG, and MAL-PEG, and branched PEGs such as PEG2-NHS and those disclosed in U.S. Pat. No. 5,932,462 and U.S. Pat. No. 5,643,575, both of which are incorporated herein by reference. Furthermore, the following publications, incorporated herein by reference, disclose useful polymer molecules and/or PEGylation chemistries: U.S. Pat. No. 5,824,778, U.S. Pat. No. 5,476,653, WO 97/32607, EP 229,108, EP 402,378, U.S. Pat. No. 4,902,502, U.S. Pat. No. 5,281,698, U.S. Pat. No. 5,122,614, U.S. Pat. No. 5,219,564, WO 92/16555, WO 94/04193, WO 94/14758, WO 94/17039, WO 94/18247, WO 94/28024, WO 95/00162, WO 95/11924, WO 95/13090, WO 95/33490, WO 96/00080, WO 97/18832, WO 98/41562, WO 98/48837, WO 99/32134, WO 99/32139, WO 99/32140, WO 96/40791, WO 98/32466, WO 95/06058, EP 439 508, WO 97/03106, WO 96/21469, WO 95/13312, EP 921 131, U.S. Pat. No. 5,736,625, WO 98/05363, EP 809 996, U.S. Pat. No. 5,629,384, WO 96/41813, WO 96/07670, U.S. Pat. No. 5,473,034, U.S. Pat. No. 5,516,673, EP 605 963, U.S. Pat. No. 5,382,657, EP 510 356, EP 400 472, EP 183 503 and EP 154 316.

The conjugation of the FVIIa polypeptide and the activated polymer molecules is conducted by use of any conventional method, e.g. as described in the following references (which also

describe suitable methods for activation of polymer molecules): R. F. Taylor, (1991), "Protein immobilisation. Fundamental and applications", Marcel Dekker, N.Y.; S. S. Wong, (1992), "Chemistry of Protein Conjugation and Crosslinking", CRC Press, Boca Raton; G. T. Hermanson et al., (1993), "Immobilized Affinity Ligand Techniques", Academic Press, N.Y.). The skilled person will be aware that the activation method and/or conjugation chemistry to be used depends on the attachment group(s) of the polypeptide (examples of which are given further above), as well as the functional groups of the polymer (e.g. being amine, hydroxyl, carboxyl, aldehyde, sulfydryl, succinimidyl, maleimide, vinysulfone or haloacetate). The PEGylation may be directed towards conjugation to all available attachment groups on the FVIIa polypeptide (i.e. such attachment groups that are exposed at the surface of the FVIIa polypeptide) or may be directed towards one or more specific attachment groups, e.g. the N-terminal amino group (U.S. Pat. No. 5,985,265). Furthermore, the conjugation may be achieved in one step or in a stepwise manner (e.g. as described in WO 99/55377).

It will be understood that the PEGylation is designed so as to produce the optimal molecule with respect to the number of PEG molecules attached, the size and form of such molecules (e.g. whether they are linear or branched), and where in the polypeptide such molecules are attached. The molecular weight of the polymer to be used will be chosen taking into consideration the desired effect to be achieved. For instance, if the primary purpose of the conjugation is to achieve a conjugate having a high molecular weight and larger size (e.g. to reduce renal clearance), one may choose to conjugate either one or a few high molecular weight polymer molecules or a number of polymer molecules with a smaller molecular weight to obtain the desired effect. Preferably, however, several polymer molecules with a lower molecular weight will be used.

It has further been found that advantageous results are obtained when the apparent size (also referred to as the "apparent molecular weight" or "apparent mass") of at least a major portion of the conjugate of the invention is at least about 50 kDa, such as at least about 55 kDa, such as at least about 60 kDa, e.g. at least about 66 kDa. This is believed to be due to the fact that renal clearance is substantially eliminated for conjugates having a sufficiently large apparent size. In the present context, the "apparent size" of a FVIIa polypeptide is determined by the SDS-PAGE method.

Furthermore, it has been reported that excessive polymer conjugation can lead to a loss of activity of the FVIIa polypeptide to which the chemical group (e.g. a non-polypeptide moiety) is conjugated (see further below). This problem can be eliminated, e.g., by removal of attachment groups located at the functional site or by reversible blocking the functional site prior to conjugation so that the functional site of the FVIIa polypeptide is blocked during conjugation. Specifically, the conjugation between the FVIIa polypeptide and the chemical

group (e.g. non-polypeptide moiety) may be conducted under conditions where the functional site of the FVIIa polypeptide is blocked by a helper molecule e.g. tissue factor capable of binding to the functional site of the FVIIa polypeptide or a serine protease inhibitor. Preferably, the helper molecule is one, which specifically recognizes a functional site of the FVIIa polypeptide, such as a receptor, in particular tissue factor, either full length or a suitably truncated form of tissue factor or two molecules, one being tissue factor the other one being a peptide or peptide inhibitor binding to and thus protecting the area around the catalytic triad (preferably defined as amino acid residues within 10 Å of any atom in the catalytic triad).

Alternatively, the helper molecule may be an antibody, in particular a monoclonal antibody recognizing the FVIIa polypeptide. In particular, the helper molecule may be a neutralizing monoclonal antibody.

The FVIIa polypeptide is preferably to interact with the helper molecule before effecting conjugation. (Often it is even advantageous to use the same helper molecule (e.g. an inhibitor) as the one used in the steps where mixed disulfides are reduced.) This ensures that the functional site of the FVIIs polypeptide is shielded or protected and consequently unavailable for derivatization by the chemical group (e.g. non-polypeptide moiety) such, as a polymer.

Following its elution from the helper molecule, the conjugate of the chemical group and the protein can be recovered with at least a partially preserved functional site.

Pharmaceutical compositions.

The dimeric and multimeric FVIIa compounds according to the present invention are applicable as pharmaceutical compositions for the treatment of bleeding disorders or bleeding episodes in a subject or for the enhancement of the normal haemostatic system. Examples of subjects in need of such treatment are i.a. subjects being treated for haemophilia A or B.

In another aspect, the present invention includes within its scope pharmaceutical compositions comprising a dimeric or multimeric FVIIa compound, as an active ingredient, or a pharmaceutically acceptable salt thereof together with a pharmaceutically acceptable carrier or diluent.

Optionally, the pharmaceutical composition of the invention may comprise a dimeric or multimeric FVIIa compound in combination with one or more other compounds exhibiting anticoagulant activity, e.g., platelet aggregation inhibitor.

The compounds of the invention may be formulated into pharmaceutical composition comprising the compounds and a pharmaceutically acceptable carrier or diluent. Such carriers include water, physiological saline, ethanol, polyols, e.g., glycerol or propylene glycol, or vegetable oils. As used herein, "pharmaceutically acceptable carriers" also encompasses any and all solvents, dispersion media, coatings, antifungal agents, preservatives, isotonic agents and the like. Except insofar as any conventional medium is incompatible with the active ingredient and its intended use, its use in the compositions of the present invention is contemplated.

The compositions may be prepared by conventional techniques and appear in conventional forms, for example, capsules, tablets, solutions or suspensions. The pharmaceutical carrier employed may be a conventional solid or liquid carrier. Examples of solid carriers are lactose, terra alba, sucrose, talc, gelatine, agar, pectin, acacia, magnesium stearate and stearic acid. Examples of liquid carriers are syrup, peanut oil, olive oil and water. Similarly, the carrier or diluent may include any time delay material known to the art, such as glyceryl monostearate or glyceryl distearate, alone or mixed with a wax. The formulations may also include wetting agents, emulsifying and suspending agents, preserving agents, sweetening agents or flavouring agents. The formulations of the invention may be formulated so as to provide quick, sustained, or delayed release of the active ingredient after administration to the patient by employing procedures well known in the art.

The pharmaceutical compositions can be sterilised and mixed, if desired, with auxiliary agents, emulsifiers, salt for influencing osmotic pressure, buffers and/or colouring substances and the like, which do not deleteriously react with the active compounds.

The route of administration may be any route, which effectively transports the active compound to the appropriate or desired site of action, such as oral or parenteral, e.g., rectal, transdermal, subcutaneous, intranasal, intramuscular, topical, intravenous, intraurethral, ophthalmic solution or an ointment, the oral route being preferred.

If a solid carrier for oral administration is used, the preparation can be tabletted, placed in a hard gelatine capsule in powder or pellet form or it can be in the form of a troche or lozenge. The amount of solid carrier may vary widely but will usually be from about 25 mg to about 1 g. If a liquid carrier is used, the preparation may be in the form of a syrup, emulsion, soft gelatine capsule or sterile injectable liquid such as an aqueous or non-aqueous liquid

suspension or solution.

For nasal administration, the preparation may contain a compound of formula (I) dissolved or suspended in a liquid carrier, in particular an aqueous carrier, for aerosol application. The carrier may contain additives such as solubilizing agents, e.g. propylene glycol, surfactants, absorption enhancers such as lecithin (phosphatidylcholine) or cyclodextrin, or preservatives such as parabenes.

For parenteral application, particularly suitable are injectable solutions or suspensions, preferably aqueous solutions with the active compound dissolved in polyhydroxylated castor oil.

Tablets, dragees, or capsules having talc and/or a carbohydrate carrier or binder or the like are particularly suitable for oral application. Preferable carriers for tablets, dragees, or capsules include lactose, corn starch, and/or potato starch. A syrup or elixir can be used in cases where a sweetened vehicle can be employed.

A typical tablet, which may be prepared by conventional tabletting techniques, contains

Core:	
Active compound (as free compound or salt thereof)	10 mg
Colloidal silicon dioxide (Areosil®)	1.5 mg
Cellulose, microcryst. (Avicel®)	70 mg
Modified cellulose gum (Ac-Di-Sol®)	7.5 mg
Magnesium stearate	
Coating:	
HPMC	approx. 9 mg
*Mywacett® 9-40 T	approx. 0.9 mg

*Acylated monoglyceride used as plasticizer for film coating.

The compounds of the invention may be administered to a mammal, especially a human in need of such treatment, prevention, elimination, alleviation or amelioration of various thrombolytic or coagulophatic diseases or disorders as mentioned above. Such mammals also include animals, both domestic animals, e.g. household pets, and non-domestic animals such as wildlife.

Usually, dosage forms suitable for oral, nasal, pulmonal or transdermal administration comprise from about 0.001 mg to about 100 mg, preferably from about 0.01 mg to about 50 mg of the compounds of formula I admixed with a pharmaceutically acceptable carrier or diluent.

The compounds may be administered concurrently, simultaneously, or together with a pharmaceutically acceptable carrier or diluent, whether by oral, rectal, or parenteral (including subcutaneous) route. The compounds are often, and preferably, in the form of an alkali metal or earth alkali metal salt thereof.

Suitable dosage ranges varies as indicated above depending upon the exact mode of administration, form in which administered, the indication towards which the administration is directed, the subject involved and the body weight of the subject involved, and the preference and experience of the physician or veterinarian in charge.

The present invention is further illustrated by the following examples which, however, are not to be construed as limiting the scope of protection. The features disclosed in the foregoing description and in the following examples may, both separately or in any combination thereof, be material for realising the invention in diverse forms thereof.

EXAMPLES

Example 1

Materials – Reduced and oxidized glutathione (GSH and GSSG, respectively), cysteine (Cys), DL-homocysteine (hCy), cysteinylglycine (CG), and γ-glutamylcysteine (γ-GC) were purchased from Sigma. Cysteamine (Cya) and 7-fluorobenzofurazan-4-sulfonic acid ammonium salt (SBD-f) were obtained from Fluka. Tris(2-carboxyethyl)phosphine (TCEP) was purchased from Calbiochem (Merck KGaA, Darmstadt, Germany). Chromogenic S-2288 substrate was obtained from Chromogenix (Milano, Italy). PEG5k-maleimide (2E2M0H01), PEG20k-maleimide (2E2M0P01), PEG40k-maleimide (2D3Y0T01), and maleimide-PEG3.4k-maleimide (2E2E0F02) were purchased from Nektar Therapeutics (Huntsville, AL). d-Phe-Phe-Arg-chloromethyl ketone was purchased from Bachem. Triphenylphosphine-3,3',3'' trisulfonic acid was obtained from Aldrich. Human plasma-derived FX and FXa were obtained from Enzyme Research Laboratories Inc. (South Bend, IN). Biotin-polyethyleneoxide-iodoacetamide (Biotin-PEO-iodoacetamide) came from Sigma (Missouri, USA). Soluble tissue factor 1-219 (sTF) was prepared according to published procedures (Freskgard et al., 1996). sTF (1-219) Glu219→Cys (E219C) was prepared essentially as described previously (Owenius et al., 1999). Expression and purification of recombinant FVIIa was performed as described previously (Thim et al., 1988; Persson and Nielsen, 1996). All other chemicals were of analytical grade or better.

Concentration determination – The concentration of GSSG in stock solutions was determined from its absorption at 248 nm using an extinction coefficient of 381 $M^{-1}cm^{-1}$ (Chau and Nelson, 1991). The concentration of GSH and other low-molecular weight thiols were determined using Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid)) and 14150 $M^{-1}cm^{-1}$ as the molar extinction coefficient of 2-nitro-5-thiobenzoic acid at 412 nm (Riddles et al., 1979).

Quantification of GSSG by HPLC – Quantification of GSSG was performed essentially as described elsewhere (Takahashi and Creighton, 1996). Briefly, 50 µl acid quenched sample were loaded onto a C_{18} reversed-phase column (Luna C18(2) 100 Å, 3 µm particle size, 4.6x50mm; Phenomenex Inc., Torrance, CA) maintained at 30°C. Following 5 min isocratic run at 100% eluent A (0.1% (v/v) trifluoroacetic acid (TFA) in water), GSSG was eluted by a linear gradient from 0-5% eluent B (0.085% (v/v) TFA in acetonitrile) in 5 min at a flow rate of 1 ml/min and detected by absorption at 214 nm. The concentration of GSSG was determined by relating the calculated peak area (Millenium32 v4.0 software, Waters) to a standard curve made with known amounts of GSSG. Linearity was observed in the range from 2-25 nmol GSSG.

Analysis of thiol-modified FVIIa 407C by HPLC – Free and thiol-modificed FVIIa 407C species were analyzed by HPLC using a C_3 reversed-phase column (Zorbax 300SB-C3, 2.1x150mm, 5-µm particle size; Agilent Technologies, Denmark) maintained at 45°C. The flow rate was 0.5 ml/min and mobile phases consisted of 0.1% (v/v) TFA in water (eluent A) and 0.085% (v/v) TFA in acetonitrile (eluent B). After injection of 25 µl acid quenched sample, the system was run isocratically at 30% eluent B for 5 min followed by linear gradients from 38-41.5% eluent B over 20 min and 41.5-55% eluent B over 20 min. The eluate was monitored by fluorescence (excitation and emission wavelengths of 280 and 348 nm, respectively).

Construction of DNA encoding FVII 407C mutant – A DNA construct encoding FVIIa 407C was constructed as described in WO 02/077218 A1

Construction of DNA encoding FVII Q250C mutant – A DNA construct encoding FVIIa Q250C was constructed as described in WO 02/077218 A1

Construction of DNA encoding FVII R396C mutant – A DNA construct encoding FVIIa R396C was constructed as described in WO 02/077218 A1

Expression and purification of FVII 407C – BHK cells were transfected essentially as previously described (Thim et al., 1988; Persson and Nielsen, 1996) to obtain expression of the FVIIa 407C variant. The Factor VII polypeptide was purified as follows:

Conditioned medium was loaded onto a 25-ml column of Q Sepharose Fast Flow (Amersham Biosciences, GE Healthcare) after addition of 5 mM EDTA, 0.1% Triton X-100 and 10 mM Tris, adjustment of pH to 8.0 and adjustment of the conductivity to 10-11 mS/cm by adding water. Elution of the protein was accomplished by a gradient from 10 mM Tris, 50 mM NaCl, 0.1% Triton X-100, pH 8.0 to 10 mM Tris, 50 mM NaCl, 25 mM $CaCl_2$, 0.1% Triton X-100, pH 7.5. The fractions containing FVIIa 407C were pooled, and applied to a 25-ml column containing the monoclonal antibody F1A2 (Novo Nordisk A/S, Bagsværd, Denmark) coupled to CNBr-activated Sepharose 4B (Amersham Biosciences, GE Healthcare). The column was equilibrated with 50 mM HEPES, pH 7.5, containing 10 mM $CaCl_2$, 100 mM NaCl and 0.02% Triton X-100. After washing with equilibration buffer and equilibration buffer containing 2 M NaCl, bound material was eluted with equilibration buffer containing 10 mM EDTA instead of $CaCl_2$. Before storage, FVIIa 407C was transferred to a 50 mM HEPES, 100 mM NaCl, 10 mM $CaCl_2$, pH 7.0 buffer by dialysis. The yield of each step was followed by factor VII ELISA measurements and the purified protein was analysed by SDS-PAGE.

Expression and purification of FVII Q250C – BHK cells were transfected essentially as previously described (Thim et al., 1988; Persson and Nielsen, 1996) to obtain expression of the FVIIa Q250C variant. The Factor VII polypeptide was purified as follows:

Conditioned medium was loaded onto a 25-ml column of Q Sepharose Fast Flow (Amersham Biosciences, GE Healthcare) after addition of 5 mM EDTA, 0.1% Triton X-100 and 10 mM Tris, adjustment of pH to 8.0 and adjustment of the conductivity to 10-11 mS/cm by adding water. Elution of the protein was accomplished by a gradient from 10 mM Tris, 50 mM NaCl, 0.1% Triton X-100, pH 8.0 to 10 mM Tris, 50 mM NaCl, 25 mM $CaCl_2$, 0.1% Triton X-100, pH 7.5. The fractions containing FVIIa 407C were pooled, and applied to a 25-ml column containing the monoclonal antibody F1A2 (Novo Nordisk A/S, Bagsværd, Denmark) coupled to CNBr-activated Sepharose 4B (Amersham Biosciences, GE Healthcare). The column was equilibrated with 50 mM HEPES, pH 7.5, containing 10 mM $CaCl_2$, 100 mM NaCl and 0.02% Triton X-100. After washing with equilibration buffer and equilibration buffer containing 2 M NaCl, bound material was eluted with equilibration buffer containing 10 mM EDTA instead of $CaCl_2$. Before storage, FVIIa Q250C was transferred to a 50 mM HEPES, 100 mM NaCl, 10 mM $CaCl_2$, pH 7.0 buffer by dialysis. The yield of each step was followed by factor VII ELISA measurements and the purified protein was analysed by SDS-PAGE.

Expression and purification of FVII R396C – Expression and purification of FVIIa R396C was performed as described in WO 02/077218 A1

Cloning and expression of glutaredoxins – DNA coding sequences for *Escherichia coli* glutaredoxin 2 (Grx2) and *Saccharomyces cerevisiae* glutaredoxin 1 (yGrx1p) were amplified by PCR using Expand High Fidelity PCR system (Roche Diagnostics Corporation, Indianapolis, IN) according to manufacturer's recommendations and primer pairs oHOJ98-f/oHOJ98-r and oHOJ11-f/oHOJ11-r, respectively, introducing flanking *Nde*I and *Xho*I restriction sites (primer sequences are listed in Table 1).

Table 1 - DNA oligos used for construction of plasmids pHOJ294, 210, and 286 expressing *E.coli* glutaredoxin 2 (Grx2), *S.cerevisiae* glutaredoxin 1 (yGrx1p), and yGrx1p C30S, respectively. *Nde*I and *Xho*I restriction sites are shown in bold face.

Primer	Plasmid	Target	Sequence (5'→3')
oHOJ11-f	pHOJ210	yGrx1p	GGGCCGCC**CATATG**GTATCTCAAGAAACTATC
oHOJ11-r	pHOJ210	yGrx1p	GCCCGGG**CTCGAG**ATTTGCAAGAATAGGTTCTAAC
oHOJ98-f	pHOJ294	Grx2	GCCGCCGG**CATATG**AAGCTATACATTTACGATCACTGCCC
oHOJ98-r	pHOJ294	Grx2	CCGCCGCC**CTCGAG**AATCGCCATTGATGATAACAAATTGATTTGTG
oHOJ88-f	pHOJ286	yGrx1p	GTTTAGGGCTGCATGCGAGTATGGACAGTACG

		C30S	
oHOJ88-r	pHOJ286	yGrx1p C30S	CGTACTGTCCATACTCGCATGCAGCCCTAAAC

Genomic template DNA for PCR reactions was prepared from *E.coli* and *S.cerevisiae* according to published procedures (Grimberg et al., 1989; Hoffman and Winston, 1987). The purified PCR products were cut with NdeI and XhoI and then ligated into the corresponding sites of pET-24a(+) (Novagen) to give pHOJ294 and pHOJ210, respectively. Since stop codons were provided by the vector, the two genes were equipped with 3' vector-derived extensions encoding C-terminal LEHHHHHH affinity tags. Plasmid pHOJ286 encoding yGrx1p Cys30→Ser (yGrx1p C30S) was constructed by QuickChange® Site-Directed Mutagenesis using primers oHOJ88-f/oHOJ88-r and pHOJ210 as template according to manufacturer's instructions (Stratagene, La Jolla, CA). The correct identity of all cloned sequences was verified by DNA sequencing.

For expression, pHOJ210, 286, and 294 plasmids were introduced into chemical competent BL21(DE3) cells (Stratagene, La Jolla, CA). Fresh overnight transformants were inoculated into 500 ml terrific broth ((Sambrook et al., 1989)) and 30 µg/ml kanamycine to an initial OD_{600} of 0.02. Cultures were grown at 37°C in baffled flasks at 230 rpm to the mid-log phase (OD_{600} 3-4) at which time the temperature was lowered to 25°C and protein expression induced by 0.1 mM isopropyl-β-D-thiogalactopyranoside (ITPG). After overnight expression, cells were harvested by centrifugation, resuspended in 50 ml lysis buffer (50 mM potassium phosphate, 300 mM NaCl, pH 8.0), and lysed by three freeze-thaw cycles. The cleared lysate was loaded onto a 20-ml Ni-NTA Superflow (Qiagen GmbH, Hilden, Germany) column equilibrated with lysis buffer at a flow rate of 5 ml/min. After washing with lysis buffer, bound protein was eluted with a linear gradient from 0-200 mM imidazole in lysis buffer. Peak fractions were pooled, treated with 20mM dithiothreitol for 20 min before extensive dialysis against 50 mM Tris-HCl, 2 mM EDTA, pH 8.0. Proteins were stored at -80°C and judged to be >90% pure by SDS-PAGE. Concentrations were estimated by absorbance at 280 nm using extinction coefficients of 5240 $M^{-1}cm^{-1}$ (yGrx1p and yGrx1p C30S) and 21740 $M^{-1}cm^{-1}$ (Grx2).

Identification of low-molecular weight thiols engaging in mixed disulfides with FVIIa 407C – HPLC detection of fluorescent SBD-derivatized low-molecular weight thiols was performed as described by Oe et al. (1998) with minor modifications. Briefly, disulfide reduction and subsequent derivatization of liberated thiols was performed by incubating 25 µl of 10 µM FVIIa 407C (or wild-type FVIIa) in 160 mM Tris-HCl, 8 mM EDTA, pH 9.6 buffer with 5 µl 14 mM TCEP (in water) and 10 µl 0.3% SBD-f (in water) at 60°C for 60 min. Subsequently, derivatization was terminated by addition of 2 µl 5 M HCl and samples were placed at 4°C

until further analysis (within 24 hr). HPLC analysis was performed by injecting 25-µl aliquots of the samples onto a reversed-phase C18(2) column (Luna, 100 Å, 3.5 µm particle size, 150×4.6 mm; Phenomenex Inc., Torrance, CA) at a flow rate of 1 ml/min. The column temperature was maintained at 30°C. SBD-derivatized thiols were separated by isocratic elution using a mobile phase consisting of 75 mM Na-Citrate, pH 2.90 and 2% methanol and detected by the fluorescence emitted at 516 nm upon excitation at 386 nm. Peak identification was performed by comparison of retention times with those of a series of known low-molecular weight thiol compounds prepared according to the procedure described above for FVIIa 407C. Calibration curves for quantification of GSH, γ-GC, GC, Cys, Hcy, and Cya were obtained by varying the concentration of each thiol from 0.4 to 3.5 µM in the final reaction mixture.

From this analysis, it can be concluded that major low-molecular weight thiols conjugated to FVIIa 407C are glutathione, cysteine, and homocysteine. Results are presented in Figure 1.

Redox titration of FVIIa – To identify conditions appropriate for selective reduction of FVIIa Cys mutants, the structural stability of FVIIa was assessed in buffers with defined redox potentials obtained by varying concentrations of GSH and GSSG essentially as described elsewhere (Loferer et al., 1995). Since reduction of the two most labile disulfide bonds in FVIIa has been shown to be associated with a loss of amidolytic activity and sTF binding (Higashi et al., 1997), the structural integrity of FVIIa was monitored by its ability to hydrolyse the chromogenic substrate S-2288 in the presence of sTF.

Redox titration of FVIIa (1 µM) was performed in 50 mM HEPES, 100 mM NaCl, 5 mM $CaCl_2$, pH 7.0 buffer (thoroughly purged with nitrogen) containing 50 µM GSSG and varying concentrations of GSH (0 – 34 mM). In addition, one series of samples contained 25 mM *p*-aminobenzamidine, an active-site inhibitor of FVIIa occupying the S_1 pocket (Sichler et al., 2002; Persson et al., 2004). To reduce the time required to reach equilibrium, reactions were performed in the presence of 1 µM yGrx1p acting as a redox catalyst (Ostergaard et al., 2004). After equilibration of the samples for 3.5 hours at 30°C under nitrogen atmosphere, residual amidolytic activity was determined as described below. At the same time, an aliquot of the reaction mixture was quenched by an equal volume of 100 mM HCl, and the equilibrium concentration of GSSG determined by HPLC as described in *materials and methods*.

For measurement of residual amidolytic activity, 20 µl of the equilibrated samples were diluted 20-fold into assay buffer (50 mM HEPES, 100 mM NaCl, 5 mM $CaCl_2$, 0.01% Tween 80, pH 7.4) containing 10 mM iodoacetamide to rapidly alkylate free thiols and prevent subsequent thiol oxidation. The activity assay was carried out in polystyrene microtiter plates

(Nunc, Denmark) in a final volume of 200 µl assay buffer containing 80 nM sTF and quenched sample to a final concentration of 10 nM FVIIa. After 15 min pre-incubation at room temperature, 1 mM chromogenic substrate S-2288 was added and the absorbance monitored continuously at 405 nm for 20 min in a SpectraMax™ 340 microplate spectrophotometer equipped with SOFTmax PRO software (v2.2; Molecular Devices Corp., Sunnyvale, CA). Amidolytic activity was reported as the slope of the linear progress curves after blank subtraction.

Data were analyzed in terms of the following reaction (Eq. 1), where FVIIa is converted into inactivated FVIIa (denoted FVIIai) by reversible reduction of a single intramolecular disulfide bond:

Eq. 1 $\quad FVIIa + 2GSH \Leftrightarrow FVIIai + GSSG$

The apparent equilibrium constant for the reverse reaction (K_{ox}) can be estimated from the following relationship (Eq. 2)

Eq. 2 $\quad f = a_{max}/(1+[GSH]^2/([GSSG]\ K_{ox}))$

where f is the residual amidolytic activity at a given $[GSH]^2/[GSSG]$, and a_{max} is the limiting amidolytic activity at low $[GSH]^2/[GSSG]$.

Fitting the redox titration data to Eq. 2 by non-linear least squares regression using Kaleidagraph software (v3.6, Synergy software) yielded apparent K_{ox}'s of 93±6 mM and 166±16 mM in the absence or presence of 25 mM *p*-aminobenzamidine, respectively (Figure 2).

Redox titration of FVIIa 407C-glutathione mixed disulfide – The stability of the mixed disulfide between glutathione and Cys407 was measured by incubating 13 µM FVIIa 407C in 50 mM HEPES, 100 mM NaCl, 10mM $CaCl_2$, pH 7.0 containing 0.5 mM GSH and varying concentrations of GSSG (5-500 µM). In addition, all samples contained 10 µM Grx2 to catalyze the reaction. After 5 hours equilibration at 30°C, a 50-µl aliquot was quenched with 100 mM HCl and the equilibrium concentration of GSSG determined by HPLC as described in *materials and methods*. To measure the relative amount of deglutathionylated FVIIa 407C, free thiols were labelled with PEG5k by combining 20 µl of each sample with 15 µl 1.6 mM PEG5k-maleimide. Following 18 min incubation at room temperature, *N*-ethylmaleimide was added to a final concentration of 25 mM to competitively suppress further (unspecific) PEGylation of the protein during subsequent processing. PEG5k-modified FVIIa 407C in each sample was detected and quantified by HPLC as described in *material and methods*.

Data were analyzed according to the following reaction (Eq. 3), where glutathionylated FVIIa 407C (FVIIa 407C-GSH) reacts with GSH to give free FVIIa 407C and GSSG

Eq. 3 FVIIa 407C-GSH + GSH ⇔ FVIIa 407C + GSSG

The apparent equilibrium constant for the reverse reaction, denoted K_{scox}, can be estimated from the following relationship (Eq. 4)

Eq. 4 $A_{407C\text{-}PEG5k} = A_{max}([GSH]/[GSSG])/([GSH]/[GSSG] + K_{scox})$

where $A_{407C\text{-}PEG5k}$ is the peak area of 5k-PEGylated FVIIa 407C at a given [GSH]/[GSSG] ratio, and A_{max} is the limiting peak area at high [GSH]/[GSSG].

A plot of the measured peak areas versus the [GSH]/[GSSG] ratio at equilibrium is shown in Figure 3. Fitting of Eq. 4 to the data by non-linear least squares regression using Kaleidagraph software (v3.6, Synergy software) gave an apparent K_{scox} of 1.0, very similar to that measured for a range of other glutathionylated proteins (Gilbert, 1995).

Identification of optimal reduction conditions – Optimal glutathione redox conditions supporting selective reduction of the FVIIa 407C- mixed disulfides were identified from plots of the following parameters as a function of [GSSG]: (1) the residual amidolytic activity in the presence or absence of *p*-aminobenzamidine using Eq. 2 and estimated K_{ox} values, and (2) the fraction of selectively reduced protein from Eq. 4 and K_{scox}. For practical reasons, the concentration of GSH was set to 0.5 mM. As shown in Figure 4, a concentration of GSSG between roughly 15 and 60 µM in the presence of 0.5 mM GSH results in >90% residual activity and >90% free Cys407. The optimal [GSSG] working range depends on several parameters, including the concentration of GSH (as exemplified in Figures 3-5), the values of K_{ox} and K_{scox} (not shown), and the allowed loss of amidolytic activity during the reduction reaction.

Selective reduction and PEG5k, PEG20k, and PEG40k modification of FVIIa 407C – Thiol modification of FVIIa 407C can be divided into three consecutive steps: (A) a glutaredoxin-catalyzed reduction reaction, (B) thiol-specific alkylation, and (C) purification. At the end of each step, a small aliquot of the reaction mixture was quenched with 10% (v/v) formic acid and analyzed by HPLC as described in *material and methods* and exemplified in Figure 7.

(A) FVIIa 407C (4.8 mg) was incubated 4.5 hours at 30°C in a total volume of 4.4 ml 50 mM HEPES, 100 mM NaCl, 10 mM $CaCl_2$, pH 7.0 buffer containing 0.5 mM GSH, 15 µM GSSG, 25 mM *p*-aminobenzamidine, and 10 µM Grx2. The initial concentration of GSSG was in the

lower end of the optimal working range (shaded area in Figure 4) to compensate for the formation of GSSG during the reaction. (B) Subsequently, free thiols were modified by addition of PEG5k-maleimide, PEG20k-maleimide, or PEG40k-maleimide (dissolved in water) to a final concentration of 0.8 mM. Thiol alkylation was allowed to proceed for 15 min at room temperature upon quenching with 0.5 mM cysteine. (C) EDTA was added in excess of calcium (20 mM final concentration) and the entire content loaded onto a 1 ml HiTrap Q FF column (Amersham Biosciences, GE Healthcare) equilibrated with buffer A (50 mM HEPES, 100 mM NaCl, 1mM EDTA, pH 7.0) to capture FVIIa 407C. After wash with buffer A, one-step elution of bound protein was performed with buffer B (10 mM GlyGly, 150 mM NaCl, 10 mM $CaCl_2$, 0.01% Tween 80, pH 7.0) directly onto a HiLoad Superdex 200 16/60 pg column (Amersham Biosciences) mounted in front of the HiTrap column. PEGylated and non-PEGylated species were separated at a flow rate of 1 ml/min and detected by absorption at 280 nm.

Selective reduction and PEG3.4 or PEG20k-crosslinking of FVIIa 407C – Selective reduction was carried out by incubating 4.8 mg FVIIa 407C at 30°C for 4.5 hours in a total volume of 4.4 ml 50 mM HEPES, 100 mM NaCl, 10 mM $CaCl_2$, pH 7.0 buffer containing 0.5 mM GSH, 10 µM GSSG, 25 mM *p*-aminobenzamidine, and 10 µM Grx2. Then, EDTA was added in excess of calcium (20 mM final concentration) and the entire content loaded onto a 1 ml HiTrap Q FF column (Amersham Biosciences, GE Healthcare) equilibrated in buffer A (50 mM HEPES, 100 mM NaCl, 1mM EDTA, pH 7.0) to capture FVIIa 407C. After wash with buffer A to remove unbound glutathione buffer and Grx2p, FVIIa 407C was eluted in one step with buffer B (50 mM HEPES, 100 mM NaCl, 10 mM $CaCl_2$, pH 7.0). The concentration of FVIIa 407C in the eluate was measured by absorbance at 280 nm using an extinction coefficient of $62 \cdot 10^3$ $M^{-1}cm^{-1}$. Cross-linking was performed in the presence of approximately 0.6 equivalent of maleimide-PEG3.4k-maleimide or maleimide-PEG20k-maleimide for 1.5 hours at room temperature. (C) EDTA was added in excess of calcium (20 mM final concentration) and the entire content loaded onto a 1 ml HiTrap Q FF column (Amersham Biosciences, GE Healthcare) equilibrated in buffer A (50 mM HEPES, 100 mM NaCl, 1mM EDTA, pH 7.0) to capture FVIIa 407C. After wash with buffer A, one-step elution of bound protein was performed with buffer B (10 mM GlyGly, 150 mM NaCl, 10 mM $CaCl_2$, 0.01% Tween 80, pH 7.0) directly onto a HiLoad Superdex 200 16/60 pg column (Amersham Biosciences) to separate PEGylated and non-PEGylated species. The flow rate was 1 ml/min and protein was detected by absorption at 280 nm.

SDS-PAGE analysis of FVIIa 407C, FVIIa 407C-PEG5k, FVIIa 407C-PEG20k, FVIIa 407C-PEG40k, and FVIIa 407C-PEG3.4k-FVIIa 407C – FVIIa 407C and 5k, 20k, 40k, and 3.4k-PEGylated compounds (approx. 1.5 µg of each) were analyzed by reducing and non-reducing SDS-PAGE on a 4-12% Bis-Tris NuPAGE® gel (Invitrogen Life Technologies, Carlsbad, CA) run

at 200 V for 35 min in MES buffer (Invitrogen Life Technologies, Carlsbad, CA) according to manufacturer's recommendations. Gels were washed with water and stained with Simply Blue™ SafeStain (Invitrogen Life Technologies, Carlsbad, CA) according to manufacturer's recommendations. Gels are shown in Figure 8.

SDS-PAGE analysis of FVIIa 407C, FVIIa 407C-PEG3.4k-FVIIa 407C, and FVIIa 407C-PEG20k-FVIIa 407C – Proteins (approx. 1 µg of each) were analyzed by reducing and non-reducing SDS-PAGE on a 4-12% Bis-Tris NuPAGE® gel (Invitrogen Life Technologies, Carlsbad, CA) run at 200 V for 35 min in MES buffer (Invitrogen Life Technologies, Carlsbad, CA) according to manufacturer's recommendations. Gels were washed with water and stained with Simply Blue™ SafeStain (Invitrogen Life Technologies, Carlsbad, CA) according to manufacturer's recommendations. Gels are shown in Figure 9.

Active-site titration of FVIIa 407C, FVIIa 407C-PEG5k, FVIIa 407C-PEG20k, FVIIa 407C-PEG40k, FVIIa 407C-PEG3.4k-FVIIa 407C, and FVIIa 407C-PEG20k-FVIIa 407C – Active site concentrations of FVIIa 407C and PEGylated compounds were determined from the irreversible loss of amidolytic activity upon titration with sub-stoichiometric levels of d-Phe-Phe-Arg-chloromethyl ketone (FFR-cmk) essentially as described elsewhere (Bock, 1992). Briefly, each protein was diluted into 50 mM HEPES, 100 mM NaCl, 10 mM $CaCl_2$, 0.01% Tween 80, pH 7.0 buffer to an approximate concentration of 300 nM using an extinction coefficient of $62 \cdot 10^3$ $M^{-1}cm^{-1}$ at 280 nm. Diluted protein (20 µl) was then combined with 20 µl 1.5 µM sTF and 20 µl 0-1.2 µM FFR-cmk (freshly prepared in buffer from a FFR-cmk stock dissolved in DMSO and stored at -80°C). After overnight incubation at room temperature, residual amidolytic activity was measured.

The activity assay was carried out in polystyrene microtiter plates (Nunc, Denmark) in a final volume of 200 µl assay buffer (50 mM HEPES, 100 mM NaCl, 5 mM $CaCl_2$, 0.01% Tween 80, pH 7.4) containing 50 nM sTF and approx. 10 nM FVIIa, corresponding to 10-fold dilutions of the samples. After 15 min pre-incubation at room temperature, 1 mM chromogenic substrate S-2288 was added and the absorbance monitored continuously at 405 nm for 20 min in a SpectraMax™ 340 microplate spectrophotometer equipped with SOFTmax PRO software (v2.2; Molecular Devices Corp., Sunnyvale, CA). Amidolytic activity was reported as the slope of the linear progress curves after blank subtraction. Active site concentrations were determined by extrapolation, as the minimal concentration of FFR-cmk completely abolishing amidolytic activity.

In Table 2 are given the measured active site concentrations relative to the absorbances of the proteins at 280 nm. Values are normalized to 100% for FVIIa 407C.

Table 2 - Specific active-site concentrations of FVIIa 407C and PEGylated variants. Specific active-site concentrations were measured as the active-site concentration by FFR-cmk titration relative to the absorbance of the protein at 280 nm. Values are normalized to 100% for FVIIa 407C.

Protein	**[Active-site]/A_{280}**
FVIIa 407C	100%
FVIIa 407C-PEG5k	90%
FVIIa 407C-PEG20k	86%
FVIIa 407C-PEG40k	91%
FVIIa 407C-PEG3.4k-FVIIa 407C	95%
FVIIa 407C-PEG20k-FVIIa 407C	90%

Surface plasmon resonance (SPR) analysis of the binding of FVIIa 407C, FVIIa 407C-PEG3.4k-FVIIa 407C, and FVIIa 407C-PEG20k-FVIIa 407C to immobilized soluble tissue factor – Binding of FVIIa 407C and PEGylated variants to immobilized soluble tissue factor was analyzed by surface plasmon resonance analysis on a Biacore 3000 instrument (Biacore AB, Uppsala, Sweden). Prior to immobilization, sTF E219C was biotinylated at its free cysteine by reacting the protein with 2mM biotin-PEO-iodoacetamide at pH 7.0 for 20 min. Excess reagent was subsequently removed by gelfiltration on a NAP™5 column (Amersham Biosciences AB, Uppsala, Sweden) equilibrated in HBS-P buffer (10mM HEPES, 150mM NaCl, 0,005% P20; Biacore AB, Uppsala, Sweden) containing 5mM $CaCl_2$. Biotinylated sTF E19C was immobilized at a density of 2.2 fmol/mm^2 (~50 RU) in flow cell 2 of a SA sensor chip (Biacore AB, Uppsala, Sweden). Kinetic analysis was performed at a flow rate of 30 µl/min in running buffer (HBS-P buffer containing 5mM $CaCl_2$) using the untreated flow cell 1 for automatic in-line reference subtraction. Serial dilutions of protein from 100nM to approx. 5nM were analyzed. Following 3 min equilibration of the flow cells in running buffer, 90 µl protein sample was injected using the KINJECT command. The dissociation phase lasted 9 min and regeneration was performed with a 3-min pulse of 20 mM EDTA in HBS-P buffer. SPR data were fitted to a 1:1 Langmuir binding model using BIAevaluation 4.1 software (Biacore AB, Uppsala, Sweden). Results are given in Table 3.

Table 3 – Surface plasmon resonance analysis of the binding of FVIIa 407C and dimeric variants to immobilized sTF. Dissociation constants (K_D) were calculated from the measured association (k_{on}) and dissociation (k_{off}) rate constants.

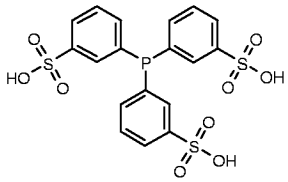
Protein	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D
FVIIa 407C	2.8×10^5	1.5×10^{-3}	5.5 nM
FVIIa 407C-PEG3.4k-FVIIa 407C	3.5×10^5	1.5×10^{-4}	0.4 nM
FVIIa 407C-PEG20k-FVIIa 407C	3.1×10^5	1.6×10^{-4}	0.5 nM

Reduction of FVIIa R396C-mixed disulfides using triphenylphosphine-3,3',3'' trisulfonic acid – Small-scale reduction of FVIIa R396C-mixed disulfides using triphenylphosphine-3,3',3'' trisulfonic acid (PPh_3S_3) was performed as follows: FVIIa R396C (4.4 µM) was treated with either 2.5 or 5.0 mM PPh_3S_3 in a total volume of 50 µl reaction buffer (50 mM HEPES, 100 mM NaCl, 10 mM $CaCl_2$, 0.05% Tween 20, pH 7.0) containing 50 mM *p*-aminobenzamidine. After 16.3 hours incubation at room temperature, reaction mixtures (30 µl) were desalted on Pro•Spin™ Spin columns (Princeton Separations, Adelphia, New Jersey) rehydrated in reaction buffer according to manufacturer's instructions to remove excess reductant. Subsequently, free thiols were alkylated with 0.2 mM PEG5k-maleimide for 10 min at room temperature. PEGylated and non-PEGylated FVIIa R396C were separated by reducing SDS-PAGE on a 4-12% Bis-Tris gel (Invitrogen Life Technologies, Carlsbad, CA) run at 200 V for 35 min in MES buffer according to manufacturer's recommendations. Gel staining with Simply Blue™ SafeStain (Invitrogen Life Technologies, Carlsbad, CA) was performed according to manufacturer's instructions. The gel is shown in Figure 10A.

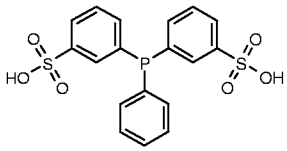
The amidolytic activity of FVIIa R396C before and after incubation with PPh_3S_3 was measured by 320-fold dilution of the reaction mixture into 200 µl (total volume) 50 mM HEPES, 100 mM NaCl, 5 mM $CaCl_2$, 1 mg/ml BSA, pH 7.4 buffer containing 50 mM sTF. After 15 min pre-incubation at room temperature, 1 mM chromogenic substrate S-2288 was added and the absorbance monitored continuously at 405 nm for 20 min in polystyrene microtiter plates (Nunc, Denmark) using a SpectraMax™ 340 microplate spectrophotometer equipped with SOFTmax PRO software (v2.2; Molecular Devices Corp., Sunnyvale, CA). Amidolytic activity was reported as the slope of the linear progress curves after blank subtraction. Results are shown in Figure 10B.

Selective Reduction of Exposed Disulfides in a Factor VII polypeptide – The commercially available triarylphosphine **1** (trisodium salt of triphenylphosphine-3,3',3''-trisulfonic acid from Aldrich) contains approximately 5% of the corresponding 3,3'-bis-sulfonic acid **2**. Thus, **1** was

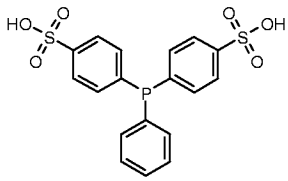
purified by standard reverse-phase HPLC, eluting with a gradient of acetonitrile in water in the presence of 0.1% trifluoroacetic acid.



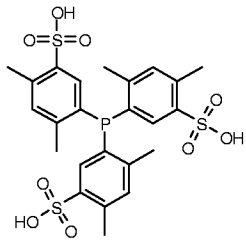
1



2



3



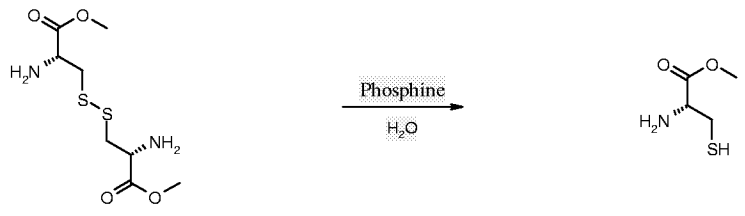
4

It has been shown that triphenylphosphine-3,3',3''-trisulfonic acid (2.5 mM) can be used in conjunction with the active site inhibitor 4-aminobenzamidine to reduce the exposed disulfide bond between glutathione and FVIIa R396C essentially without loss of amidolytic activity. Reductive cleavage of the mixed disulfide bond was demonstrated by subsequent modification of the liberated cysteine with PEG5k-maleimide.

Triarylphosphines **1**-**3** (10 mM) were individually incubated with rFVIIa for 1 h at room temperature. In the presence of **1**, rFVIIa retained most of its activity. In contrast, the 3,3'-bis-sulfonic acid **2** caused a rapid decrease in the enzyme's amidolytic activity, much like the analogous 4,4'-bis-sulfonic acid **3** (dipotassium salt from Aldrich), and was therefore not considered optimal for reduction of rFVIIa.

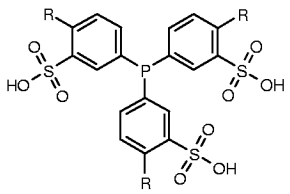
Furthermore, **1** was tested for its ability to reduce cystine dimethyl ester **5**. The reaction was conducted at room temperature at 15 mM concentration of **1** in water. The substrate was present at 5 mM concentration. It was demonstrated by LC-MS analyses that the disulfide bond in **5** was reduced under the given conditions.

Scheme 1



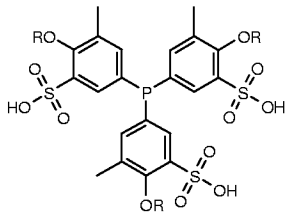
5 6

The more sterically hindered triarylphosphine **4** (trisodium salt from Strem) was found to be almost unreactive towards rfVIIa, suggesting the feasibility of developing a more selective reducing agent. Compounds **9**-**11** represent non-limiting examples of triarylphosphines which are expected to be selective disulfide reducing agents.



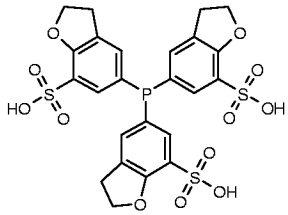
R = H, Me, Et, Pr, 2-Pr, Bu, MeO, EtO, PrO, 2-PrO, BuO.

9



R = Me, Et, Pr, 2-Pr, Bu.

10



11

Example 2

Materials – FVIII deficient plasma was obtained from George King Bio-Medical Inc. (Kansas, USA; Prod. no. 0800). Lyophilised human thrombocytes were purchased from Helena Biosciences (UK, Prod. no. 5371). Thrombocyte reconstitution buffer: Tris Buffered Saline was obtained from Helena Biosciences (UK; Prod. no. 5365). Innovin was obtained from Dade Behring (Liederbach, Germany; Prod. no. B4212-50). Thrombin specific substrate: Z-Gly-Gly-Arg-AMC HCl Fluorophor was obtained from Bachem (Weil am Rhein, Germany; Prod. no. I-1140). Expression and purification of recombinant FVIIa (rFVIIa) was performed as described previously (Thim et al., 1988; Persson and Nielsen, 1996). All other chemicals were of analytical grade or better.

Determination of rFVIIa-like activity of FVII-analogues using an Endogenous Thrombin Potential (ETP) bioassay - The endogenous thrombin potential (ETP) assay is based on a real time determination of thrombin generation in a selected plasma sample. The plasma sample contains lyophilized thrombocytes as a physiological lipid surface source for the assembly of the Xase and prothrombinase complexes in the coagulation cascade. The real time thrombin activity is determined by continuous detection of an appearing fluorescent product from a thrombin specific substrate (Hemker et al., 2000; Hemker & Béguin, 2000).

The concentration of FVIIa and PEGylated variants were determined by absorbance measurements at 280 nm. A value of 1.36 was taken as the absorbance of a 1 mg/ml protein solution (E0.1%).

One vial of lyophilized thrombocytes was dissolved in 0.73 ml reconstitution buffer (Tris Buffered Saline) and further diluted to 75.000 thrombocytes/µl in FVIII deficient plasma. Innovin, recombinant FVIIa (rFVIIa) as well as test samples were diluted in assay buffer (20 mM Hepes, 150 mM NaCl, pH 7.35, 1.5% bovine serum albumin (BSA)). Thrombin specific

substrate, Z-Gly-Gly-Arg-AMC, was dissolved in 60% dimethylsulfoxide (DMSO) and further diluted in calcium-containing assay buffer (100mM $CaCl_2$, 20 mM Hepes, 150 mM NaCl, pH 7.35). In a Costar microtiter plate (96 wells, Prod. No. 3631) 10 µl rFVIIa (final 0 – 10000 ng/ml), or test sample, or blank (assay buffer) (10 µl) were added to respective wells. Innovin (10 µl, final 0.12 pM), and thrombocyte containing plasma (80 µl, final 50.000 thrombocytes/µl) were added to respective wells of a Costar plate (96 wells, Prod. No. 3631). The plate was incubated for 10 min at 37ºC in Thermo Fluoroscan Ascent (Thermo Electron Corporation, Cambridge, UK). Substrate (20 µl, final concentrations: 0.4 mM Thrombin specific substrate, 0.5% DMSO and 16.7 mM $CaCl_2$) was immediately added and fluorescence was continuously recorded for 60 minutes (excitation 390 nm, emission 460 nm).

Concentration-response curves were generated for rFVIIa and FVII-analogues based on the signal (Area under the fluorescent unit-time curve for 60 min). The ETP activity of the analogue relative to rFVIIa was calculated from the curves and given in Table 4.

Table 4 - ETP activities of dimeric FVIIa 407C molecules relative to rFVIIa. The relative activities are given per FVIIa 407C protomer and, thus, the total activities of the dimeric molecules are twice the reported values.

Protein	**ETP relative activity**
FVIIa 407C-PEG3.4k-FVIIa 407C	620%
FVIIa 407C-PEG20k-FVIIa 407C	270%

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CLAIMS

1. Dimeric or multimeric FVIIa compound comprising at least two FVIIa polypeptides covalently connected such as to retain the intrinsic catalytic activity of the FVIIa polypeptides.

2. The compound according to claim 1, wherein said compound has an active site concentration which is at least 70%, preferably at least 80%, as measured by the assay disclosed herein.

3. The compound according to claim 1, wherein said compound has an increased biological activity as compared to the biological activity of the constituent FVIIa polypeptides.

4. The compound according to any one of the preceding claims, wherein two FVIIa polypeptides are covalently connected to form a dimeric FVIIa compound.

5. The compound according to any one of the preceding claims, wherein said at least two FVIIa polypeptides are covalently connected via engineered cysteines in said FVIIa polypeptides.

6. The compound according to any one of the preceding claims, wherein said FVIIa polypeptides covalently connected are identical FVIIa polypeptides.

7. The compound according to any one of the preceding claims, wherein at least two different FVIIa polypeptides are covalently connected.

8. The compound according to any one of the preceding claims, wherein said dimeric or multimeric FVIIa compound comprises a linker between the constituent FVIIa polypeptides.

9. The compound according to claim 8, wherein said linker has the structure that results from a reaction involving a bivalent cysteine reactive PEG.

10. The compound according to claim 8, wherein said linker has the structure that results from a reaction involving maleimide-PEG-maleimide.

11. The compound according to claim 10, wherein said maleimide-PEG-maleimide is selected from the group consisting of maleimide-PEG2kD-maleimide, maleimide-PEG3.4kD-maleimide, maleimide-PEG5kD-maleimide, maleimide-PEG10kD-maleimide, and maleimide-PEG20kD-maleimide.

12. The compound according to claim 8, wherein said linker comprises an amino acid sequence.

13. The compound according to any one of the preceding claims, wherein said at least two FVIIa polypeptides are covalently connected via free amines in said FVIIa polypeptides.

14. The compound according to any one of the preceding claims, wherein said at least two FVIIa polypeptides are covalently connected via natural or engineered glycans attached to said FVIIa polypeptides.

15. The compound according to any one of the preceding claims, wherein at least two FVIIa polypeptides are covalently connected via a chemical structure resulting from an enzyme catalyzed coupling reaction, e.g. by transglutaminase or sortase.

16. The compound according to any one of the preceding claims, wherein said FVIIa polypeptide is FVIIa 407C

17. The compound according to any one of the preceding claims, wherein said FVIIa polypeptide is a FVIIa 407C variant.

18. The compound according to any one of the preceding claims, wherein said FVIIa polypeptide is FVIIa P406C or a FVIIa P406C variant.

19. The compound according to any one of the preceding claims, wherein said FVIIa polypeptide is FVIIa R396C or a FVIIa R396C variant.

20. The compound according to any one of the preceding claims, wherein said FVIIa polypeptide is FVIIa Q250C or a FVIIa Q250C variant.

21. The compound according to any one of the preceding claims, wherein said FVIIa polypeptides are FVIIa variants having optimized GLA domains, e.g. combinations of Y4 insertion, P10Q, K32E, D33F, D33E, and A34E.

22. The compound according to any one of the preceding claims, wherein said FVIIa polypeptides are FVIIa variants having increased intrinsic proteolytic activity, comprising substitutions selected from the group consisting of M298Q, V158D/E296V/M298Q, V158D/E296V/M298Q/K337A, F374Y/L305V, F374Y/L305V/S314E/K337A, F374Y/L305V/S314E, F374Y/L305V/K337A, L305V/K337A, V158D/E296V/M298Q/L305V,

V158D/E296V/M298Q/L305V/K337A, V158T/M298Q, E296V/M298Q, V158D/E296V, V158D/M298Q and the 407C variants thereof.

23. The compound according to any one of claims 1-20, wherein said FVIIa variant comprises substitutions selected from the group consisting of L305V, L305V/M306D/D309S, L305I, L305T, F374P, V158T/M298Q, V158D/E296V/M298Q, K337A, M298Q, V158D/M298Q, L305V/K337A, V158D/E296V/M298Q/L305V, V158D/E296V/M298Q/K337A, V158D/E296V/M298Q/L305V/K337A, K157A, E296V, E296V/M298Q, V158D/E296V, V158D/M298K, S336G, L305V/K337A, L305V/V158D, L305V/E296V, L305V/M298Q, L305V/V158T, L305V/K337A/V158T, L305V/K337A/M298Q, L305V/K337A/E296V, L305V/K337A/V158D, L305V/V158D/M298Q, L305V/V158D/E296V, L305V/V158T/M298Q, L305V/V158T/E296V, L305V/E296V/M298Q, L305V/V158D/E296V/M298Q, L305V/V158T/E296V/M298Q, L305V/V158T/K337A/M298Q, L305V/V158T/E296V/K337A, L305V/V158D/K337A/M298Q, L305V/V158D/E296V/K337A, L305V/V158D/E296V/M298Q/K337A, L305V/V158T/E296V/M298Q/K337A, S314E/K316H, S314E/K316Q, S314E/L305V, S314E/K337A, S314E/V158D, S314E/E296V, S314E/M298Q, S314E/V158T, K316H/L305V, K316H/K337A, K316H/V158D, K316H/E296V, K316H/M298Q, K316H/V158T, K316Q/L305V, K316Q/K337A, K316Q/V158D, K316Q/E296V, K316Q/M298Q, K316Q/V158T, S314E/L305V/K337A, S314E/L305V/V158D, S314E/L305V/E296V, S314E/L305V/M298Q, S314E/L305V/V158T, S314E/L305V/K337A/V158T, S314E/L305V/K337A/M298Q, S314E/L305V/K337A/E296V, S314E/L305V/K337A/V158D, S314E/L305V/V158D/M298Q, S314E/L305V/V158D/E296V, S314E/L305V/V158T/M298Q, S314E/L305V/V158T/E296V, S314E/L305V/E296V/M298Q, S314E/L305V/V158D/E296V/M298Q, S314E/L305V/V158T/E296V/M298Q, S314E/L305V/V158T/K337A/M298Q, S314E/L305V/V158T/E296V/K337A, S314E/L305V/V158D/K337A/M298Q, S314E/L305V/V158D/E296V/K337A, S314E/L305V/V158D/E296V/M298Q/K337A, S314E/L305V/V158T/E296V/M298Q/K337A, K316H/L305V/K337A, K316H/L305V/V158D, K316H/L305V/E296V, K316H/L305V/M298Q, K316H/L305V/V158T, K316H/L305V/K337A/V158T, K316H/L305V/K337A/M298Q, K316H/L305V/K337A/E296V, K316H/L305V/K337A/V158D, K316H/L305V/V158D/M298Q, K316H/L305V/V158D/E296V, K316H/L305V/V158T/M298Q, K316H/L305V/V158T/E296V, K316H/L305V/E296V/M298Q, K316H/L305V/V158D/E296V/M298Q, K316H/L305V/V158T/E296V/M298Q/K337A, K316Q/L305V/K337A, K316Q/L305V/V158D, K316Q/L305V/E296V, K316Q/L305V/M298Q, K316Q/L305V/V158T, K316Q/L305V/K337A/V158T, K316Q/L305V/K337A/M298Q, K316Q/L305V/K337A/E296V, K316Q/L305V/K337A/V158D, K316Q/L305V/V158D/M298Q, K316Q/L305V/V158D/E296V, K316Q/L305V/V158T/M298Q, K316Q/L305V/V158T/E296V, K316Q/L305V/E296V/M298Q,

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24. The compound according to any one of the preceding claims, wherein said FVIIa polypeptides are PEGylated.

25. A compound which is a conjugate between human serum albumin or a variant thereof and a dimeric or multimeric FVIIa compound according to any one of the preceding claims.

26. A pharmaceutical composition comprising a compound according to any one of claims 1-25 and a pharmaceutically acceptable carrier or excipient.

27. Use of a compound according to any one of claims 1-25 for the manufacture of a medicament for the treatment of bleeding disorders or bleeding episodes in a subject or for the enhancement of the normal haemostatic system.

28. A method for preparation of a compound according to any one of claims 1-25, said method comprising the steps:
a) synthesis and purification of said FVIIa polypeptides;
b) synthesis of said compound by coupling said FVIIa polypeptides;
c) isolation of said dimeric or multimeric FVIIa compound.

29. A method for the preparation of a compound according to any one of claims 5, 9-11 and 15-25, said method comprising the steps:
a) synthesis and purification of said FVIIa polypeptides;
b) selective reduction of the cysteine residue on the FVIIa polypeptides which are to be linked;
c) synthesis of said dimeric or multimeric FVIIa compound by coupling said FVIIa polypeptides with reduced cysteine residues in the presence of an activated linker;
d) isolation of said dimeric or multimeric FVIIa compound.

30. The method according to claim 29, wherein step b) comprises selective reduction of the cysteine residue on the FVIIa polypeptide by reaction with a redox buffer or a triarylphosphine reducing agent.

Figure 1

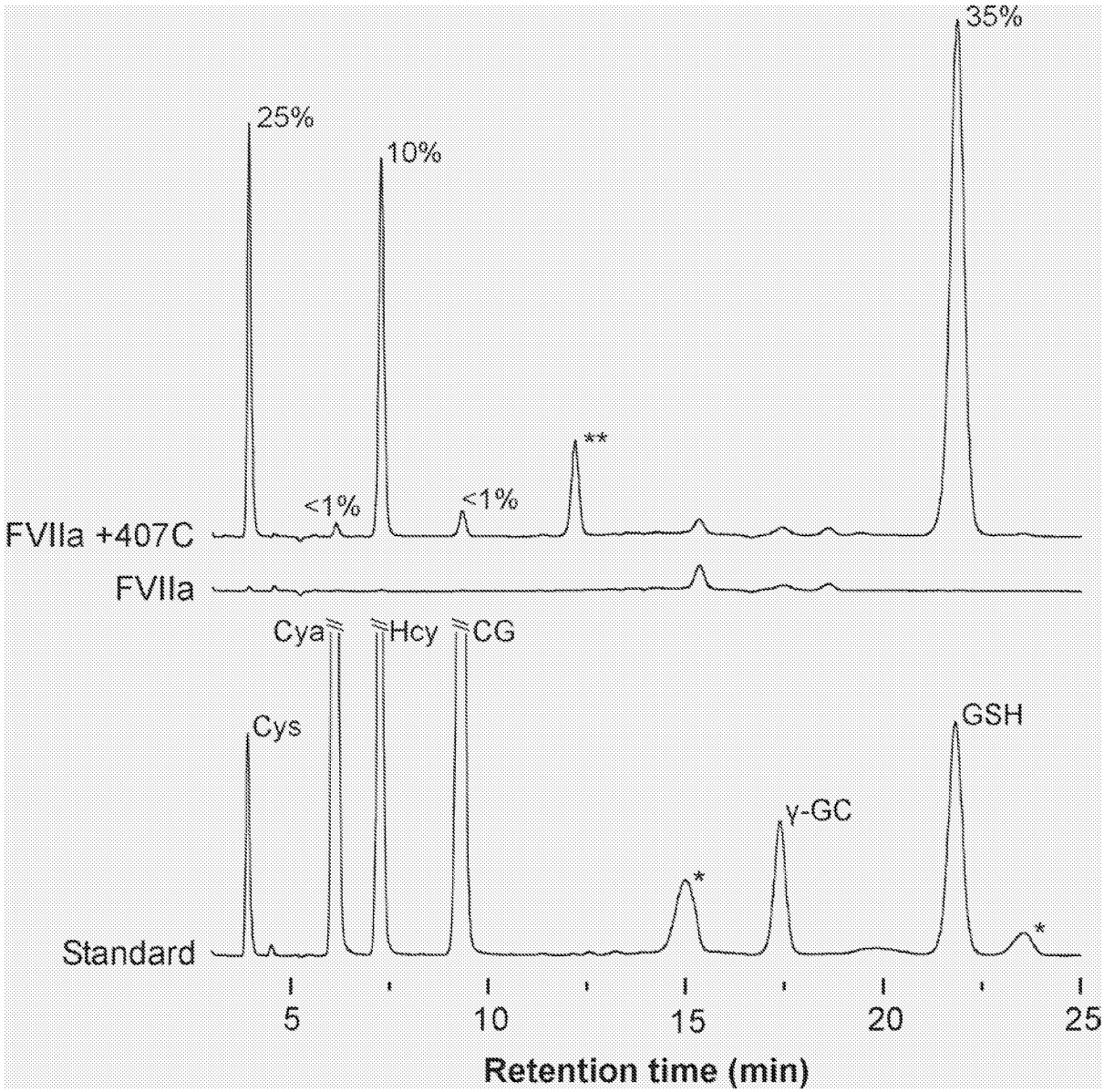

35%
25%
10%
**
<1%
<1%
FVIIa +407C
FVIIa
Cya
Hcy
CG
Cys
GSH
γ-GC
*
*
Standard
5
10
15
20
25
Retention time (min)

Figure 2

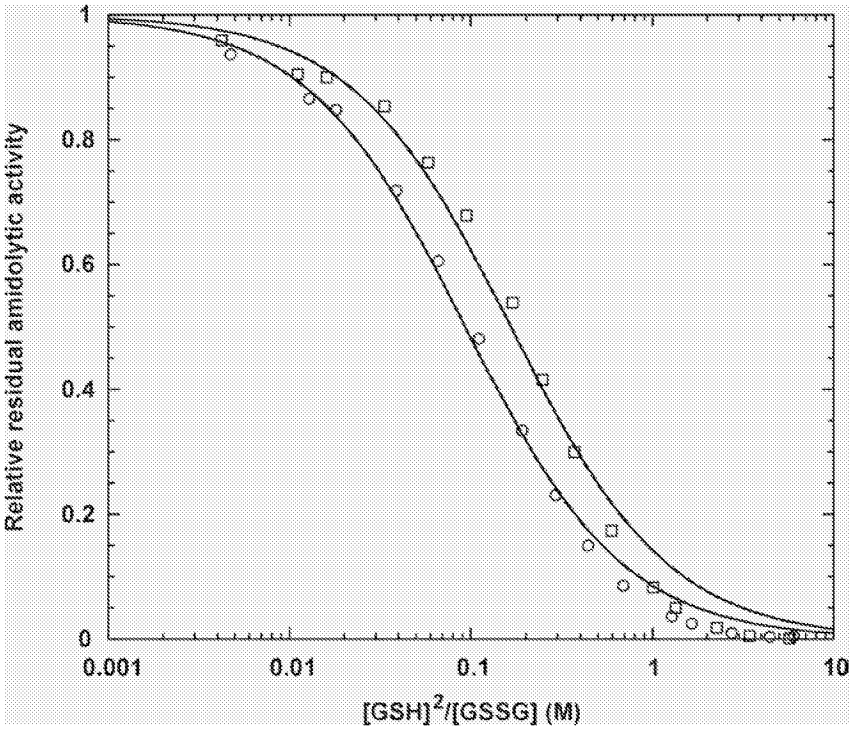

Relative residual amidolytic activity
1
0.8
0.6
0.4
0.2
0
0.001
0.01
0.1
1
10
[GSH]2/[GSSG] (M)

Figure 3

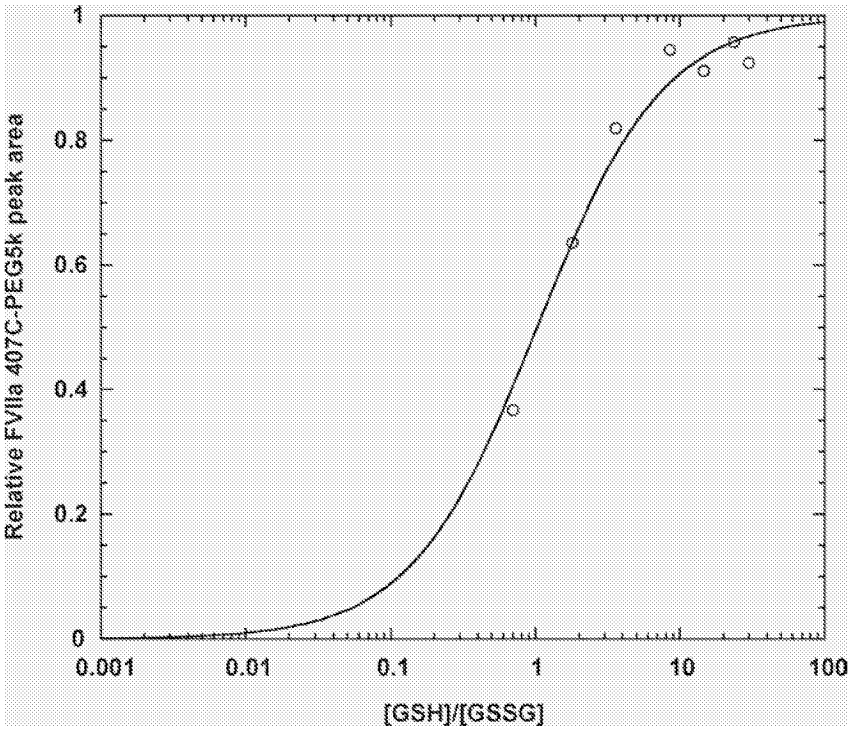

Relative FVIIa 407C-PEG5k peak area
1
0.8
0.6
0.4
0.2
0
0.001
0.01
0.1
1
10
100
[GSH]/[GSSG]

Figure 4

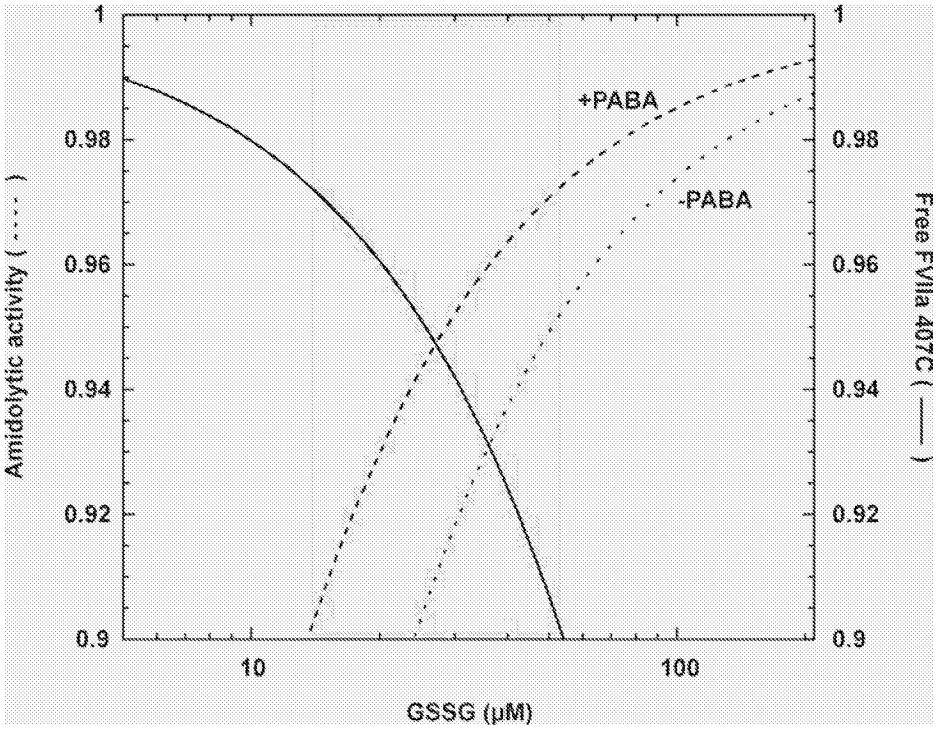

1
0.98
0.96
0.94
0.92
0.9
Amidolytic activity (- - - -)
+PABA
-PABA
10
100
GSSG (μM)
Free FVIIa 407C (——)

Figure 5

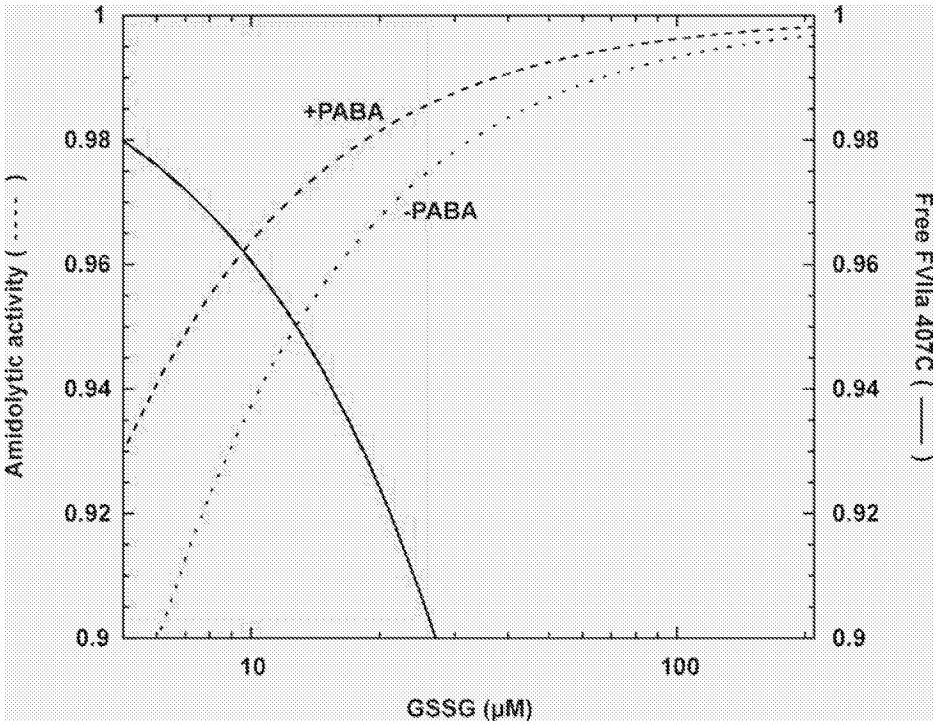

1
0.98
0.96
0.94
0.92
0.9
+PABA
-PABA
Amidolytic activity (- - - -)
Free FVIIa 407C (——)
10
100
GSSG (µM)

Figure 6

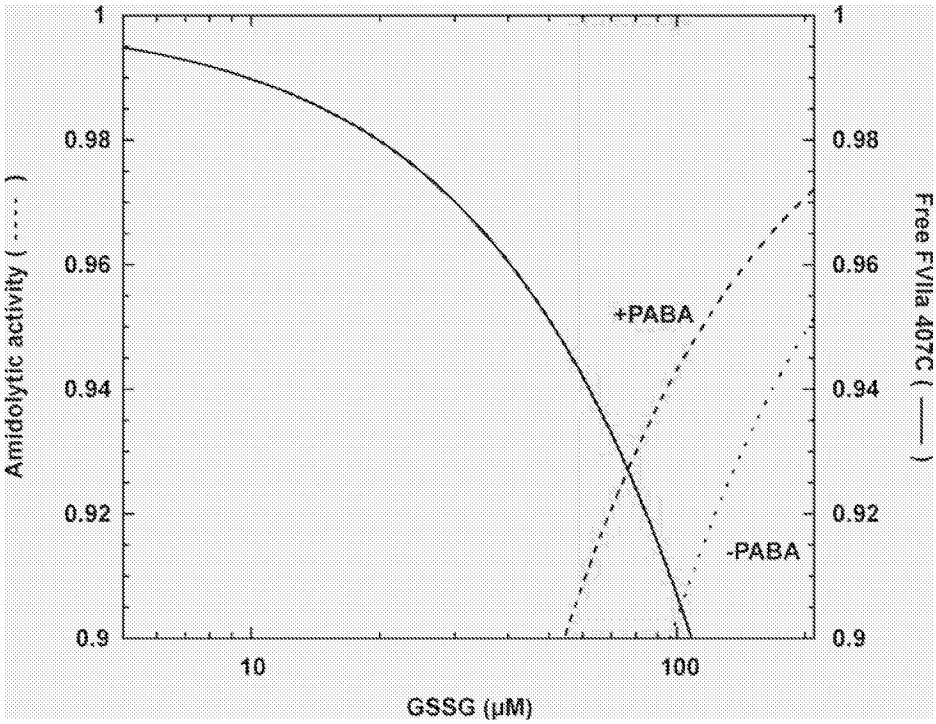

1
0.98
0.96
0.94
0.92
0.9
Amidolytic activity (- - - -)
Free FVIIa 407C (——)
+PABA
-PABA
10
100
GSSG (µM)

Figure 7

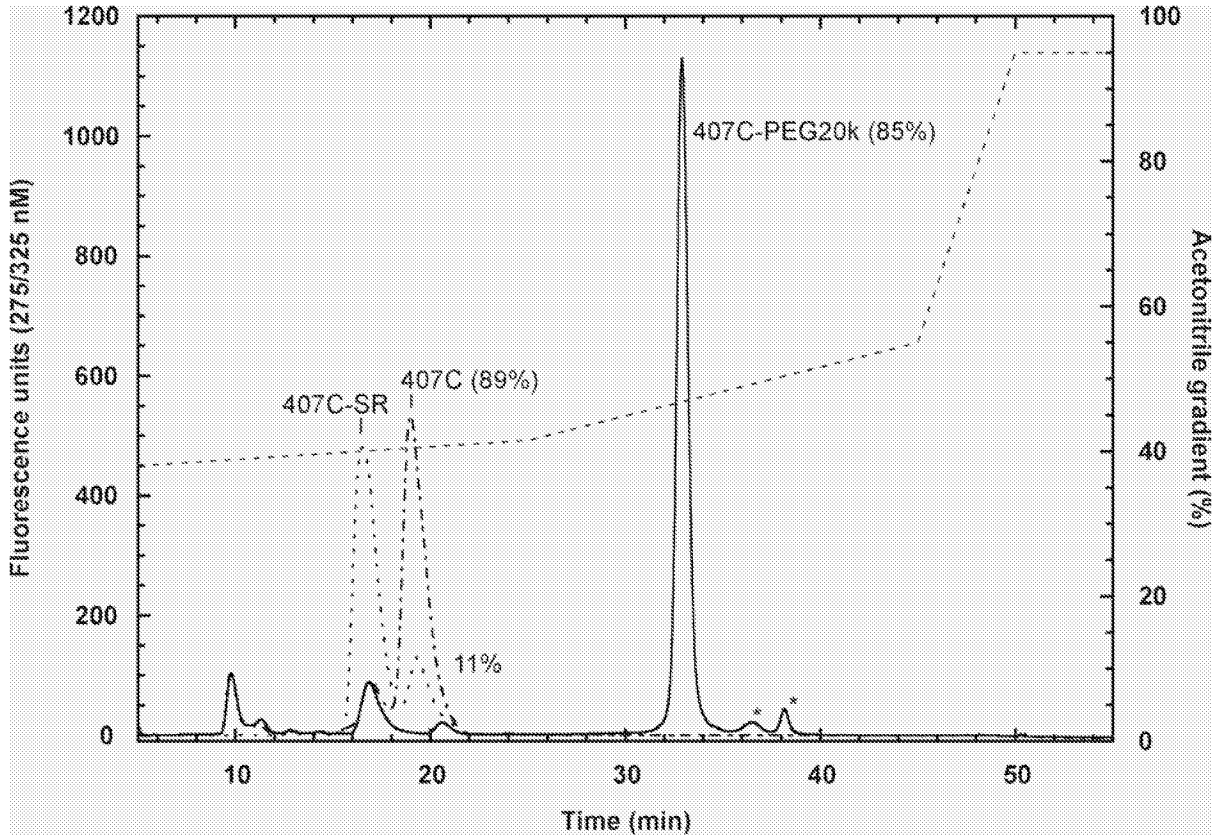

1200
1000
800
600
400
200
0
Fluorescence units (275/325 nM)
100
80
60
40
20
0
Acetonitrile gradient (%)
10
20
30
40
50
Time (min)
407C-PEG20k (85%)
407C (89%)
407C-SR
11%
*
*

Figure 8

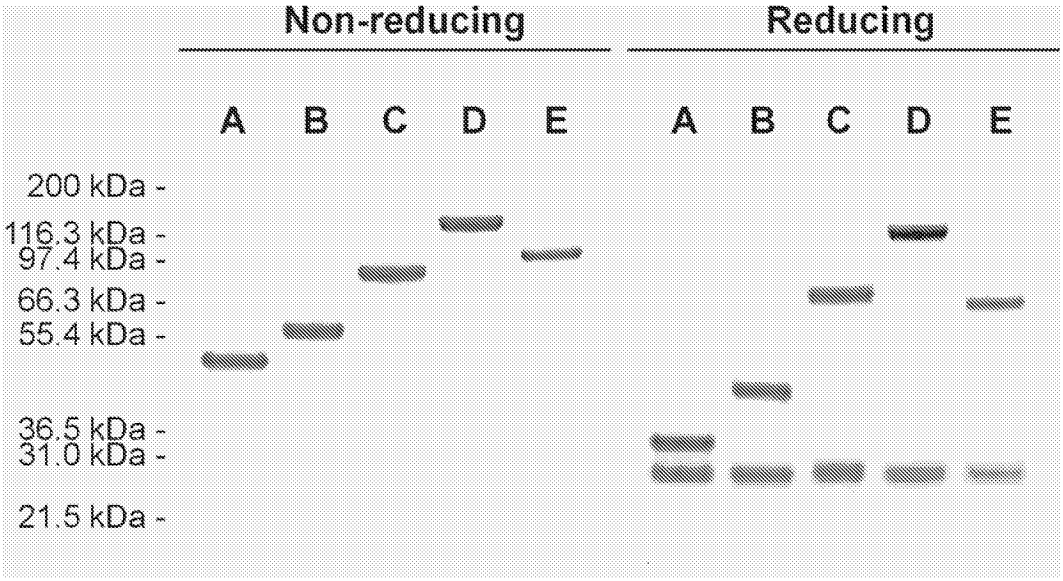

Non-reducing
Reducing
A
B
C
D
E
A
B
C
D
E
200 kDa -
116.3 kDa -
97.4 kDa -
66.3 kDa -
55.4 kDa -
36.5 kDa -
31.0 kDa -
21.5 kDa -

Figure 9

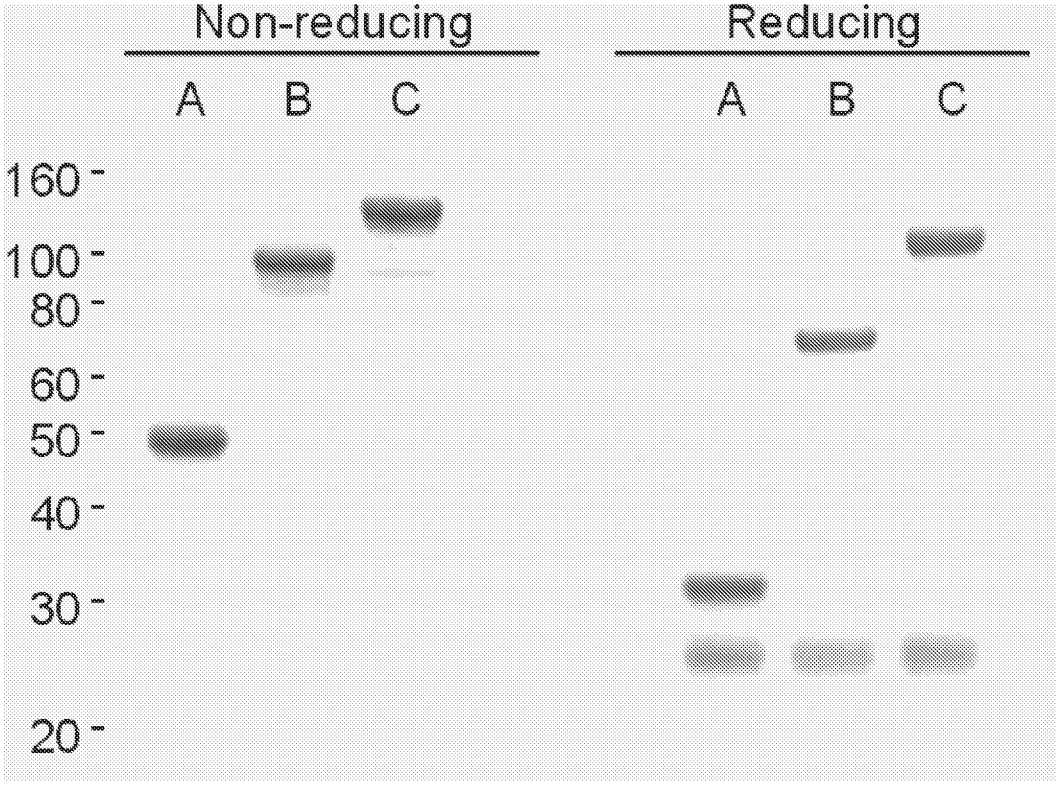
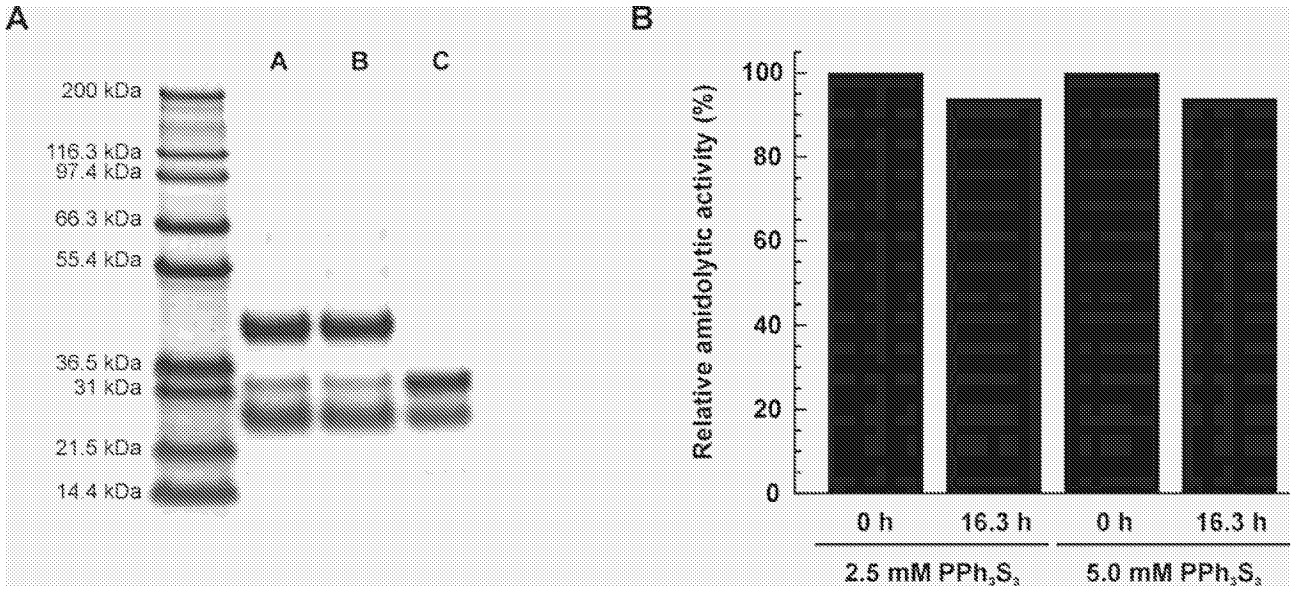


Figure 10


A
A
B
C
200 kDa
116.3 kDa
97.4 kDa
66.3 kDa
55.4 kDa
36.5 kDa
31 kDa
21.5 kDa
14.4 kDa
B
Relative amidolytic activity (%)
100
80
60
40
20
0
0 h
16.3 h
0 h
16.3 h
2.5 mM PPh3S3
5.0 mM PPh3S3

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WO 2006/134174 A3

(54) Title: DIMERIC AND MULTIMERIC FVIIA COMPOUND

(57) Abstract: The present invention relates to dimeric or multimeric FVIIa compounds comprising at least two FVIIa polypeptides covalently connected such as to retain the intrinsic catalytic activity of the FVIIa polypeptides.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2006/063311

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K1/113 C12N9/64

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 02/077218 A (NOVO NORDISK AS [DK]) 3 October 2002 (2002-10-03) cited in the application the whole document	
A	WO 03/076461 A2 (NOVO NORDISK AS [DK]) 18 September 2003 (2003-09-18) cited in the application page 22, last paragraph - page 23, paragraph 1 page 16, line 5 - line 12	

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Schmitz, Till

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2006/063311

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PERSSON E ET AL: "Augmented intrinsic activity of Factor VIIa by replacement of residues 305, 314, 337 and 374: evidence of two unique mutational mechanisms of activity enhancement" BIOCHEMICAL JOURNAL, THE BIOCHEMICAL SOCIETY, LONDON, GB, vol. 379, no. Pt 2, 15 April 2004 (2004-04-15), pages 497-503, XP002398830 ISSN: 0264-6021 cited in the application the whole document	
A	WO 01/21661 A (NOVO NORDISK AS [DK]; FRESKGAARD PER OLA [SE]; JAKOBSEN PALLE [DK]) 29 March 2001 (2001-03-29) example 6	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2006/063311

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 02077218 A	03-10-2002	BR 0208203 A	19-04-2005
		CA 2441580 A1	03-10-2002
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		EP 1485476 A2	15-12-2004
		JP 2005538936 T	22-12-2005
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WO 2007/022784 A2

(54) **Title:** LIQUID FACTOR VII COMPOSITION

(57) **Abstract:** Storage- stable aqueous pharmaceutical compositions comprising a Factor VII or Factor Vila polypeptide, a buffering agent, and zinc ions (Zn^{2+}) as a stabilizer.

LIQUID FACTOR VII COMPOSITION

FIELD OF THE INVENTION

The present invention relates to storage-stable aqueous compositions of Factor VII or Factor VIIa comprising zinc ions as a stabilizer.

BACKGROUND OF THE INVENTION

Factor VII (FVII), an important protein in the blood coagulation cascade, is a vitamin K-dependent plasma protein synthesized in the liver and secreted into the blood as a single-chain glycoprotein with a molecular weight of 53 kDa. The FVII zymogen is converted into an activated form (FVIIa) by proteolytic cleavage at a single site, R152-I153, resulting in two chains linked by a single disulfide bridge. Recombinant human FVIIa is commercially available from Novo Nordisk under the name NovoSeven® and is used for the treatment of bleeding episodes, e.g. in hemophilia or trauma. Recombinantly produced variants of human FVII or FVIIa have also been reported.

NovoSeven® is a freeze-dried FVIIa product which must be reconstituted before use. A vial (1.2 mg) of NovoSeven® contains 1.2 mg recombinant human FVIIa (rhFVIIa), 5.84 mg NaCl, 2.94 mg $CaCl_2,2H_2O$, 2.64 mg glycylglycine, 0.14 mg polysorbate 80 and 60.0 mg mannitol; it is reconstituted to pH 5.5 by 2.0 ml water for injection. When reconstituted, the protein is stable for use for 24 hours. No liquid ready-for-use or concentrated FVII products are currently commercially available.

A stable liquid preparation of FVIIa would provide the advantage of easier handling for patients, medical care personnel, etc. Further, in case of emergencies such as severe bleeding caused e.g. by acute trauma or surgery, a ready-to-use soluble formulation of FVIIa would be able to be administered quicker than a dry formulation that must first be reconstituted with water, which could potentially be life-saving and/or prevent complications caused by loss of blood.

Attempts to prevent proteolytic degradation by site-directed mutagenesis at major proteolytic sites are disclosed in WO 88/10295, and attempts to prepare stabilized liquid compositions of FVIIa are disclosed in WO 03/055511, WO 03/055512, WO 2004/082708, WO 2004/112828 and WO 2005/016365.

It is known that zinc ions can function as an *in vivo* modulator of FVIIa activity. Pedersen et al. (*Thromb. Haemost.* 65(5): 528-31, 1991) described how the amidolytic and proteolytic activity of FVIIa is inhibited by zinc ions. They found that the amidolytic activity

of FVIIa was inhibited by micromolar concentrations of zinc ions in the presence of physiological levels of calcium, with their results suggesting a weak competition between calcium and zinc for a putative zinc binding site on FVIIa. In contrast to the inhibitory effect observed on FVIIa, Pedersen et al. found that zinc ions did not inhibit other vitamin K-dependent proteases tested.

A later study by Petersen et al. (*Protein Science* 9: 859-866, 2000) similarly found that binding of zinc ions to FVIIa resulted in decreased amidolytic activity and a slightly reduced affinity for tissue factor. The authors suggested that zinc inhibits the activity of FVIIa by specific binding to the calcium binding loop (amino acid residues 210-220).

Bajaj et al. (*J. Biol. Chem.*, Vol. 281 (34):24873-24888, August 25, 2006; originally published online on June 5, 2006) obtained *p*-aminobenzamidine-VIIa/soluble tissue factor crystals that were used to determine the Ca^{2+}, Mg^{2+}, Na^{+} and Zn^{2+} sites of FVIIa. They describe that the protease domain has two Zn^{2+} sites unique to FVIIa.

WO 94/22905 discloses a method for the purification of Factor VII in which zinc ions are present in at least one chromatographic purification step in order to purify FVII in its single chain form.

WO 2005/002615 discloses liquid aqueous pharmaceutical compositions comprising a Factor VII polypeptide, a buffer, a non-ionic surfactant, and at least one metal-containing agent selected from the first transition series metals of oxidation state +II, except zinc. According to WO 2005/002615, zinc has properties that are different from the remaining metals of the first transition metal series and is therefore not considered useful in connection with the aqueous FVII compositions described therein.

In spite of the significant clinical advantages that would be provided by a stable liquid formulation of FVIIa, such formulations are not yet available.

BRIEF DISCLOSURE OF THE INVENTION

The object of the present invention is to provide a stabilized formulation of a Factor VII or VIIa polypeptide, preferably a liquid, aqueous formulation having a sufficient stability to allow storage in liquid form for a desired length of time, without the need for e.g. freezing or freeze-drying, while maintaining a sufficient level of protein activity. This would e.g. make it possible to rapidly administer the polypeptide to a patient without first having to reconstitute a freeze-dried powder in water, thus saving valuable time in acute clinical situations.

One aspect of the invention thus relates to a liquid, aqueous pharmaceutical composition comprising a Factor VII or Factor VIIa polypeptide, a buffering agent, and zinc (Zn^{2+}).

Another aspect of the invention relates to a method for producing a storage-stable aqueous composition comprising a Factor VII or Factor VIIa polypeptide, the method comprising mixing the Factor VII or Factor VIIa polypeptide with a buffering agent, zinc (Zn^{2+}), and water.

Further aspects of the invention relate to a method of treating or preventing a condition treatable by administration of Factor VIIa, comprising administering to a patient in need thereof a therapeutically effective amount of a composition of the invention, as well as use of a composition of the invention for the manufacture of a medicament for treating or preventing a condition treatable by administration of Factor VIIa.

DESCRIPTION

Definitions

In the description and claims below, the follow definitions apply:

The term "FVII" or "FVII polypeptide" refers to a FVII molecule provided in single chain form.

The term "FVIIa" or "FVIIa polypeptide" refers to a FVIIa molecule provided in its activated two-chain form, wherein the peptide bond between R152 and I153 of the single-chain form has been cleaved.

The terms "rFVII" and "rFVIIa" refer to FVII and FVIIa molecules produced by recombinant techniques, respectively. These may have the wild-type human sequence or may be variants of the human sequence.

The terms "hFVII" and "hFVIIa" refer to wild-type human FVII and FVIIa, respectively. The sequence of human FVII/FVIIa is well-known and is disclosed e.g. in US 4,784,950 and in Swiss-Prot under accession number P08709; it is also reproduced below as SEQ ID NO:1.

Unless it is indicated otherwise or apparent from the context, the terms "FVII", "FVII protein", "FVII polypeptide" and "Factor VII" as used herein are intended to include both the non-activated and activated forms of FVII, and to include the recombinant wild-type sequence of human FVII as well as variants thereof.

The term "parent" is intended to indicate the FVII or FVIIa molecule from which a FVII or FVIIa variant is derived by way of e.g. substitution, insertion or deletion. Although

the parent polypeptide may be any FVII or FVIIa polypeptide, and thus be derived from any origin, e.g. a non-human mammalian origin, the parent polypeptide is normally hFVII or hFVIIa.

A "variant" is a polypeptide which differs in one or more amino acid residues from its parent polypeptide, normally in 1-15 amino acid residues (e.g. in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acid residues), such as in 1-10 amino acid residues, e.g. in 1-8, 1-6, 1-5 or 1-3 amino acid residues, where the difference between the parent and the variant is e.g. a substitution, insertion and/or deletion.

The terminology used for identifying amino acid positions is illustrated as follows: G124 indicates that position 124 is occupied by a glycine residue in the amino acid sequence of human FVII. G124R indicates that the glycine residue of position 124 has been substituted with an arginine residue. Alternative substitutions are indicated with a "/". Multiple substitutions are indicated with a "+", e.g. K143N+N145S/T means an amino acid sequence which comprises a substitution of the lysine residue in position 143 with an asparagine residue and a substitution of the asparagine residue in position 145 with a serine or a threonine residue. Insertion of an additional amino acid residue, e.g. insertion of an alanine residue after G124, is indicated by G124GA.

Detailed description

According to the present invention, a Factor VII or VIIa polypeptide is formulated in a liquid, aqueous composition containing at least one buffering agent and zinc ions. Preferably, the composition contains the polypeptide in its activated form, i.e. recombinant human Factor VIIa or a variant thereof.

Zinc

The zinc used in compositions of the invention will normally be in the form of a zinc salt, in particular selected form the group consisting of zinc acetate, zinc bromide, zinc chloride, zinc iodide, zinc fluoride and zinc sulfate. Examples of preferred zinc salts are zinc chloride and zinc acetate.

The zinc salt will generally be present in the compositions in a concentration (calculated as the concentration of the Zn^{2+} ion) of at least about 1 μM, typically at least about 2 μM, such as at least about 5 μM, at least about 10 μM, at least about 20 μM or at least about 50 μM, and up to a maximum of about 100 mM, typically a maximum of about 50 mM, such as a maximum of about 20 mM, a maximum of about 10 mM, a maximum of

about 5 mM, a maximum of about 1 mM, a maximum of about 500 μM or a maximum of about 200 μM. The zinc concentration will thus be within the range of 1 μM – 100 mM, typically in the range of 1 μM – 50 mM, such as 10 μM – 10 mM. In one embodiment, the zinc concentration is in the range of 1 μM – 1 mM, such as 10-500 μM, e.g. 20-200 μM, for example about 25 μM, about 50 μM, about 100 μM or about 150 μM.

Alternatively, the amount of zinc may be expressed as a molar ratio between zinc (Zn^{2+}) and the FVII or FVIIa polypeptide. In this case, the molar ratio will between zinc and the polypeptide will be at least about 0.1, typically at least about 0.2 or at least about 0.5, such as at least about 1, at least about 2 or at least about 5, and up to a maximum of about 1000, typically a maximum of about 500, a maximum of about 200, a maximum of about 150 or a maximum of about 100, such as a maximum of about 50, a maximum of about 25 or a maximum of about 10. Expressed in this manner, the molar ratio between zinc and polypeptide may e.g. be in the range of 0.1-1000, such as 0.2-500, 0.5-200 or 1-100, for example in the range of 1-50, e.g. in the range of 2-25.

pH

The pH of the compositions of the invention is preferably selected so as to minimize pain or discomfort upon administration to patient, and taking into consideration the influence of pH on e.g. stability of the polypeptide and solubility of the zinc salt. However, since FVIIa may be used to treat acute and potentially life-threatening bleeding episodes, e.g. in the case of trauma or intracerebral hemorrhage (ICH), it may in some cases be desirable to formulate the polypeptide using a relatively acidic pH, e.g. down to about 3.0 or perhaps even lower, such as down to about 2.5. The pH will thus generally be in the range of from about 2.5 to about 9.0. Suitable pH ranges may, for example, be from about 2.5 to about 4.0, e.g. about 3.0 or 3.5; from about 4.0 to about 8.0, e.g. from about 5.0 to about 7.0, such as about 5.5, 6.0 or 6.5; or from about 5.0 to about 9.0, e.g. from about 6.0 to about 8.0, such as about 6.5, 7.0 or 7.5.

Buffering agents

Suitable buffering agents for use with the present invention include both organic and inorganic acids and salts thereof. Examples of suitable buffering agents include citrate buffers (e.g., monosodium citrate-disodium citrate mixture, citric acid-trisodium citrate mixture, citric acid-monosodium citrate mixture, etc.), succinate buffers (e.g., succinic acid-monosodium succinate mixture, succinic acid-sodium hydroxide mixture, succinic acid-

disodium succinate mixture, etc.), tartrate buffers (e.g., tartaric acid-sodium tartrate mixture, tartaric acid-potassium tartrate mixture, tartaric acid-sodium hydroxide mixture, etc.), fumarate buffers (e.g., fumaric acid-monosodium fumarate mixture, fumaric acid-disodium fumarate mixture, monosodium fumarate-disodium fumarate mixture, etc.), gluconate buffers (e.g., gluconic acid-sodium glyconate mixture, gluconic acid-sodium hydroxide mixture, gluconic acid-potassium glyuconate mixture, etc.), oxalate buffers (e.g., oxalic acid-sodium oxalate mixture, oxalic acid-sodium hydroxide mixture, oxalic acid-potassium oxalate mixture, etc.), lactate buffers (e.g., lactic acid-sodium lactate mixture, lactic acid-sodium hydroxide mixture, lactic acid-potassium lactate mixture, etc.) and acetate buffers (e.g., acetic acid-sodium acetate mixture, acetic acid-sodium hydroxide mixture, etc.). Additional possibilities are phosphate or maleate buffers; amino acid buffers such as histidine, glycine, glycylglycine, lysine or arginine; trimethylamine salts such as TRIS, as well as MES, PIPES, ACES, BES, TES and HEPES; or imidazole. The buffering agent will generally be present in a concentration of 1-100 mM, such as about 1-50 mM, typically about 1-25 mM, e.g. about 2-20 mM.

Additional ingredients

In addition to the polypeptide, the buffering agent and zinc, the compositions of the invention may include one or more additional pharmaceutically acceptable excipients such as a non-ionic surfactant, a tonicity modifying agent, an antioxidant or a preservative.

Non-ionic surfactants or detergents (also known as "wetting agents") may be present to help solubilize the polypeptide as well as to protect the polypeptide against agitation-induced aggregation, which also reduces the risk of denaturation of the polypeptide upon exposure to shear surface stress. Suitable non-ionic surfactants include, for example, polysorbates (polyoxyethylene sorbitol esters, e.g. polysorbate 20, polysorbate 80, etc.), polyoxamers (polyoxypropylene-polyoxyethylene block copolymers, e.g. poloxamer 184, poloxamer 188, etc.), Pluronic□ polyols, and polyoxyethylene sorbitan monoethers (Tween□ -20, Tween□ -80, etc.).

Isotonicifiers or tonicity modifying agents are added to ensure isotonicity or to otherwise adjust the tonicity of liquid compositions to a desired level and include polyhydric sugar alcohols, preferably trihydric or higher sugar alcohols, such as glycerin, erythritol, arabitol, xylitol, sorbitol and mannitol, or sugars such as sucrose or trehalose. Also suitable for use as a tonicity modifying agent are neutral salts such as sodium salts, potassium salts, calcium salts or magnesium salts, e.g. with chloride as the counterion. In some cases, it may

be advantageous to have a high ionic strength in the composition, e.g. at least 50 mM, at least 100 mM or at least 200 mM, e.g. up to about 500 mM or up to about 1000 mM, for example provided by use of a neutral salt. Such high ionic strength compositions of FVII are described in WO 2004/112828 .

Preservatives may be added to retard microbial growth, and are typically added in amounts of e.g. about 0.1%-2% (w/v). Suitable preservatives for use with the present invention include phenol, benzyl alcohol, meta-cresol, ortho-cresol, para-cresol, methyl paraben, propyl paraben, octadecyldimethylbenzyl ammonium chloride, benzalkonium halides (e.g. benzalkonium chloride, bromide or iodide), hexamethonium chloride, alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol and 3-pentanol.

In addition, the compositions may comprise miscellaneous excipients such as an antioxidant, for example ascorbic acid, methionine or vitamin E; a bulking agent or filler, e.g. starch; or a chelating agent, e.g. EDTA. The composition may further comprise calcium or magnesium ions, e.g. in the form of calcium chloride or magnesium chloride, for example in a concentration of 1-20 mM, such as about 2-15 mM.

Stability

The aqueous compositions of the invention are contemplated to be suitable for long-term storage, e.g. in single-use vials that can be stored at a temperature of about 2-8°C. The terms "long-term storage" and "storage-stable" refer to the fact that the aqueous compositions are able to be stored at 2-8°C, such as about 5°C, for an extended period of time, typically a period of at least about 6 months, preferably at least about 1 year, such as up to about 2 years, without suffering any substantial damage to the polypeptide (e.g. proteolysis or other degradation, or aggregation or denaturation, where the degradation, aggregation or denaturation results in a composition that is unsuitable for therapeutic use due to e.g. reduced efficacy or risk of immunogenicity). The storage stability of a polypeptide may e.g. be measured in terms of activity as compared to a reference composition subjected to the same storage conditions, or compared to the same composition that has not been subject to storage or has been subjected to low-temperature storage at e.g. about -80°C. The activity measured may, for example, be amidolytic activity or clotting activity. Suitable assays for determining the amidolytic or clotting activity of FVIIa polypeptides as well as other FVIIa assays are known in the art and are described e.g. in WO 01/58935 and WO 03/093465.

The storage stability may alternatively or additionally be measured in terms of the level of aggregate formation by methods known in the art, for example using size-exclusion chromatography. The improved storage stability of the compositions of the invention is thus intended to comprise physical stability, e.g. reduced aggregate formation, and/or chemical stability, e.g. reduced degradation.

Freeze-dried compositions

Although the compositions of the invention are generally designed for long-term storage in liquid form, it may in some cases be desirable to have a zinc-containing composition according to the invention in lyophilized form, typically in the form of a powder. This could be advantageous e.g. for use under circumstances where maintaining a liquid FVIIa composition at a reduced temperature of about 2-8°C is difficult, for example for military use where refrigerated storage of a liquid composition may not be possible. The lyophilized composition may e.g. be packaged in single-use vials for reconstitution with water shortly before use. For ease of administration, a freeze-dried zinc-containing FVIIa composition according to the invention may alternatively be packaged in a pre-filled, two-compartment syringe wherein one of the compartments contains the freeze-dried FVIIa composition and the other compartment contains sterile water, thus allowing the dry FVIIa composition to be quickly and easily mixed with water in an appropriate amount immediately prior to administration.

FVII/FVIIa polypeptides

The FVII or FVIIa polypeptides that may be formulated according to the present invention include in particular human recombinant FVII or FVIIa as well as variants thereof, and preferably in the activated form. Factor VII or VIIa variants of interest include, for example, those described in WO 01/58935, WO 03/093465, WO 2004/029091, WO 2004/111242, WO 99/20767, WO 00/66753, WO 88/10295, WO 92/15686, WO 02/29025, WO 01/70763, WO 01/83725, WO 02/02764, WO 02/22776, WO 02/38162, WO 02/077218, WO 03/027147, WO 03/037932, WO 2004/000366, WO 2004/029090, and WO 2004/108763.

The FVII or FVIIa variants may include one or more substitutions, insertions or deletions compared to wild-type human FVII, for example resulting in a variant that differs in 1-15 amino acid residues from the amino acid sequence of wild-type human FVII, typically in 1-10 amino acid residues, e.g. in 1-8 or 1-6 amino acid residues, where the differences in

amino acid sequence from the wild-type are typically substitutions. Such substitutions may be performed e.g. with the aim of introducing one or more *in vivo* glycosylation sites, typically N-glycosylation sites, or introducing one or more PEGylation sites into the protein, and/or for improving or otherwise modifying the clotting activity of the wild-type protein, for example by way of one or more amino acid substitutions in the Gla domain (amino acid residues 1-45 of hFVIIa) to improve clotting activity. Such variants are discussed below and are described in more detail in WO 01/58935, WO 03/093465, WO 2004/029091 and WO 2004/111242. Alternatively, where a FVII or FVIIa variant is designed to function as an anti-coagulant, the composition of the invention may comprise a FVII or FVIIa variant having reduced clotting activity.

Preferred FVII or FVIIa variants suitable for use in the compositions of the invention include at least one modification in the Gla domain (residues 1-45 of human Factor VII) and/or at least one amino acid modification that introduces an attachment site for a non-polypeptide moiety. Non-limiting examples of such modifications are provided in the following.

Modification of the Gla domain: In one embodiment, the FVII or FVIIa variant includes at least one modification in the Gla domain, in particular at least one modification that results in increased phospholipid membrane binding affinity compared to a similar polypeptide without said modification in the Gla domain. Such modifications in the Gla domain are disclosed e.g. in WO 99/20767, WO 00/66753 and WO 03/093465, and include modifications in one or more of positions 10, 11, 28, 32, 33 and 34 relative to SEQ ID NO:1. Preferably, the variant includes modifications in at least position 10 or 32, preferably both. The variant may thus include substitution of a glutamine, a glutamic acid, an aspartic acid or an asparagine residue in position 10, preferably a glutamine residue; and/or substitution of a glutamic acid or an aspartic acid residue in position 32, preferably a glutamic acid. Preferably, the variant includes substitutions at both of positions 10 and 32, more preferably the substitutions P10Q+K32E.

In other embodiments the variant may include substitution of a glutamic acid or a phenylalanine residue at position 28; or substitution of a hydrophobic amino acid residue in position 33, the substitution being selected from the group consisting of D33I, D33L, D33M, D33V, D33F, D33Y and D33W, in particular D33F.

In another embodiment the variant includes substitution of a negatively charged residue in position 34, i.e. A34E or A34D, preferably A34E. Alternatively, the variant may include a hydrophobic amino acid residue introduced by substitution in position 34. In this

case, the hydrophobic amino acid residue to be introduced in position 34 may be selected from the group consisting of I, L, M, V, F, Y and W. When position 34 is modified, the substitution A34E will generally be preferred.

In another embodiment the variant includes an amino acid substitution in position 36. Preferably, the amino acid residue to be introduced by substitution in position 36 is a negatively charged amino acid residue, i.e. R36E or R36D, in particular R36E.

In another embodiment the variant includes an amino acid substitution in position 38, in particular a negatively charged amino acid residue introduced by substitution in position 38, i.e. K38E or K38D, in particular K38E.

In another embodiment the variant includes an insertion of at least one (typically one) amino acid residue between position 3 and 4. The inserted amino acid residue is preferably a hydrophobic amino acid residue. Most preferably the insertion is A3AY.

Specific examples of variants having multiple substitutions in the Gla domain include: P10Q+K32E; P10Q+K32E+A34E; P10Q+K32E+R36E; P10Q+K32E+K38E; P10Q+K32E+A34E+R36E; P10Q+K32E+R36E+K38E; P10Q+K32E+A34E+K38E; P10Q+K32E+A34E+R36E+K38E; P10Q+K32E+A34L; P10Q+K32E+A34L+R36E; P10Q+K32E+A34L+K38E; and P10Q+K32E+A34L+R36E+K38E.

Introduction of in vivo glycosylation sites: In another embodiment, the variants used in the compositions of the invention comprise one or more modifications, typically substitutions, that introduce an *in vivo* N-glycosylation site compared to hFVII with the wild-type sequence. An N-glycosylation site has the sequence N-X-S/T/C, wherein X is any amino acid residue except proline, N is asparagine and S/T/C is either serine, threonine or cysteine, preferably serine or threonine, and most preferably threonine. Attachment sites for *in vivo* N-glycosylation can therefore be introduced by modification, typically substitution, of one or two amino acid residues in order to obtain the necessary N-X-S/T/C triplet.

Variants having introduced N-glycosylation sites relative to hFVIIa will typically comprise 1-5 additional *in vivo* N-glycosylation sites, such as 1-4 or 1-3 additional *in vivo* N-glycosylation sites, e.g. 1, 2 or 3 additional *in vivo* N-glycosylation sites relative to the native sequence. Human FVII has four naturally occurring glycosylation sites at positions N145, N322, S52 and S60, where S52 and S60 are O-glycosylation sites and N145 and N322 are N-glycosylation sites. Attachment of sugar moieties to one or more *in vivo* N-glycosylation sites is preferably performed by expression of the polypeptide variant in a host cell capable of *in vivo* glycosylation.

Specific examples of substitutions creating an *in vivo* N-glycosylation site include substitutions selected from the group consisting of A51N, G58N, T106N, K109N, G124N, K143N+N145T, A175T, I205S, I205T, V253N, T267N, T267N+S269T, S314N+K316S, S314N+K316T, R315N+V317S, R315N+V317T, K316N+G318S, K316N+G318T, G318N and D334N. Preferred substitutions include one or more of T106N, I205T and V253N.

In one embodiment, only one *in vivo* N-glycosylation site has been introduced by substitution. In another embodiment, two or more *in vivo* N-glycosylation sites have been introduced by substitution. Preferred substitutions creating two *in vivo* N-glycosylation sites include substitutions are selected from the group consisting of T106N+A175T, T106N+I205T, T106N+V253N, T106N+T267N+S269T, A175T+I205T, A175T+V253N, A175T+T267N+S269T, I205T+V253N, I205T+T267N+S269T and V253N+T267N+S269T, more preferably from the group consisting of T106N+I205T, T106N+V253N and I205T+V253N.

In a further embodiment, three or more *in vivo* N-glycosylation sites have been introduced by substitution. Examples of preferred substitutions creating three *in vivo* N-glycosylation sites include substitutions selected from the group consisting of I205T+ V253N+T267N+S269T and T106N+I205T+V253N.

Variants having a modification in the Gla domain and an introduced N-glycosylation site: In a further embodiment, the compositions of the invention may comprise a FVII or FVIIa variant having at least one modification in the Gla domain and at least one introduced *in vivo* N-glycosyation site as described in the respective sections above.

Specific examples of "combined" variants having multiple substitutions in the Gla domain and at least one introduced N-glycosylation site include: P10Q+K32E+T106N; P10Q+K32E+A34E+T106N; P10Q+K32E+R36E+T106N; P10Q+K32E+A34E+R36E+T106N; P10Q+K32E+I205T; P10Q+K32E+A34E+I205T; P10Q+K32E+R36E+I205T; P10Q+K32E+A34E+R36E+I205T; P10Q+K32E+V253N; P10Q+K32E+A34E+V253N; P10Q+K32E+R36E+V253N; P10Q+K32E+A34E+R36E+V253N; P10Q+K32E+ T106N+I205T; P10Q+K32E+A34E+T106N+I205T; P10Q+K32E+R36E+T106N+I205T; P10Q+K32E+A34E+R36E+T106N+I205T; P10Q+K32E +T106N+V253N; P10Q+K32E+A34E+T106N+V253N; P10Q+K32E+R36E+T106N+V253N; P10Q+K32E+A34E+R36E+T106N+V253N; P10Q+K32E+I205T+V253N; P10Q+K32E+A34E+I205T+V253N; P10Q+K32E+R36E+I205T+V253N; P10Q+K32E+A34E+R36E+I205T+V253N; P10Q+K32E+T106N+I205T+V253N;

P10Q+K32E+A34E+T106N+I205T+V253N; P10Q+K32E+R36E+T106N+I205T+V253N; P10Q+K32E+A34E+R36E+T106N+I205T+V253N; P10Q+K32E+A34L+T106N; P10Q+K32E+A34L+I205T; P10Q+K32E+A34L+V253N; P10Q+K32E+A34L+T106N+I205T; P10Q+K32E+A34L+T106N+V253N; P10Q+K32E+A34L+I205T+V253N; P10Q+K32E+A34L+T106N+I205T+V253N; P10Q+K32E+A34L+R36E+T106N; P10Q+K32E+A34L+R36E I205T; P10Q+K32E+A34L+R36E+V253N; P10Q+K32E+A34L+R36E+T106N+I205T; P10Q+K32E+A34L+R36E+T106N+V253N; P10Q+K32E+A34L+R36E+I205T+V253N; and P10Q+K32E+A34L+R36E+T106N+I205T+V253N.

Variants with modifications in the tissue factor binding site: In another embodiment, the compositions of the invention may comprise a FVII or FVIIa variant comprising a substitution in at least one position selected from the group consisting of L39, I42, S43, K62, L65, F71, E82 and F275. These amino acid substitutions in the tissue factor (TF) binding site of the FVII molecule result in an improved clotting activity. Preferred substitutions in these positions in the TF binding site include the following: L39E, L39Q or L39H; I42R; S43Q; K62E or K62R; L65Q or L65S; F71D, F71E, F71N, F71Q or F71Y; E82Q or E82N; F275H. The variant of this embodiment may e.g. comprise one, two or three of these substitutions. Preferred substitutions include one or more of S43Q, K62E, L65Q and F71Y, in particular one or more of S43Q, K62E and L65Q. Further information about variants of this type having modifications in the TF binding site may be found in WO 2004/029091. It will be understood that these substitutions in the TF binding site may if desired be combined with one or more of the other types of modifications described elsewhere herein, e.g. the modifications in the Gla domain as described above, introduction of at least one *in vivo* N-glycosylation site, and/or conjugation with a PEG polymer as described below.

Variants having an introduced PEGylation site: In a further embodiment, the compositions of the invention may comprise a FVII or FVIIa variant having at least one polymer molecule, in particular a polyethylene glycol (PEG) or other polyalkylene oxide, conjugated to an attachment group selected from the group consisting of a lysine residue, a cysteine residue, an aspartic acid residue, a glutamic acid residue, a histidine residue, and a tyrosine residue, preferably a cysteine or a lysine residue.

Methods for conjugating various polypeptides with a polyethylene glycol moiety ("PEGylation") are known in the art. For example, WO 01/58935 describes methods by which PEG moieties may be attached to a FVII or FVIIa variant which has been modified

relative to hFVII so as to have at least one introduced and/or removed attachment site for PEGylation, for example one or more introduced lysine residues, optionally in combination with removal of one or more lysine residues in positions where PEGylation is not desired, or one or more introduced cysteine residues, in this case optionally in combination with removal of one or more cysteine residues. Additional information regarding PEGylation may be found, e.g., in WO 02/02764, which discloses vitamin K-dependent polypeptides such as FVIIa linked to a PEG polymer, for example wild-type human FVIIa and a variant of FVIIa having the substitutions P10Q and K32E, in WO 96/11953, which describes methods for preparing N-terminally PEGylated proteins, and in the Nektar Advanced PEGylation Catalog 2004, "Polyethylene Glycol and Derivatives for Advanced PEGylation" (Nektar Therapeutics). It will be understood that the FVII or FVIIa variants for use in the compositions of the invention may, in addition to the attachment of one or more PEG polymers, also include one or more of the amino acid modifications otherwise described herein to provide e.g. an increased phospholipid membrane binding affinity and/or an increased tissue factor independent activity, and/or to provide one or more introduced *in vivo* N-glycosylation sites.

Other modifications: In a further embodiment, variants for use in the compositions of the invention may comprise, in addition to one or more of the modifications described above, at least one further amino acid substitution in a position selected from the group consisting of position 74, 77 and 116, in particular P74S, E77A and/or E116D. In a still further embodiment, the FVII or FVIIa variant may contain mutations known to increase the intrinsic activity of the polypeptide, for example those described in WO 02/22776. For example, the variant may comprise at least one modification in a position selected from the group consisting of 157, 158, 296, 298, 305, 334, 336, 337 and 374. Examples of such substitutions include one or more of V158D, E296D, M298Q, L305V and K337A.

The FVII/FVIIa protein or variant thereof may be produced by any suitable organism, e.g. in mammalian, yeast or bacterial cells, although eukaryotic cells are preferred, more preferably host cells capable of *in vivo* glycosylation, in particular mammalian cells such as CHO cells, HEK cells or BHK cells. Methods for production of recombinant FVII/FVIIa as well as variants thereof using e.g. mammalian cells are well-known in the art, as are methods for subsequent purification and isolation of the recombinant polypeptides. See, for example, WO 01/58935, WO 03/093465 and WO 2005/002615.

The concentration of the polypeptide in the compositions of the invention may vary and will typically be in the range of about 0.1-5 mg/ml, such as about 0.2-2 mg/ml, e.g. about

0.5-1.5 mg/ml. For example, the concentration may be similar to that of NovoSeven□ after reconstitution with water, which is about 0.6 mg/ml, or slightly higher such as about 1.0 mg/ml. For variants of FVII/FVIIa that have an improved clotting activity compared to rhFVIIa, the concentration of the polypeptide may in some cases be slightly lower, e.g. about 0.1-0.5 mg/ml, such as about 0.2-0.4 mg/ml.

Additional aspects

In a further aspect, the invention relates to a method for producing a storage-stable aqueous composition comprising a Factor VII or Factor VIIa polypeptide, the method comprising mixing the Factor VII or Factor VIIa polypeptide with a buffering agent, zinc, and water. It will be understood that the nature and amounts of the buffering agent, zinc, and optional additional components used in this method for stabilizing a FVII/FVIIa composition will be as described above.

In still further aspects, the invention relates to a method of treating or preventing a condition treatable by administration of Factor VIIa, comprising administering to a patient in need thereof a therapeutically effective amount of a composition of the invention as described above, as well as use of a composition of the invention as described above for the manufacture of a medicament for treating or preventing a condition treatable by administration of Factor VIIa. As described above, the composition of the invention will generally be a liquid, aqueous composition, but may in some cases alternatively be in the form of a freeze-dried composition that is reconstituted with water prior to administration.

Conditions treatable by administration of Factor VIIa include blood factor deficiencies, in particular hemophilia A or B, as well as bleeding associated with trauma (both blunt and penetrative trauma), intracerebral hemorrhage (ICH), traumatic brain injury (TBI), burns, variceal bleeds, gastrointestinal bleeding, surgical bleeds, transplantation, fibrinolytic treatment, anticoagulant treatment, postpartum hemorrhage, viral-induced hemorrhage, Von Willebrand disease and thrombocytopenia.

The Factor VII or VIIa compositions of the invention may be administered as single or multiple injections (bolus or transfusion), and may e.g. be administered subcutaneously, intramuscularly or intravenously, but will typically be administered intravenously. The compositions will generally be provided in single-dose form, e.g. in the form of a single-use vial or a pre-filled syringe. The dosage of the polypeptide will e.g. depend on the condition being treated and the weight of the patient, but will often be similar to dosages used for

rhFVIIa (NovoSeven□), for example from about 20 to about 300 μg/kg, typically from about 25 to about 150 μg/kg, such as from about 40 to about 120 μg/kg

EXAMPLES

The invention is further described by the following non-limiting examples, which illustrate Factor VIIa compositions according to the invention. In these examples, the zinc ions can e.g. be in the form of zinc chloride or zinc acetate.

Example 1

Ingredient	Concentration
rhFVIIa or variant	0.3-1.0 mg/ml, e.g. 0.6 mg/ml (about 12 μM)
glycylglycine	1.0-1.5 mg/ml, e.g. 1.3 mg/ml
Zn^{2+}	50-200 μM, e.g. 100 μM
Polysorbate 80	0.05-0.2 mg/ml, e.g. 0.1 mg/ml
sodium chloride	2-4 mg/ml, e.g. 3 mg/ml
calcium chloride dihydrate	1-2 mg/ml, e.g. 1.5 mg/ml
mannitol	25-35 mg/ml, e.g. 30 mg/ml
pH	5.5-7.5, e.g. 6.5

Example 2

Ingredient	Concentration
rhFVIIa or variant	0.8-1.2 mg/ml, e.g. 1.0 mg/ml
PIPES	12-18 mg/ml, e.g. 15 mg/ml
Zn^{2+}	10-100 μM, e.g. 50 μM
Poloxamer 188	0.5-1.5 mg/ml, e.g. 1.0 mg/ml
sodium chloride	2-4 mg/ml, e.g. 3 mg/ml
calcium chloride dihydrate	1-2 mg/ml, e.g. 1.5 mg/ml
methionine	0.25-0.75 mg/ml, e.g. 0.5 mg/ml
sucrose	60-70 mg/ml, e.g. 65 mg/ml
pH	6.0-7.0, e.g. 6.5

Example 3

Ingredient	Concentration
rhFVIIa or variant	0.8-1.2 mg/ml, e.g. 1.0 mg/ml
PIPES	12-18 mg/ml, e.g. 15 mg/ml
Zn^{2+}	10-100 µM, e.g. 50 µM
Poloxamer 188	0.5-1.5 mg/ml, e.g. 1.0 mg/ml
sodium chloride	20-40 mg/ml, e.g. 30 mg/ml
calcium chloride dihydrate	1-2 mg/ml, e.g. 1.5 mg/ml
pH	6.0-7.0, e.g. 6.5

Example 4

Ingredient	Concentration
rhFVIIa or variant	0.1-0.5 mg/ml, e.g. 0.3 mg/ml
glycylglycine	1.0-1.5 mg/ml, e.g. 1.3 mg/ml
Zn^{2+}	10-100 µM, e.g. 50 µM
Poloxamer 188	0.5-1.5 mg/ml, e.g. 1.0 mg/ml
sodium chloride	2-4 mg/ml, e.g. 3 mg/ml
mannitol	25-35 mg/ml, e.g. 30 mg/ml
pH	2.5-3.5, e.g. 3.0

Example 5 (freeze-dried composition; amounts below are upon reconstitution)

Ingredient	Concentration
rhFVIIa or variant	0.3-1.0 mg/ml, e.g. 0.6 mg/ml
glycylglycine	1.0-1.5 mg/ml, e.g. 1.3 mg/ml
Zn^{2+}	20-200 µM, e.g. 100 µM
Polysorbate 80	0.05-0.2 mg/ml, e.g. 0.1 mg/ml
calcium chloride dihydrate	1-2 mg/ml, e.g. 1.5 mg/ml
mannitol	10-30 mg/ml, e.g. 20 mg/ml
sucrose	30-70 mg/ml e.g. 50 mg/ml
pH	6.5-7.5, e.g. 7.0

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be clear to one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention. It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, patent applications, and/or other documents cited in this application are incorporated herein by reference in their entirety for all purposes to the same extent as if each individual publication, patent, patent application, and/or other document were individually indicated to be incorporated herein by reference in its entirety for all purposes.

Sequence listing

SEQ ID NO:1: Human Factor VII

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Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu
1               5                  10                  15

Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys
            20                  25                  30

Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp
        35                  40                  45

Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln
    50                  55                  60

Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn
65                  70                  75                  80

Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly
                85                  90                  95

Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys
            100                 105                 110

Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr
        115                 120                 125

Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg
    130                 135                 140

Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro
145                 150                 155                 160

Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln
                165                 170                 175

Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala
            180                 185                 190

His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu
        195                 200                 205

Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg
    210                 215                 220

Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn
225                 230                 235                 240

His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp
                245                 250                 255
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His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr
            260                 265                 270

Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu
        275                 280                 285

Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg
    290                 295                 300

Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser
305                 310                 315                 320

Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser
                325                 330                 335

Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr
            340                 345                 350

Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys
        355                 360                 365

Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile
    370                 375                 380

Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu
385                 390                 395                 400

Leu Arg Ala Pro Phe Pro
                405
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CLAIMS

1. A liquid, aqueous pharmaceutical composition comprising a Factor VII or Factor VIIa polypeptide, a buffering agent, and zinc (Zn^{2+}).

2. The composition of claim 1, wherein the Factor VII or Factor VIIa polypeptide is recombinant human Factor VIIa or a variant thereof that differs in 1-15 amino acid residues from the amino acid sequence of human Factor VIIa.

3. The composition of claim 1 or 2, wherein the zinc is in the form of a zinc salt selected form the group consisting of zinc acetate, zinc bromide, zinc chloride, zinc iodide, zinc fluoride and zinc sulfate.

4. The composition of any of claims 1-3, comprising zinc in a concentration of 1 μM – 1 mM.

5. The composition of any of claims 1-3, wherein the molar ratio between zinc and the polypeptide is in the range of 0.1-1000.

6. The composition of any of the preceding claims, having a pH in the range of from about 2.5 to about 9.0.

7. The composition of any of the preceding claims, wherein the buffering agent comprises at least one acid or salt of a component selected from the group consisting of citrate, succinate, tartrate, fumarate, gluconate, oxalate, lactate, acetate, phosphate, maleate histidine, glycine, glycylglycine, lysine, arginine, MES, PIPES, ACES, BES, TES, HEPES, TRIS or imidazole.

8. The composition of any of the preceding claims, wherein the buffering agent is present in a concentration of 1-100 mM.

9. The composition of any of the preceding claims, wherein the concentration of the polypeptide is in the range of 0.1-5 mg/ml.

10. The composition of any of the preceding claims, further comprising at least one component selected from a non-ionic surfactant, a tonicity modifying agent, an antioxidant, a preservative, calcium ions and magnesium ions.

11. A method for producing a storage-stable liquid, aqueous composition comprising a Factor VII or Factor VIIa polypeptide, the method comprising mixing the Factor VII or Factor VIIa polypeptide with a buffering agent, zinc (Zn^{2+}), and water.

12. A method of treating or preventing a condition treatable by administration of Factor VIIa, comprising administering to a patient in need thereof a therapeutically effective amount of a composition according to any of claims 1-10.

13. Use of a composition according to any of claims 1-10 for the manufacture of a medicament for treating or preventing a condition treatable by administration of Factor VIIa.

14. A lyophilized pharmaceutical composition comprising a Factor VII or Factor VIIa polypeptide, a buffering agent, and zinc (Zn^{2+}).

15. A method for producing a lyophilized pharmaceutical composition comprising a Factor VII or Factor VIIa polypeptide, a buffering agent, and zinc (Zn^{2+}), comprising freeze-drying a composition according to any of claims 1-10.

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WO 2007/022784 A3

(54) Title: LIQUID FACTOR VII COMPOSITION

(57) Abstract: Storage- stable aqueous pharmaceutical compositions comprising a Factor VII or Factor Vila polypeptide, a buffering agent, and zinc ions (Zn^{2+}) as a stabilizer.

INTERNATIONAL SEARCH REPORT

International application No
PCT/DK2006/050036

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/36 A61P7/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, EMBASE, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2004/082708 A2 (NOVO NORDISK AS [DK]; JENSEN MICHAEL BECH [DK]; HANSEN BIRTHE LYKKEGAA) 30 September 2004 (2004-09-30) claims 1-5,9-11,47,48,50,60-62	1-15
X	US 2003/096338 A1 (PEDERSEN ANDERS HJELHOLT [DK] ET AL) 22 May 2003 (2003-05-22) column 19 column 23 column 245 column 249 - column 254 column 281 claims 63-67	1-15

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search	Date of mailing of the international search report
6 February 2007	21/02/2007

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Ganschow, Silke

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK2006/050036

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claim 12 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/DK2006/050036

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2004082708 A2	30-09-2004	AU 2004222625 A1	30-09-2004
		CA 2518327 A1	30-09-2004
		EP 1605968 A2	21-12-2005
		JP 2006524195 T	26-10-2006
		MX PA05009914 A	09-01-2006
US 2003096338 A1	22-05-2003	US 2006019336 A1	26-01-2006

Severe factor VII deficiency caused by a novel point mutation (Arg353Pro) combined with a rare Cys22Arg mutation

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Dear Sir,

Inherited human coagulation factor VII (FVII) deficiency is a rare autosomal recessive hemorrhagic disorder characterized by wide genetic heterogeneity and a poor correlation between FVII activity and severity of the hemorrhagic features (1).

We describe here the molecular basis of severe FVII deficiency in a girl and her relatives. The proband is a 14-year-old Chinese female with non-consanguineous parents who presented with epistaxis since three years of age and menorrhagia at puberty. She was found to have a prolonged prothrombin time (PT, 67.4 seconds [s]) and no FVII inhibitors in the plasma. Her FVII activity (FVII: C) (normal range 80–130%, FVII: C was tested in a one-stage assay using rabbit brain thromboplastin) was 2%, FVII antigen (FVII:Ag) 10%.The activated partial thromboplastin time, fibrinogen levels, FII:C, FV:C, FVIII:C, FIX:C, FX:C were normal. She was diagnosed with a severe FVII deficiency. The patient's parents, sister and two uncles were asymptomatic with normal PT.

In order to identify the genetic variation involved, FVII coding regions, the exon-intron boundaries and the 5', 3'flanking regions were amplified by polymerase chain reaction (2) and directly sequenced. Annotation of FVII nucleotide sequences are as per O'¯Hara et al. Molecular modeling was performed using the Swiss Protein Data Bank Deepviewer Version 3.7. The coordinate of FVII was obtained from the Protein Data Bank (code 1DAN).

Two missense mutations were found in the coding regions. An exon 2 T4407 →C heterozygous mutation resulted in a codon change of TGC to CGC, leading to an amino-acid change of Cys→Arg at residue 22. A novel exon 8 G11496→C heterozygous mutation resulted in a codon change of CGG to CCG, leading to an amino-acid change of Arg353→Pro. The proband with these two heterozygous mutations showed markedly decreased FVII:C and FVII:Ag levels, whereas her parents and sister with either heterozygous mutation showed moderately reduced FVII:C and FVII:Ag level (Fig. 1).

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Among more than 130 FVII gene lesions in the literature, about 90 missense mutations were found (3). The first reported polymorphism to affect FVII levels was the Arg353Gln polymorphism within exon 8. Heterozygosity for this polymorphism was associated with an approximately 25% reduction in FVII:C and FVII:Ag levels while homozygous individuals had 50% reduction (4). The Gln353 substitution that changes an amino acid with a positively charged side group to an uncharged one likely alters the conformation of the protein that affects the processing of FVII in hepatocytes resulting in reduced secretion of the protein (5). Therefore, the Arg amino acid at residue 353 might be important for FVII secretion. We report here a novel heterozygous mutation for Arg353Pro. Using molecular modeling, we found that hydrogen bonds between amino acid 353 and Glu94/Cys98 disrupted with Pro or Gln substitution. Although Pro is an uncharged amino acid as is Gln, proline might break the α-helix or β-sheet that comes from its side chain constraints and sterics. Therefore, the local conformation with proline substitution changed more dramatically than glutamine which might diminish FVII protein secretion to a greater extent. The result that the proband's mother, only with this heterozygous mutation, had a 45% reduction in FVII:Ag is consistent with this hypothesis.

Another heterozygous mutation for Cys22Arg here is a rare mutation. Fromovich first reported a severe FVII deficiency in a patient with heterozygous Cys22Arg mutation and their in-vitro results suggested that Cys22Arg substitution abolished the structure of the molecule and impacted its secretion (6). Our results that the patient's father and sister only with heterozygous Cys22Arg mutation had reduced FVII:C and FVII:Ag at different levels confirmed that this mutation might affect FVII secretion. On the other hand, the Gla domain is part of the interface between tissue factor (TF) and FVII, and any change in its structure affects the overall interaction with TF. Cys17 and Cys22 are conserved residues in vitamin K-dependent coagulation factors forming a disulfide bond important for holding together two Gla residues in a proper position. This position enables capturing Ca^{2+} ions that are critical for proper function of the FVII (7). Modeling of the replacement of cysteine by Arg at residue 22 showed that the disulfide bond between residue 22 and cysteine 17 was disrupted, while three hydrogen bonds between Arg22 and Cys17 and two hydrogen bonds between Arg22 and Ser12/Leu13 formed, respectively. The local conformation changed and then possibly impaired the interaction with TF. A low level of dysfunctional FVII-22Arg molecules in the plasma might help to explain the relatively higher FVII:Ag level (10%) as compared to FVII:C level in the proband. Thus, mutation for Cys22Arg might affect FVII both in secretion and in interaction with TF. A heterozygous mutation for Cys22Phe in Gla domain

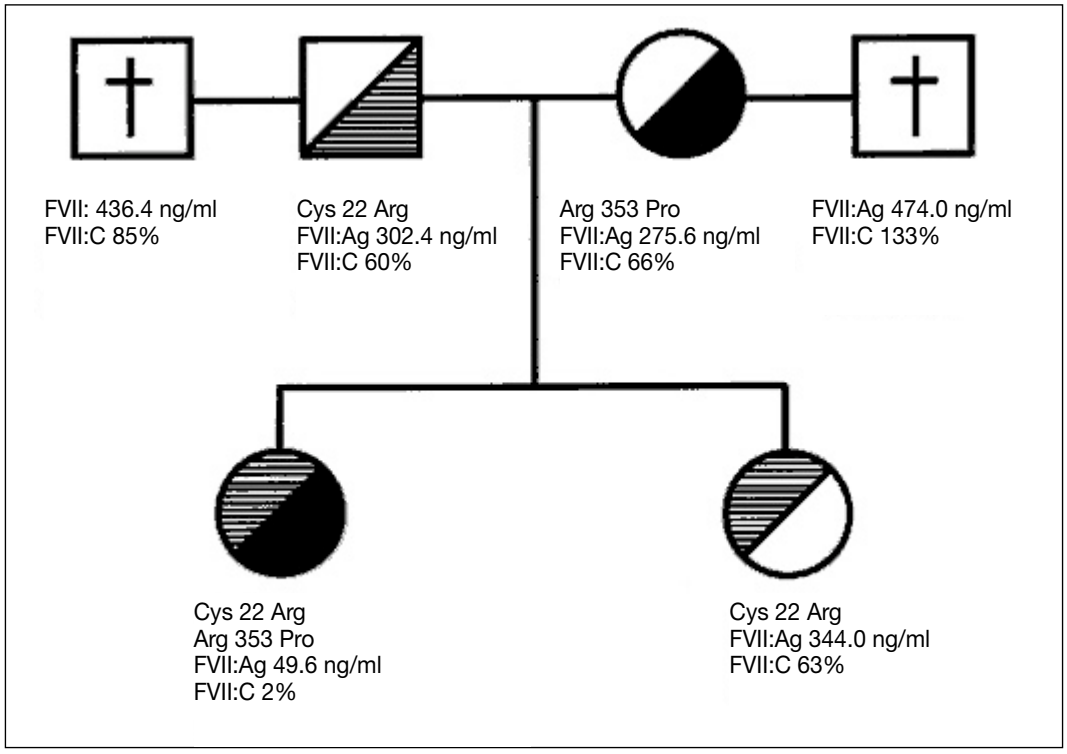


Figure 1: Pedigree of a Chinese family with factor VII deficiency.

has been previously described in a patient with mild bleeding (see FVII mutation database).

For the proband with combined heterozygous mutations, it can be speculated that the bleeding tendency resulted from markedly reduced FVII protein levels yielding less efficient secretion and interaction with tissue factor. The exact consequences of these FVII mutations require further study by in-vitro expression.

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US 20060270001A1

(19) **United States**

(12) **Patent Application Publication** (10) **Pub. No.: US 2006/0270001 A1**

Haaning et al. (43) **Pub. Date: Nov. 30, 2006**

(54) **FACTOR VII OR VIIA POLYPEPTIDE VARIANTS**

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(60) Provisional application No. 60/376,679, filed on Apr. 30, 2002.

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C12P 21/04	(2006.01)

(52) **U.S. Cl.** **435/69.6**; 435/320.1; 435/325; 514/12; 530/383; 536/23.5

(57) **ABSTRACT**

The present invention relates to novel polypeptide variants of factor VII (FVII) or factor VIIa (FVIIa) polypeptides, where said variants comprise an amino acid substitution in position 10 and 32 and where said variants further comprise a sugar moiety covalently attached to an introduced in vivo N-glycosylation site located outside of the Gla domain. Such polypeptide variants are useful in therapy, in particular for the treatment of a variety of coagulation-related disorders, such as trauma.

FACTOR VII OR VIIA POLYPEPTIDE VARIANTS

FIELD OF THE INVENTION

[0001] The present invention relates to novel polypeptide variants of factor VII (FVII) or factor VIIa (FVIIa) polypeptides, where said variants comprise an amino acid substitution in position 10 and 32 and where said variants further comprise a sugar moiety covalently attached to an introduced in vivo N-glycosylation site.

[0002] The present invention also relates to use of such polypeptide variants in therapy, in particular for the treatment of a variety of coagulation-related disorders.

BACKGROUND OF THE INVENTION

[0003] Blood coagulation is a process consisting of a complex interaction of various blood components (or factors) that eventually results in a fibrin clot. Generally, the blood components participating in what has been referred to as the "coagulation cascade" are proenzymes or zymogens, i.e. enzymatically inactive proteins that are converted into an active form by the action of an activator. One of these coagulation factors is FVII.

[0004] FVII is a vitamin K-dependent plasma protein synthesized in the liver and secreted into the blood as a single-chain glycoprotein with a molecular weight of 53 kDa (Broze & Majerus, J. Biol. Chem 1980; 255:1242-1247). The FVII zymogen is converted into an activated form (FVIIa) by proteolytic cleavage at a single site, R152-I153, resulting in two chains linked by a single disulfide bridge. FVIIa in complex with tissue factor (FVIIa complex) is able to convert both factor IX and factor X into their activated forms, followed by reactions leading to rapid thrombin production and fibrin formation (Østerud & Rapaport, Proc Natl Acad Sci USA 1977; 74:5260-5264).

[0005] FVII undergoes post-translational modifications, including vitamin K-dependent carboxylation resulting in ten γ-carboxyglutamic acid residues in the N-terminal region of the molecule. Thus, residue number 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35 shown in SEQ ID NO:2 are γ-carboxyglutamic acids residues in the Gla domain important for FVII activity. Other post-translational modifications include sugar moiety attachment at two naturally occurring N-glycosylation sites at position 145 and 322, respectively, and at two naturally occurring O-glycosylation sites at position 52 and 60, respectively.

[0006] The gene coding for human FVII (hFVII) has been mapped to chromosome 13 at q34-qter 9 (de Grouchy et al., Hum Genet 1984; 66:230-233). It contains nine exons and spans 12.8 Kb (O'Hara et al., Proc Natl Acad Sci USA 1987; 84:5158-5162). The gene organisation and protein structure of FVII are similar to those of other vitamin K-dependent procoagulant proteins, with exons 1a and 1b encoding for signal sequence; exon 2 the propeptide and Gla domain; exon 3 a short hydrophobic region; exons 4 and 5 the epidermal growth factor-like domains; and exon 6 through 8 the serine protease catalytic domain (Yoshitake et al., Biochemistry 1985; 24: 3736-3750).

[0007] Reports exist on experimental three-dimensional structures of hFVIIa (Pike et al., PNAS. U.S.A., 1999; 96:8925-30 and Kemball-Cook et al., J. Struct. Biol, 1999; 127:213-223); of hFVIIa in complex with soluble tissue factor using X-ray crystallographic methods (Banner et al., Nature, 1996; 380:41 and Zhang et al., J. Mol. Biol, 1999; 285: 2089); and of smaller fragments of hFVII (Muranyi et al., Biochemistry, 1998; 37:10605 and Kao et al., Biochemistry, 1999; 38:7097).

[0008] Some protein-engineered variants of FVII have been reported. See, e.g., Dickinson & Ruf, J Bio Chem, 1997;272:19875-19879, Kemball-Cook et al., J Biol Chem, 1998; 273:8516-8521, Bharadwaj et al., J Biol Chem, 1996; 271:30685-30691, Ruf et al., Biochemistry, 1999; 38:1957-1966; WO 99/20767; WO 00/11416; WO 02/22776; WO 02/38162; WO 01/83725; WO 01/58935; U.S. Pat. No. 5,580,560.

[0009] Reports exist on expression of FVII in BHK or other mammalian cells (WO 92/15686, WO 91/11514 and WO 88/10295) and co-expression of FVII and kex2 endoprotease in eukaryotic cells (WO 00/28065).

[0010] Commercial preparations of human recombinant FVIIa are sold as NovoSeven®. NovoSeven® is indicated for the treatment of bleeding episodes in hemophilia A or B patients. NovoSeven® is the only rhFVIIa for effective and reliable treatment of bleeding episodes available on the market.

[0011] An inactive form of FVII in which arginine 152 and/or isoleucine 153 is/are modified has been reported in WO91/1154. These amino acids are located at the activation site. WO 96/12800 describes inactivation of FVIIa by a serine proteinase inhibitor; inactivation by carbamylation of FVIIa at the α-amino acid group 1153 has been described by Petersen et al., Eur J Biochem, 1999;261:124-129. The inactivated form is capable of competing with wild-type FVII or FVIIa for binding to tissue factor and inhibiting clotting activity. The inactivated form of FVIIa is suggested to be used for treatment of patients being in hypercoagulable states, such as patients with sepsis, in risk of myocardial infarction or of thrombotic stroke.

[0012] A circulating rhFVIIa half-life of 2.3 hours was reported in "Summary Basis for Approval for NovoSeven®", FDA reference number 96-0597. Relatively high doses and frequent administration are necessary to reach and sustain the desired therapeutic or prophylactic effect. As a consequence adequate dose regulation is difficult to obtain and the need of frequent intravenous administrations imposes restrictions on the patient's way of living.

[0013] In connection with treatment of uncontrolled bleedings, such as trauma, it is believed that factor VIIa is capable of activating factor X to factor Xa without binding to tissue factor, and this activation reaction is believed to occur primarily on activated blood platelets (Hedner et al. Blood Coagulation & Fibrinolysis, 2000;11;107-111). However, hFVIIa or rhFVIIa has a low activity towards factor X in the absence of tissue factor and, consequently, treatment of uncontrolled bleeding, for example in trauma patients, requires relatively high and multiple doses of hFVIIa or rhFVIIa. Therefore, in order to treat uncontrolled bleedings more efficiently (to minimize blood loss) there is need for improved FVIIa molecules, which possess a high activity toward factor X in the absence of tissue factor. Such improved FVIIa molecules will exhibit a lowered clotting time (or faster action) as compared to rhFVIIa when administered in connection with uncontrolled bleedings.

[0014] A molecule with a longer circulation half-life would decrease the number of necessary administrations. Given the association of current the rhFVIIa product with frequent injections, and the potential for obtaining more optimal therapeutic FVIIa levels with concomitant enhanced therapeutic effect, there is a clear need for improved FVII- or FVIIa-like molecules.

[0015] One way to increase the circulation half-life of a protein is to ensure that renal clearance of the protein is reduced. This may be achieved by conjugating the protein to a chemical moiety, which is capable of conferring reduced renal clearance to the protein.

[0016] Furthermore, attachment of a chemical moiety to the protein or substitution of amino acids exposed to proteolysis may effectively block a proteolytic enzyme from contact leading to proteolytic degradation of the protein. Polyethylene glycol (PEG) is one such chemical moiety that has been used in the preparation of therapeutic protein products. WO 98/32466 suggests that FVII, among many other proteins, may be PEGylated but does not contain any further information in this respect. WO 01/58935 discloses a new strategy for developing FVII or FVIIa molecules having inter alia an increased half-life.

[0017] As indicated above, another problem in current rhFVIIa treatment is the relative instability of the molecule with respect to proteolytic degradation. Proteolytic degradation is a major obstacle for obtaining a preparation in solution as opposed to a lyophilized product. The advantage of obtaining a stable soluble preparation lies in easier handling for the patient, and, in the case of emergencies, quicker action, which potentially can become life saving. Attempts to prevent proteolytic degradation by site directed mutagenesis at major proteolytic sites have been disclosed in WO 88/10295.

[0018] One object of the present invention is to provide improved FVII or FVIIa molecules (FVII or FVIIa variants) with a longer circulation half-life (thereby decreasing the number of necessary administrations) and which are capable of activating factor X to factor Xa (without binding to tissue factor) more efficiently than hFVIIa or rhFVIIa (thereby being able to treat uncontrolled bleadings, such as a trauma, more efficiently).

[0019] Another object of the present invention is to provide improved FVII or FVII molecules (FVII or FVIIa variants) with an increased bioavailability (such as an increased Area Under the Curve as compared to rhFVIIa when administered intravenously) and which are capable of activating factor X to factor Xa (without binding to tissue factor) more efficiently than hFVIIa or rhFVIIa (thereby being able to treat uncontrolled bleadings, such as a trauma, more efficiently).

[0020] These objects are met by the FVII or FVIIa variants provided herein.

BRIEF DISCLOSURE OF THE INVENTION

[0021] In its broadest aspect the present invention relates to a FVII or FVIIa polypeptide variant having an amino acid sequence comprising 3-15 amino acid modifications relative to hFVII or hFVIIa having the amino acid sequence shown in SEQ ID NO:2, wherein said amino acid sequence of the variant comprises an amino acid substitution in position 10 and 32 and wherein a sugar moiety is covalently attached to an introduced in vivo N-glycosylation site located outside the Gla domain.

[0022] Another aspect of the invention relates to a nucleotide sequence encoding the polypeptide variant of the invention.

[0023] In a further aspect the invention relates to an expression vector comprising the nucleotide sequence of the invention.

[0024] In a still further aspect the invention relates to a host cell comprising the nucleotide sequence of the invention or the expression vector of the invention.

[0025] In an even further aspect the invention relates to a pharmaceutical composition comprising the polypeptide variant of the invention, and a pharmaceutical acceptable carrier or excipient.

[0026] Still another aspect of the invention relates to the polypeptide variant of the invention, or the pharmaceutical composition of the invention, for use as a medicament.

[0027] Further aspects of the present invention will be apparent from the below description as well as from the appended claims.

DETAILED DISCLOSURE OF THE INVENTION

Definitions

[0028] In the context of the present application and invention the following definitions apply:

[0029] The term "conjugate" (or interchangeably "conjugated polypeptide") is intended to indicate a heterogeneous (in the sense of composite or chimeric) molecule formed by the covalent attachment of one or more polypeptide(s) to one or more non-polypeptide moieties such as polymer molecules, lipophilic compounds, sugar moieties or organic derivatizing agents. Preferably, the conjugate is soluble at relevant concentrations and conditions, i.e. soluble in physiological fluids such as blood. Examples of conjugated polypeptides of the invention include glycosylated and/or PEGylated polypeptides.

[0030] The term "covalent attachment" or "covalently attached" means that the polypeptide variant and the non-polypeptide moiety are either directly covalently joined to one another, or else are indirectly covalently joined to one another through an intervening moiety or moieties, such as a bridge, spacer, or linkage moiety or moieties.

[0031] The term "non-polypeptide moiety" is intended to mean a molecule, different from a peptide polymer composed of amino acid monomers and linked together by peptide bonds, which molecule is capable of conjugating to an attachment group of the polypeptide variant of the invention. Preferred examples of such molecules include polymer molecules, sugar moieties, lipophilic compounds or organic derivatizing agents. When used in the context of a conjugated variant of the invention it will be understood that the non-polypeptide moiety is linked to the polypeptide part of the conjugated variant through an attachment group of the polypeptide. As explained above, the non-polypeptide moiety can be directly covalently joined to the attachment group or it can be indirectly covalently joined to the attachment

group through an intervening moiety or moieties, such as a bridge spacer or linker moiety or moieties.

[0032] A "polymer molecule" is a molecule formed by covalent linkage of two or more monomers, wherein none of the monomers is an amino acid residue, except where the polymer is human albumin or another abundant plasma protein. The term "polymer" may be used interchangeably with the term "polymer molecule". The term is also intended to cover carbohydrate molecules attached by in vitro glycosylation, i.e. a synthetic glycosylation performed in vitro normally involving covalently linking a carbohydrate molecule to an attachment group of the polypeptide variant, optionally using a cross-linking agent. In vitro glycosylation is discussed in detail further below.

[0033] The term "sugar moiety" is intended to indicate a carbohydrate-containing molecule comprising one or more monosaccharide residues, capable of being attached to the polypeptide variant (to produce a polypeptide variant conjugate in the form of a glycosylated polypeptide variant) by way of in vivo glycosylation. The term "in vivo glycosylation" is intended to mean any attachment of a sugar moiety occurring in vivo, i.e. during posttranslational processing in a glycosylating cell used for expression of the polypeptide variant, e.g. by way of N-linked and O-linked glycosylation. The exact oligosaccharide structure depends, to a large extent, on the glycosylating organism in question.

[0034] An "N-glycosylation site" has the sequence N-X-S/T/C, wherein X is any amino acid residue except proline, N is asparagine and S/T/C is either serine, threonine or cysteine, preferably serine or threonine, and most preferably threonine. Preferably, the amino acid residue in position +3 relative to the asparagines residue is not a proline residue.

[0035] An "O-glycosylation site" is the OH-group of a serine or threonine residue.

[0036] The term "attachment group" is intended to indicate a functional group of the polypeptide variant, in particular of an amino acid residue thereof or a carbohydrate moiety, capable of attaching a non-polypeptide moiety such as a polymer molecule, a lipophilic molecule, a sugar moiety or an organic derivatizing agent. Useful attachment groups and their matching non-polypeptide moieties are apparent from the table below.

Attachment group	Amino acid	Examples of non-polypeptide moiety	Conjugation method/-Activated PEG	Reference
—NH_2	N-terminal, Lys	Polymer, e.g. PEG, with amide or imine group	mPEG-SPA Tresylated mPEG	Shearwater Inc. Delgado et al, critical reviews in Therapeutic Drug Carrier Systems 9(3, 4): 249–304 (1992)
—COOH	C-terminal, Asp, Glu	Polymer, e.g. PEG, with ester or amide group	mPEG-Hz	Shearwater Inc.
		Carbohydrate moiety	In vitro coupling	
—SH	Cys	Polymer, e.g. PEG, with disulfide, maleimide or vinylsulfone group	PEG-vinylsulphone PEG-maleimide	Shearwater Inc. Delgado et al, critical reviews in Therapeutic Drug Carrier Systems 9(3, 4): 249–304 (1992)
		Carbohydrate moiety	In vitro coupling	
—OH	Ser, Thr, Lys, OH—	Sugar moiety PEG with ester, ether, carbamate, carbonate	In vivo O-linked glycosylation	
—$CONH_2$	Asn as part of an N-glycosylation site	Sugar moiety Polymer, e.g. PEG	In vivo N-glycosylation	
Aromatic residue	Phe, Tyr, Trp	Carbohydrate moiety	In vitro coupling	
—$CONH_2$	Gln	Carbohydrate moiety	In vitro coupling	Yan and Wold, Biochemistry, 1984, Jul 31; 23(16): 3759–65
Aldehyde Ketone	Oxidized oligosaccharide	Polymer, e.g. PEG, PEG-hydrazide	PEGylation	Andresz et al., 1978, Makromol. Chem. 179: 301, WO 92/16555, WO 00/23114
Guanidino	Arg	Carbohydrate moiety	In vitro coupling	Lundblad and Noyes, Chimical Reagents for Protein Modification, CRC Press Inc., Florida, USA
Imidazole ring	His	Carbohydrate moiety	In vitro coupling	As for guanidine

[0037] For in vivo N-glycosylation, the term "attachment group" is used in an unconventional way to indicate the amino acid residues constituting a N-glycosylation site (with the sequence N-X-S/T/C, wherein X is any amino acid residue except proline, N is asparagine and S/T/C is either serine, threonine or cysteine, preferably serine or threonine, and most preferably threonine). Although the asparagine residue of the N-glycosylation site is the one to which the sugar moiety is attached during glycosylation, such attachment cannot be achieved unless the other amino acid residues of the N-glycosylation site is present.

[0038] Accordingly, when the non-polypeptide moiety is a sugar moiety and the conjugation is to be achieved by in vivo N-glycosylation, the term "amino acid residue comprising an attachment group for a non-polypeptide moiety" as used in connection with alterations of the amino acid sequence of the polypeptide is to be understood as meaning that one or more amino acid residues constituting an in vivo N-glycosylation site are to be altered in such a manner that a functional in vivo N-glycosylation site is introduced into the amino acid sequence.

[0039] In the present application, amino acid names and atom names (e.g. CA, CB, CD, CG, SG, NZ, N, O, C, etc) are used as defined by the Protein DataBank (PDB) (www-.pdb.org) based on the IUPAC nomenclature (IUPAC Nomenclature and Symbolism for Amino Acids and Peptides (residue names, atom names, etc.), *Eur. J. Biochem.,* 138, 9-37 (1984) together with their corrections in *Eur. J. Biochem.,* 152, 1 (1985)).

[0040] The term "amino acid residue" is intended to indicate an amino acid residue contained in the group consisting of alanine (Ala or A), cysteine (Cys or C), aspartic acid (Asp or D), glutamic acid (Glu or E), phenylalanine (Phe or F), glycine (Gly or G), histidine (His or H), isoleucine (Ile or I), lysine (Lys or K), leucine (Leu or L), methionine (Met or M), asparagine (Asn or N), proline (Pro or P), glutamine (Gln or Q), arginine (Arg or R), serine (Ser or S), threonine (Thr or T), valine (Val or V), tryptophan (Trp or W), and tyrosine (Tyr or Y) residues.

[0041] The terminology used for identifying amino acid positions is illustrated as follows: I205 indicates that position 205 is occupied by an isoleucine residue in the amino acid sequence shown in SEQ ID NO:2. I205T indicates that the isoleucine residue of position 205 has been substituted with a threonine residue. Alternative substitutions are indicated with a "/", e.g. I205SIT means an amino acid sequence in which isoleucine in position 205 is substituted with either serine or threonine. Multiple substitutions are indicated with a "+", e.g. K143N+N145T means a substitution of the lysine residue in position 143 with an asparagine residue and a substitution of the asparagine residue in position 145 with a threonine residue. Insertion of an additional amino acid residue is indicated in the following way: Insertion of a tyrosine residue after A3 (i.e. in position 4) is indicated by A3AY (leading to insertion of a tyrosine residue in position 4). A deletion of an amino acid residue is indicated by an asterix. For example, deletion of a valine residue in position 172 is indicated by V172*. Simultaneous insertion and substitution are indicated in the following way: Substitution of an alanine residue in position 175 with a threonine residue followed by insertion of a leucine residue after position 175 is indicated A175TL.

[0042] Unless otherwise indicated, the numbering of amino acid residues made herein is made relative to the amino acid sequence of the hFVII/hFVIIa polypeptide (SEQ ID NO:2).

[0043] The term "differs from" as used in connection with specific mutations is intended to allow for additional differences being present apart from the specified amino acid difference. For instance, in addition to the introduction of in vivo N-glycosylation sites (located outside the Gla domain), the polypeptide may comprise other modifications that are not related to introduction of such amino acid residues. In a similar way, in addition to the modifications performed in the Gla domain aiming at increasing the phospholipid membrane binding affinity, the polypeptide may contain other modifications that are not necessarily related to this effect.

[0044] Thus, in addition to the amino acid modifications disclosed herein, it will be understood that the amino acid sequence of the polypeptide variant of the invention may, if desired, contain other alterations, i.e. other substitutions, insertions or deletions. These may, for example, include truncation of the N- and/or C-terminus by one or more amino acid residues (e.g. by 1-10 amino acid residues), or addition of one or more extra residues at the N- and/or C-terminus, e.g. addition of a methionine residue at the N-terminus or introduction of a cysteine residue near or at the C-terminus, as well as "conservative amino acid substitutions", i.e. substitutions performed within groups of amino acids with similar characteristics, e.g. small amino acids, acidic amino acids, polar amino acids, basic amino acids, hydrophobic amino acids and aromatic amino acids.

[0045] Examples of such conservative substitutions are shown in the below table.

1	Alanine (A)	Glycine (G)	Serine (S)	Threonine (T)
2	Aspartic acid (D)	Glutamic acid (E)		
3	Asparagine (N)	Glutamine (Q)		
4	Arginine (R)	Histidine (H)	Lysine (K)	
5	Isoleucine (I)	Leucine (L)	Methionine (M)	Valine (V)
6	Phenylalanine (F)	Tyrosine (Y)	Tryptophan (W)	

[0046] Still other examples of additional modifications are disclosed in the section entitled "Other modifications outside the Gla domain" below.

[0047] The term "nucleotide sequence" is intended to indicate a consecutive stretch of two or more nucleotide molecules. The nucleotide sequence may be of genomic, cDNA, RNA, semisynthetic, synthetic origin, or any combinations thereof.

[0048] The term "polymerase chain reaction" or "PCR" generally refers to a method for amplification of a desired nucleotide sequence in vitro, as described, for example, in U.S. Pat. No. 4,683,195. In general, the PCR method involves repeated cycles of primer extension synthesis, using oligonucleotide primers capable of hybridising preferentially to a template nucleic acid.

[0049] The term "vector" refers to a plasmid or other nucleotide sequences that are capable of replicating within a host cell or being integrated into the host cell genome, and as such, are useful for performing different functions in

conjunction with compatible host cells (a vector-host system): to facilitate the cloning of the nucleotide sequence, i.e. to produce usable quantities of the sequence, to direct the expression of the gene product encoded by the sequence and to integrate the nucleotide sequence into the genome of the host cell. The vector will contain different components depending upon the function it is to perform.

[0050] "Cell", "host cell", "cell line" and "cell culture" are used interchangeably herein and all such terms should be understood to include progeny resulting from growth or culturing of a cell.

[0051] "Transformation" and "transfection" are used interchangeably to refer to the process of introducing DNA into a cell.

[0052] "Operably linked" refers to the covalent joining of two or more nucleotide sequences, by means of enzymatic ligation or otherwise, in a configuration relative to one another such that the normal function of the sequences can be performed. For example, the nucleotide sequence encoding a presequence or secretory leader is operably linked to a nucleotide sequence coding for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide: a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the nucleotide sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, then synthetic oligonucleotide adaptors or linkers are used, in conjunction with standard recombinant DNA methods.

[0053] In the context of the present invention the terms "modification" or "amino acid modification" is intended to cover replacement of an amino acid side chain, substitution of an amino acid residue, deletion of an amino acid residue and/or insertion of an amino acid residue.

[0054] The terms "mutation" and "substitution" are used interchangeably herein.

[0055] The term "introduce" refers to introduction of an amino acid residue by substitution of an existing amino acid residue or, alternatively, by insertion of an additional amino acid residue.

[0056] The term "remove" refers to removal of an amino acid residue by substitution of the amino acid residue to be removed by another amino acid residue or, alternatively, by deletion (without substitution) of the amino acid residue to be removed

[0057] The term "FVII" or "FVII polypeptide" refers to a FVII molecule provided in single chain form. One example of a FVII polypeptide is wild-type human FVII (hFVII) shown in SEQ ID NO:2. It should be understood, however, that the term "FVII polypeptide" also covers hFVII-like molecules, such as fragments or variants of SEQ ID NO:2, in particular variants where the sequence comprises at least one, such as 1-15, e.g., 1-10, amino acid modifications as compared to SEQ ID NO:2.

[0058] The term "FVIIa" or "FVIIa polypeptide" refers to a FVIIa molecule provided in its activated two-chain form. When the amino acid sequence of SEQ ID NO:2 is used to describe the amino acid sequence of FVIIa it will be understood that the peptide bond between R152 and I153 of the single-chain form has been cleaved, and that one of the chains comprises amino acid residues 1-152, the other chain amino acid residues 153-406.

[0059] The terms "rFVII" and "rFVIIa" refer to FVII and FVIIa polypeptides produced by recombinant techniques.

[0060] The terms "hFVII" and "hFVIIa" refer to human wild-type FVII and FVIIa, respectively, having the amino acid sequence shown in SEQ ID NO:2

[0061] The terms "rhFVII" and "rhFVIIa" refer to human wild-type FVII and FVIIa, having the amino acid sequence shown in SEQ ID NO:2, produced by recombinant means. An example of rhFVIIa is NovoSeven®.

[0062] When used herein, the term "Gla domain" is intended to cover amino acid residues no. 1 to 45 of SEQ ID NO:2.

[0063] Accordingly, the term "located outside the Gla domain" covers amino acid residue no. 46-406 of SEQ ID NO:2.

[0064] The abbreviations "TF" and "TFPI" mean Tissue Factor and Tissue Factor Pathway Inhibitor, respectively.

[0065] The term "protease domain" is used about residues 153-406 counted from the N-terminus.

[0066] The term "catalytic site" is used to mean the catalytic triad consisting of S344, D242 and H193 of the polypeptide variant.

[0067] The term "parent" is intended to indicate the molecule to be modified/improved in accordance with the present invention. Although the parent polypeptide to be modified by the present invention may be any FVII or FVIIa polypeptide, and thus be derived from any origin, e.g. a non-human mammalian origin, it is preferred that the parent polypeptide is hFVII or hFVIIa

[0068] A "variant" is a polypeptide, which differs in one or more amino acid residues from its parent polypeptide, normally in 3-15 amino acid residues (e.g. in 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acid residues), such as in 3-10 amino acid residues, e.g. in 3-8 or 3-5 amino acid residues. In other words, a "variant" typically contains 3-15 anmino acid modifications (for example 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acid modifications), such as 3-10 amino acid modifications, e.g. 3-8 or 3-5 amino acid modifications relative to the parent polypeptide. In the present context, the term "modification" encompasses insertions, deletions, substitutions and combinations thereof. It will be understood that a polypeptide variant according to the present invention will be modified in at least three positions, namely in at least position 10 and 32 (located in the Gla domain) and in at least one position located outside the Gla domain, where said at least one modification creates an in vivo N-glycosylation site.

[0069] The term "clotting activity" is used to mean the activity measured in the "Clotting Assay" described herein. In order to exhibit "clotting activity" a variant of the invention, in its activated form, should have at least 10% of the clotting acitivty of rhFVIIa when assayed in the "Clotting Assay" described herein. In a preferred embodiment of

the invention the variant, in its activated form, has at least 20% of the clotting activity of rhFVIIa, such as at least 30%, e.g. at least 40%, more preferably at least 50%, such as at least 60%, e.g. at least 70%, even more preferably at least 80%, such as at least 90% of the clotting activity of rhFVIIa when assayed in the "Clotting Assay" described herein. In an interesting embodiment the variant, in its activated form, has substantially the same clotting activity as rhFVIIs, such as a clotting activity of 75-125% of the clotting acitivity of rhFVIIa.

[0070] The term "amidolytic activity" is used to mean the activity measured in the "Amidolytic Assay" described herein. In order to exhibit "amidolytic activity" a variant of the invention, in its activated form, should have at least 10% of the amidolytic acitivty of rhFVIIa when assayed in the "Amidolytic Assay" described herein. In a preferred embodiment of the invention the variant, in its activated form, has at least 20% of the amidolytic activity of rhFVIIa, such as at least 30%, e.g. at least 40%, more preferably at least 50%, such as at least 60%, e.g. at least 70%, even more preferably at least 80%, such as at least 90% of the amidolytic activity of rhFVIIa when assayed in the "Amidolytic Assay" described herein. In an interesting embodiment the variant, in its activated form, has substantially the same amidolytic activity as rhFVIIs, such as an amidolytic activity of 75-125% of the amidolytic acitivity of rhFVIIa.

[0071] In the present context, the term "activity" is also used in connection with the variants capability of activating FX to FXa. This activity is also denoted "FX activation activity" or "FXa generation activity".

[0072] The term "increased FX activiation activity" or "increased FXa generation activity" is used to indicate that a variant of the invention, in its activated form, has a statistically signifycantly increased capability to activate FX to FXa as compared to rhFVIIa or. To what extent a variant of the invention (in its activated form) has an increased FX activation activity may conveniently be determined in the "TF-independent Factor X Activation Assay" described herein.

[0073] The term "stronger clot" or "increased clot strength" is used to indicate that the strength of the clot generated by the polypeptide variant is statistically significantly increased relative to that generated by rhFVIIa as determined under comparable conditions. This effect may be determined as the Area Under the Curve (AUC_{throm}) generated by the variant of the invention, in its activated form, when assayed in the "Thrombogram Assay" disclosed herein. In a similar way, the term "increased AUC_{throm}" is used to indicate that the Area Under the Curve generated by the variant (in its activated form) is statistically significantly increased relative to that generated by rhFVIIa as determined under comparable conditions and when measured in the "Thrombogram Assay" described herein

[0074] The term "T_{max}" is used about the time it takes to obtain the maximum thrombin activity level in the "Thrombogram Assay".

[0075] The term "immunogenicity" as used in connection with a given substance is intended to indicate the ability of the substance to induce a response from the immune system. The immune response may be a cell or antibody mediated response (see, e.g., Roitt: Essential Immunology (8th Edition, Blackwell) for further definition of immunogenicity). Normally, reduced antibody reactivity will be an indication of reduced immunogenicity. The reduced immunogenicity may be determined by use of any suitable method known in the art, e.g. in vivo or in vitro.

[0076] The term "functional in vivo half-life" is used in its normal meaning, i.e. the time at which 50% of the biological activity of the polypeptide is still present in the body/target organ, or the time at which the activity of the polypeptide is 50% of the initial value.

[0077] As an alternative to determining functional in vivo half-life, "serum half-life" may be determined, i.e. the time at which 50% of the polypeptide circulates in the plasma or bloodstream prior to being cleared. Determination of serum half-life is often more simple than determining the functional in vivo half-life and the magnitude of serum half-life is usually a good indication of the magnitude of functional in vivo half-life. Alternatively terms to serum half-life include "plasma half-life", "circulating half-life", "serum clearance", "plasma clearance" and "clearance half-life". The polypeptide is cleared by the action of one or more of the reticuloendothelial systems (RES), kidney, spleen or liver, by tissue factor, SEC receptor or other receptor mediated elimination, or by specific or unspecific proteolysis. Normally, clearance depends on size (relative to the cutoff for glomerular filtration), charge, attached carbohydrate chains, and the presence of cellular receptors for the protein. The functionality to be retained is normally selected from procoagulant, proteolytic or receptor binding activity. The functional in vivo half-life and the serum half-life may be determined by any suitable method known in the art.

[0078] The term "increased" as used about the functional in vivo half-life or serum half-life is used to indicate that the relevant half-life of the polypeptide variant is statistically significantly increased relative to that of rhFVIIa as determined under comparable conditions (typically determined in an experimental animal, such as rats, rabbits, pigs or monkeys).

[0079] The term "AUC_{iv}" or "Area Under the Curve when administered intravenously" is used in its normal meaning, i.e. as the area under the activity in serum-time curve, where the polypeptide variant has been administered intravenously, in particular when administered intravenously in rats. Typically, the activity measured is the "clotting activity" as defined hereinbefore. Once the experimental activity-time points have been determined, the AUC_{iv}, may conveniently be calculated by a computer program, such as GraphPad Prism 3.01.

[0080] It will be understood that in order to make a direct comparison between the AUC_{iv}-values of different molecules (e.g. between the variants of the invention and a reference molecule such as rhFVIIa) the same amount of activity should be administered. Consequently, the AUC_{iv}-values are typically normalized (i.e. corrected for differences in the injected dose) and expressed as AUC_{iv}/dose administered.

[0081] The term "reduced sensitivity to proteolytic degradation" is primarily intended to mean that the polypeptide variant has reduced sensitivity to proteolytic degradation in comparison to hFVIIa or rhFVIIa as determined under comparable conditions. Preferably, the proteolytic degrada-

tion is reduced by at least 10% (e.g. by 10-25% or by 10-50%), such as at least 25% (e.g. by 25-50%, by 25-75% or by 25-100%), more preferably by at least 35%, such as at least 50%, (e.g. by 50-75% or by 50-100%) even more preferably by at least 60%, such as by at least 75% (e.g. by 75-100%) or even at least 90%. Most preferably, the proteolytic degradation is reduced by at least 100%.

[0082] The term "renal clearance" is used in its normal meaning to indicate any clearance taking place by the kidneys, e.g. by glomerular filtration, tubular excretion or degradation in the tubular cells. Renal clearance depends on physical characteristics of the polypeptide, including size (diameter), hydrodynamic volume, symmetry, shape/rigidity, and charge. Normally, a molecular weight of about 67 kDa is considered to be a cut-off-value for renal clearance. Renal clearance may be established by any suitable assay, e.g. an established in vivo assay. Typically, renal clearance is determined by administering a labelled (e.g. radiolabelled or fluorescence labelled) polypeptide to a patient and measuring the label activity in urine collected from the patient. Reduced renal clearance is determined relative to a corresponding reference polypeptide, e.g. rhFVIIa, under comparable conditions. Preferably, the renal clearance rate of the polypeptide variant is reduced by at least 50%, preferably by at least 75%, and most preferably by at least 90% compared to rhFVIIa.

[0083] The terms "at least 25% of its side chain exposed to the surface of the molecule" and "at least 50% of its side chain exposed to the surface of the molecule" are defined with reference to Example 1, where the calculations, etc. are described in detail.

[0084] It should be noted that when the terms "at least 25% of its side chain exposed to the surface of the molecule" and "at least 50% of its side chain exposed to the surface of the molecule" are used in connection with introduction of an in vivo N-glycosylation site these terms refer to the surface accessibility of the amino acid side chain in the position where the sugar moiety is actually attached. In many cases it will be necessary to introduce a serine or a threonine residue in position +2 relative to the asparagine residue to which the sugar moiety is actually attached (unless, of course, this position is already occupied by a serine or a threonine residue) and these positions, where the serine or threonine residues are introduced, are allowed to be buried, i.e. to have less than 25% or 50% of their side chains exposed to the surface of the molecule.

[0085] The terms "tissue factor binding site", "active site region" and "ridge of the active site binding cleft" are defined with reference to Example 1 herein, wherein the above-mentioned sites/regions are determined.

[0086] The term "hydrophobic amino acid residue" includes the following amino acid residues: isoleucine (I), leucine (L), methionine (M), valine (V), phenylalanine (F), tyrosine (Y) and tryptophan (W).

[0087] The term "negatively charged amino acid residue" includes the following amino acid residues: Aspartic acid (D) and glutamic acid (E).

[0088] The term "positively charged amino acid residue" includes the following amino acid residues: Lysine (K), arginine (R) and histidine (H).

Variants of the Invention

[0089] In its broadest aspect the present invention relates to a FVII or FVIIa polypeptide variant having an amino acid sequence comprising 3-15 amino acid modifications relative to hFVII or hFVIIa having the amino acid sequence shown in SEQ ID NO:2, wherein said amino acid sequence of the variant comprises an amino acid substitution in position 10 and 32 and wherein a sugar moiety is covalently attached to an introduced in vivo N-glycosylation site located outside the Gla domain.

[0090] The modifications perfomed in the two above-indicated regions of the parent FVII polypeptide serve the following purposes:

[0091] The modifications performed in positions located outside the Gla domain (introduction of in vivo N-glycosylation site(s)) are preferably of such nature that the AUC_{iv}, the functional in vivo half-life and/or the serum half-life of the resulting variant is increased as compared to rhFVIIa.

[0092] The modifications perfomed in the Gla domain of the parent polypeptide are preferably of such nature that an increased phospholipid membrane binding affinity of the resulting molecule is achieved and/or of such nature the resulting molecule has an improved capability to activate FX to FXa and/or of such nature that a stronger clot is formed.

[0093] Without being limited by any particular theory, it is presently believed that the enhanced membrane affinity results in a higher local concentration of the activated polypeptide variants in close proximity to the other coagulation factors, particularly FX. Thus, the rate of activation of FX to FXa will be higher, simply due to a higher molar ratio of the activated FVII variant to FX. The increased activation rate of FX then results in a higher amount of active thrombin, and thus a higher rate of cross-linking of fibrin.

[0094] Thus, in preferred embodiments of the invention the parent FVII or FVIIa polypeptide has been modified so that the resulting activated polypeptide variant has (as compared to rhFVIIa):

[0095] i) an increased bioavailability (AUC_{iv}) and an increased phospholipid membrane binding affinity;

[0096] ii) an increased bioavailability (AUC_{iv}) and an increased capability to activate FX to FXa;

[0097] iii) an increased bioavailability (AUC_{iv}) and is capable of generating a stronger clot (increased AUC_{throm});

[0098] iv) an increased bioavailability (AUC_{iv}) and a reduced T_{max}; v) an increased functional in vivo half-life and an increased phospholipid membrane binding affinity;

[0099] vi) an increased functional in vivo half-life and an increased capability to activate FX to FXa;

[0100] vii) an increased functional in vivo half-life and is capable of generating a stronger clot (increased AUC_{throm});

[0101] viii) an increased functional in vivo half-life and a reduced T_{max};

[0102] ix) an increased serum half-life and an increased phospholipid membrane binding affinity;

[0103] x) an increased serum half-life and an increased capability to activate FX to FXa;

[0104] xi) an increased serum half-life and is capable of generating a stronger clot (increased AUC_{throm}); and/or

[0105] xii) an increased serum half-life and a reduced T_{max}.

[0106] Consequently, medical treatment with a polypeptide variant according to the invention offers a number of advantages over the currently available rhFVIIa compound (NovoSeven®), such as administration of lower dosage, longer duration between injections, increased clot strength and/or faster action.

[0107] Thus, preferred variants of the invention are such variants which, in their activated forms and when compared to rhFVIIa, generates in increased Area Under the Curve when administered intravenously (AUC_{iv}), in particular when administered intravenously in rats. More particularly, variants of the present invention, which are preferred, are such variants where the ratio between the AUC_{iv}, of said variant, in its actvated form, and the AUC_{iv} of rhFVIIa is at least 1.25, such as at least 1.5, e.g. at least 1.75, more preferably at least 2, such as at least 3, even more preferably at least 4, such as at least 5, in particular when administered (intravenously) in rats.

[0108] This effect may in turn (but do not necessarily do so) correspond to an increased functional in vivo half-life and/or an increased serum half-life as compared to rhFVIIa. Accordingly, in another preferred embodiment of the invention, the ratio between the functional in vivo half-life or the serum half-life for the variant, in its activated form, and the functional in vivo half-life or the serum half-life for rhFVIIa is at least 1.25. More preferably, the ratio between the relevant half-life for the variant, in its activated form, and the relevant half-life for hFVIIa or rhFVIIa is at least 1.5, such as at least 1.75, e.g. at least 2, even more preferably at least 3, such as at least 4, e.g. at least 5.

[0109] As will be understood, the variants of the invention also possess, in addition to the above-mentioned functionality (i.e. increased AUC_{iv}, increased functional in vivo half-life and/or increased serum half-life), an increased phospholipid membrane binding affinity as compared to rhFVII, an an increased capability to activate FX to Fxa, a capability of generating a stronger clot (increased AUC_{throm}) and/or a reduced T_{max}.

[0110] Thus, in one preferred embodiment of the invention, the polypeptide variant has (in addition to an increased AUC_{iv}, increased functional in vivo half-life and/or increased serum half-life) an increased phospholipid membrane binding affinity relative to rhFVIIa. Membrane binding affinity may be measured by methods known in the art, such as by the Biacore® assay described in K. Nagata and H. Handa (Ads.), Real-Time Analysis of Biomolecular Interactions, Springer-Verlag, Tokyo, 2000, Chapter 6 entitled "Lipid-Protein Interactions". Alternatively, the membrane binding affinity may be measured as described in Example 1 in WO 99/20767.

[0111] In another preferred embodiment of the invention, the polypeptide variant (in addition to an increased AUC_{iv}, increased functional in vivo half-life and/or increased serum half-life), has an increased FX activation activity as compared to rhFVIIa, in particular when assayed in a TF-independent assay, such as the "TF-independent Factor X Activation Assay" disclosed herein. More particularly, it is preferred that the ratio between the FX activation activity of the polypeptide variant, in its activated form, and the FX activation activity of rhFVIIa is at least 1.25 when assayed in the "TF-independent Factor X Activation Assay" disclosed herein. More preferably, the ratio between the FX activation activity of the variant, in its activated form, and the PX activation activity of rhFVIIa is at least 1.5, such as at least 1.75, e.g. at least 2, even more preferably at least 3, such as at least 4, e.g. at least 5, still more preferably at least 6, such as at least 7, e.g. at least 8, most preferably at least 9, such as at least 10, when assayed in the "TF-independent Factor X Activation Assay" described herein.

[0112] In still another preferred embodiment of the invention, the polypeptide variant is (in addition to an increased AUC_{iv}, increased functional in vivo half-life and/or increased serum half-life), in its activated form, capable of generating a stronger clot as compared to rhFVIIa. This effect may be determined in the "Thrombogram Assay" described herein as an increase in the Area Under the Curve (AUC_{throm}). The AUC_{throm} is also sometimes denoted "total thrombin work" and constitutes a measure for the strength of the clot formed. More particularly, it is preferred that the ratio between the AUC_{throm}, generated by the variant in its activated form, and the AUC_{throm} generated by rhFVIIa is at least 1.15 when assayed in the "Thrombogram Assay" described herein. More preferably, the ratio is at least 1.2, such as at least 1.25, e.g. at least 1.3, even more preferably at least 1.4, such as at least 1.5, e.g. at least 1.6, most preferably at least 1.7, such as at least 1.8, e.g. at least 1.9 or at least 2.

[0113] In even another preferred embodiment of the invention the polypeptide variant has (in addition to an increased AUC_{iv}, increased functional in vivo half-life and/or increased serum half-life), in its activated form, a faster action. This effect may be determined in the "Thrombogram Assay" described herein as a reduction in the time needed to reach maximum thrombin level (T_{max}). Accordingly, preferred variants are such variants where the ratio between T_{max} for the variant, in its activated form, and T_{max} for rhFVIIa is at the most 0.95 when assayed in the "Thrombogram Assay" described herein. Preferably, the ratio is at the most 0.9, such as at the most 0.8, e.g. at the most 0.7, more preferably at the most 0.6, such as at the most 0.5.

Introduction of In Vivo N-Glucosylation Sites Located Outside the Gla Domain

[0114] A number of suitable modifications leading to an increase in AUC_{iv}, functional in vivo half-life and/or serum half-life is disclosed in WO 01/58935. The variants disclosed in WO 01/58935 are the result of a generally new strategy for developing improved FVII or FVIIa molecules, which may also be used for the parent FVII or FVIIa polypeptide of the present invention.

[0115] The position to be modified is preferably selected from a part of the FVII or FVIIa molecule that is located outside the tissue factor binding site, and/or outside the active site region, and/or outside the ridge of the active site binding cleft. These sites/regions are identified in Example 1 herein. It should be emphasized, however, that in certain situations, e.g. in case an inactivated polypeptide variant is

desired, it may be advantageous to perform modifications in or close to such regions. For example, it is contemplated that one or more in vivo N-glycosylation sites may advantageously be introduced in the active site region or at the ridge of the active site binding cleft of the FVII or FVIIa molecule. The active site region, the tissue factor binding site and the ridge of the active site binding cleft are defined in Example 1 herein and are constituted by the following residues:

[0116] I153, Q167, V168, L169, L170, L171, Q176, L177, C178, G179, G180, T181, V188, V189, S190, A191, A192, H193, C194, F195, D196, K197, I198, W201, V228, I229, I230, P231, S232, T233, Y234, V235, P236, G237, T238, T239, N240, H241, D242, I243, A244, L245, L246, V281, S282, G283, W284, G285, Q286, T293, T324, E325, Y326, M327, F328, D338, S339, C340, K341, G342, D343, S344, G345, G346, P347, H348, L358, T359, G360, 1361, V362, S363, W364, G365, C368, V376, Y377, T378, R379, V380, Q382, Y383, W386, L387, L400 and F405 (active site region);

[0117] L13, K18, F31, E35, R36, L39, F40, I42, S43, S60, K62, D63, Q64, L65, I69, C70, F71, C72, L73, P74, F76, E77, G78, R79, E82, K85, Q88, I90, V92, N93, E94, R271, A274, F275, V276, R277, F278, R304, L305, M306, T307, Q308, D309, Q312, Q313, E325 and R379 (tissue factor binding site); and

[0118] N173, A175, K199, N200, N_2O_3, D289, R290, G291, A292, P321 and T370 (the ridge of the active site binding cleft).

[0119] The total number of amino acid residues to be modified outside the Gla domain in the parent FVII or FVIIa polypeptide (as compared to the amino acid sequence shown in SEQ ID NO:2) will typically not exceed 10. Preferably, the FVII or FVIIa variant comprises an amino acid sequence which differs in 1-10 amino acid residues from amino acid residues 46-406 shown in SEQ ID NO:2, typically in 1-8 or in 2-8 amino acid residues, e.g. in 1-5 or in 2-5 amino acid residues, such as in 1-4 or in 1-3 amino acid residues, e.g. in 1, 2 or 3 amino acid residues from amino acid residues 46-406 shown in SEQ ID NO:2.

[0120] Thus, the polypeptide variant of the invention may contain 1-10 (additional or introduced) in vivo N-glycosylation sites, typically 1-8 or 2-8 (additional or introduced) in vivo N-glycosylation sites, preferably 1-5 or 2-5 (additional or introduced) in vivo N-glycosylation sites, such as 1-4 or 1-3 (additional or introduced) in vivo N-glycosylation sites, e.g. 1, 2 or 3 (additional or introduced) in vivo N-glycosylation sites. Analogously, the polypeptide variant of the invention may contain 1-10 (additional or introduced) sugar moieties, typically 1-8 or 2-8 (additional or introduced) sugar moieties, preferably 1-5 or 2-5 (additional or introduced) sugar moieties, such as 1-4 or 1-3 (additional or introduced) sugar moieties, e.g. 1, 2 or 3 (additional or introduced) sugar moieites. It will be understood that the introduced sugar moiety/moieties will be covalently attached to the introduced in vivo N-glycosylation site(s)

[0121] When used in the present context, the term "naturally occurring glycosylation site" covers the glycosylation sites at postions N145, N322, S52 and S60. In a similar way, the term "naturally occurring in vivo O-glycosylation site" includes the positions S52 and S60, whereas the term "naturally occurring in vivo N-glycosylation site" includes positions N145 and N322.

[0122] It will be understood that in order to prepare a polypeptide variant, wherein the polypeptide variant comprises one or more sugar moieties covalently attached to one or more in vivo N-glycosylation sites, the polypeptide variant must be expressed in a host cell capable of attaching sugar (oligosaccharide) moieties at the glycosylation site(s) or alternatively subjected to in vitro glycosylation. Examples of glycosylating host cells are given in the section further below entitled "Coupling to a sugar moiety".

[0123] Examples of positions, wherein the in vivo N-glycosylation sites may be introduced include, but is not limited to, positions comprising an amino acid residue having an amino acid residue having at least 25% of its side chain exposed to the surface (as defined in Example 1 herein), such as in a position comprising an amino acid residue having at least 50% of its side chain exposed to the surface (as defined in Example 1 herein). In general, it is preferred that the in vivo N-glycosylation site is introduced by substitution, although insertion is also contemplated. The position is preferably selected from a part of the molecule that is located outside the tissue factor binding site and/or the active site region and/or outside the ridge of the active site cleft. These sites/regions are identified in Example 1 herein. It should be understood that when the term "at least 25% (or at least 50%) of its side chain exposed to the surface" is used in connection with introduction of an in vivo N-glycosylation site this term refers to the surface accessibility of the amino acid side chain in the position where the sugar moiety is actually attached. In many cases it will be necessary to introduce a serine or a threonine residue in position +2 relative to the asparagine residue to which the sugar moiety is actually attached (unless, of course, this position is already occupied by a serine or a threonine residue) and these positions, where the serine or threonine residues are introduced, are allowed to be buried, i.e. to have less than 25% of their side chains exposed to the surface.

[0124] Specific and preferred examples of such substitutions creating an in vivo N-glycosylation site include a substitution selected from the group consisting of A51N, G58N, T106N, K109N, G124N, K143N+N145T, A175T, I205S, I205T, V253N, T267N, T267N+S269T, S314N+K316S, S314N+K316T, R315N+V317S, R315N+V317T, K316N+G318S, K316N+G318T, G318N, D334N and combinations thereof. More preferably, the in vivo N-glycosylation site is introduced by a substitution selected from the group consisting of A51N, G58N, T106N, K109N, G124N, K143N+N145T, A175T, I205T, V253N, T267N+S269T, S314N+K316T, R315N+V317T, K316N+G318T, G318N, D334N and combinations thereof. Even more preferably, the in vivo N-glycosylation site is introduced by a substitution selected from the group consisting of T106N, A175T, I205T, V253N, T267N+S269T and combinations thereof, in particular I205T.

[0125] In one embodiment, only one in vivo N-glycosylation site has been introduced by substitution. In another embodiment, two or more (such as two) in vivo N-glycosylation sites have been introduced by substitution. Examples of preferred substitutions creating two in vivo N-glycosylation sites include substitutions selected from the group consisting of A51N+G58N, A51N+T106N, A51N+K109N, A51N+G124N, A51N+K143N+N145T, A51N+A175T, A51N+I205T, A51N+V253N, A51N+T267N+S269T, A51N+S314N+K316T, A51N+R315N+V317T,

A51N+K316N+G318T, A51N+G318N, A51N+D334N, G58N+T106N, G58N+K109N, G58N+G124N, G58N+K143N+N145T, G58N+A175T, G58N+I205T, G58N+V253N, G58N+T267N+S269T, G58N+S314N+K316T, G58N+R315N+V317T, G58N+K316N+G318T, G58N+G318N, G58N+D334N, T106N+K109N, T106N+G124N, T106N+K143N+N145T, T106N+A175T, T106N+I205T, T106N+V253N, T106N+T267N+S269T, T106N+S314N+K316T, T106N+R315N+V317T, T106N+K316N+G318T, T106N+G318N, T106N+D334N, K109N+G124N, K109N+K143N+N145T, K109N+A175T, K109N+I205T, K109N+V253N, K109N+T267N+S269T, K109N+S314N+K316T, K109N+R315N+V317T, K109N+K316N+G318T, K109N+G318N, K109N+D334N, G124N+K143N+N145T, G124N+A175T, G124N+I205T, G124N+V253N, G124N+T267N+S269T, G124N+S314N+K316T, G124N+R315N+V317T, G124N+K316N+G318T, G124N+G318N, G124N+D334N, K143N+N145T+A175T, K143N+N145T+I205T, K143N+N145T+V253N, K143N+N145T+T267N+S269T, K143N+N145T+S314N+K316T, K143N+N145T+R315N+V317T, K143N+N145T+K316N+G318T, K143N+N145T+G318N, K143N+N145T+D334N, A175T+I205T, A175T+V253N, A175T+T267N+S269T, A175T+S314N+K316T, A175T+R315N+V317T, A175T+K316N+G318T, A175T+G318N, A175T+D334N, I205T+V253N, I205T+T267N+S269T, I205T+S314N+K316T, I205T+R315N+V317T, I205T+K316N+G318T, I205T+G318N, I205T+D334N, 253N+T267N+S269T, V253N+S314N+K316T, V253N+R315N+V317T, 253N+K316N+G318T, V253N+G318N, V253N+D334N, T267N+S269T+S314N+K316T, 267N+S269T+R315N+V317T, T267N+S269T+K316N+G318T, T267N+S269T+G318N, T267N+S269T+D334N, S314N+K316T+R315N+V317T, S314N+K316T+G318N, S314N+K316T+D334N, R315N+V317T+K316N+G318T, R315N+V317T+G318N, R315N+V317T+D334N and G318N+D334N. More preferably, the substitutions are selected from the group consisiting of T106N+A175T, T106N+I205T, T106N+V253N, T106N+T267N+S269T, A175T+I205T, A175T+V253N, A175T+T267N+S269T, I205T+V253N, I205T+T267N+S269T and V253N+T267N+S269T, even more preferably from the group consisiting of T106N+I205T, T106N+V253N and I205T+T267N+S269T.

[0126] In an even further embodiment, three or more (such as three) in vivo N-glycosylation sites have been introduced by substitution. Examples of preferred substitutions creating three in vivo N-glycosylation sites include substitutions selected from the group consisiting of I205T+V253N+T267N+S269T and T106N+I205T+V253N.

[0127] As discussed above, it is preferred that the in vivo N-glycosylation site is introduced in a position which does neither form part of the tissue factor binding site nor form part of the active site region and the ridge of the active site binding cleft as defined herein. It is envisaged that such glycosylation variants will primarily belong to the class of active polypeptide variants as defined hereinbefore.

[0128] It will be understood that as an alternative to introduction of in vivo N-glycosylation sites in the above-discussed positions one may introduce (either by substitution or by insertion) cysteine residues in the same positions, where the introduced cysteine residue is then subsequently covalently attached to a non-polypeptide moiety, such as PEG, in particular mPEG. Thus, examples of positions, wherein a cysteine residue may be introduced include, but is not limited to, positions comprising an amino acid residue having an amino acid residue having at least 25% of its side chain exposed to the surface (as defined in Example 1 herein), such as in a position comprising an amino acid residue having at least 50% of its side chain exposed to the surface (as defined in Example 1 herein). The position is preferably selected from a part of the molecule that is located outside the tissue factor binding site and/or the active site region and/or outside the ridge of the active site cleft. These sites/regions are identified in Example 1 herein. Thus, the above disclosure concerning introduction of in vivo N-glycosylation sites applies mutatis mutandis to introduction of cysteine residues.

[0129] It will be understood that the modifications in positions located outside the Gla domain discussed in the above section should be combined with one or more modifications in the Gla domain (see the section entitled "Modifications in the Gla domain" below).

Modifications in the Gla Domain

[0130] As will be understood the variants of the present invention comprises, in addition to at least one introduced in vivo N-glycosylation site located outside the Gla domain (cf. above), at least two substitutions in the Gla domain, namely a substitution in position 10 and position 32.

[0131] A number of suitable modifications leading to increased phospholipid membrane binding affinity is disclosed in WO 99/20767 and WO 00/66753.

[0132] In a preferred embodiment of the invention the substitution in position 10 is P10Q. In another preferred embodiment of the invention the substitution in position 32 is K32E. In a particularly preferred embodiment of the invention the variant comprises the following substitutions P10Q+K32E.

[0133] In an interesting embodiment of the invention the variant comprises, in addition to the substitutions in position 10 and 32, such as in addition to the substitutions P10Q+K32E, at least one further modification in the Gla domain.

[0134] In one preferred embodiment of the invention the further modification in the Gla domain comprises an amino acid substitution in position 33. Preferably, a hydrophobic amino acid residue is introduced by substitution in position 33, such as D33I, D33L, D33M, D33V, D33F, D33Y or D33W, in particular D33F. Accordingly, in one very interesting embodiment of the invention, the variant comprises the following substitutions P10Q+K32+D33F.

[0135] In another preferred embodiment of the invention the further modification in the Gla domain comprises an insertion of at least one (such as one) amino acid residue between position 3 and 4. It is preferred that the inserted amino acid residue is a hydrophobic amino acid residue. Most preferably the insertion is A3AY. Accordingly, in another very interesting embodiment of the invention, the variant comprises the following modifications A3AY+P10Q+K32E or A3AY+P10Q+K32E+D33F.

[0136] In still another preferred embodiment of the invention the further modification in the Gla domain comprises a substitution in position 34. It is preferred that a negatively charged amino acid residue is introduced by substitution in position 34. Most preferably the substitution is A34E. Accordingly, in still another very interesting embodiment of

the invention, the variant comprises the following modifications P10Q+K32E+A34E, P10Q+K32E+D33F+A34E, A3AY+P10Q+K32E+A34E or A3AY+P10Q+K32E+D33F+A34E.

[0137] The Gla domain may also contain modifications in other positions, in particular in positions 8, 11 and 28, such as R28F or R28E. On the other hand it should be understood that the Gla domain should not be modified to such an extent that the membrane binding properties are impaired. Accordingly, it is preferred that no modifications are made in the residues that become γ-carboxylated, i.e. it is preferred that no modifications are made in residues 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35. In a similar way, it is in general not preferred that non-polypeptide moieties, such as sugar moieties and/or PEG groups, are introduced in the Gla domain. Consequently, it is preferred that no modifications are made in the Gla domain that creates an in vivo N-glycosylation site.

[0138] Finally, it will be understood that the modifications in the Gla domain discussed in this section must be combined with one or more of the modifications disclosed in the section entitled "Introduction of in vivo N-glycosylation sites located outside the Gla domain" above.

[0139] Specific examples of such "combined" variants are given below.

[0140] In one embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+T106N.

[0141] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A175T.

[0142] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+I205T. In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+V253N.

[0143] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+T267+S269T.

[0144] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+T1106N+I205T.

[0145] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+T106N+V253N.

[0146] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+I205T+T267N+S269T.

[0147] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+T106N.

[0148] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A175T.

[0149] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+I205T.

[0150] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+V253N.

[0151] In a further preferred embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+T267+S269T.

[0152] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+T106N+I205T.

[0153] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+T106N+V253N.

[0154] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+I205T+T267N+S269T.

[0155] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+T106N.

[0156] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A175T.

[0157] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+I205T.

[0158] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+V253N.

[0159] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+T267+S269T.

[0160] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+T106N+I205T.

[0161] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+T106N+V253N.

[0162] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+I205T+T267N+S269T.

[0163] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+T106N.

[0164] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A175T.

[0165] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+T205T.

[0166] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+V253N.

[0167] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+T267+S269T.

[0168] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+T106N+I205T.

[0169] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+T106N+V253N.

[0170] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+I205T+T267N+S269T.

[0171] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+T106N.

[0172] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+A175T.

[0173] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+I205T.

[0174] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+V253N.

[0175] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+T267+S269T.

[0176] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+T106N+I205T.

[0177] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+T106N+V253N.

[0178] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+A34E+I205T+T267N+S269T.

[0179] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E+T106N.

[0180] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E A175T.

[0181] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E I205T.

[0182] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E V253N.

[0183] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E T267+S269T.

[0184] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E T106N+I205T.

[0185] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E T106N+V253N.

[0186] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: P10Q+K32E+D33F+A34E I205T+T267N+S269T.

[0187] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E+T106N.

[0188] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E A175T.

[0189] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E I205T.

[0190] In a farther embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E V253N.

[0191] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E T267+S269T.

[0192] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E T106N+I205T.

[0193] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E T106N+V253N.

[0194] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+A34E I205T+T267N+S269T.

[0195] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E+T106N.

[0196] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E A175T.

[0197] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E I205T.

[0198] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E V253N.

[0199] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+IK32E+D33F+A34E T267+S269T.

[0200] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E T106N+I205T.

[0201] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E T106N+V253N.

[0202] In a further embodiment of the invention said FVII or FVIIa variant comprises the following modifications: A3AY+P10Q+K32E+D33F+A34E I205T+T267N+S269T.

Other Modifications Outside the Gla Domain

[0203] In a further embodiment of the present invention, the FVII or FVIIa variant may, in addition to the modifications described in the sections above, also contain mutations,

which are already known to increase the intrinsic activity of the polypeptide, e.g. such as those described in WO 02/22776

[0204] Examples of preferred substitutions include substitutions selected from the group consisting of V158D, E296D, M298Q, L305V and K337A. More preferably, said substitutions are selected from the group consisting of V158D+E296D+M298Q+L305V+K337A, V158D+E296D+M298Q+K337A, V158D+E296D+M298Q+L305V, V158D+E296D+M298Q, M298Q, L305V+K337A, L305V and K337A.

[0205] In a further embodiment of the present invention, the FVII or FVIIa variant may, in addition to the modifications described in the sections above, also contain mutations, which are already known to cause a decreased inhibition by TFPI. One example includes the substitution K341Q disclosed by Neuenschwander et al, Biochemistry, 1995; 34:8701-8707.

[0206] Moreover, the variant may contain modifications which are belived to increase the TF binding affinity. Examples of such modifications include substitutions selected from the group consisting of L39E, L39Q, L39H, I42K, I42R, S43H, S43Q, K62E, K62R, L65Q, L65S, F71D, F71Y, F71E, F71Q, F71N, E82Q, E82N, E82K and F275H

[0207] As already indicated above, the variant may also contain conservative amino acid substitutions.

The Non-Polypeptide Moiety

[0208] Based on the present disclosure the skilled person will be aware that amino acid residues comprising other attachment groups may be introduced by substitution into the parent polypeptide, using the same approach as that illustrated above with in vivo N-glycosylation sites. For instance, one or more amino acid residues comprising an acid group (glutamnic acid or aspartic acid), tyrosine or lysine may be introduced into the positions discussed above. In particular, one or more cysteine residues may be introduced in the positions discussed above.

[0209] As indicated further above the non-polypeptide moiety of the conjugated variant is preferably selected from the group consisting of a polymer molecule, a lipophilic compound, a sugar moiety (by way of in vivo glycosylation) and an organic derivatizing agent. All of these agents may confer desirable properties to the variant polypeptide, in particular increased AUC_{iv}, increased functional in vivo half-life and/or increased plasma half-life. The variant polypeptide is normally conjugated to only one type of non-polypeptide moiety, but may also be conjugated to two or more different types of non-polypeptide moieties, e.g. to a polymer molecule and a sugar moiety, to a lipophilic group and a sugar moiety, to an organic derivatizing agent and a sugar moiety, to a lipophilic group and a polymer molecule, etc. The conjugation to two or more different non-polypeptide moieties may be done simultaneous or sequentially.

Methods of Preparing a Conjugated Variant of the Invention

[0210] In the following sections "Conjugation to a lipophilic compound", "Conjugation to a polymer molecule", "Conjugation to a sugar moiety" and "Conjugation to an organic derivatizing agent" conjugation to specific types of non-polypeptide moieties is described. In general, a conjugated variant according to the invention may be produced by culturing an appropriate host cell under conditions conducive for the expression of the variant polypeptide, and recovering the variant polypeptide, wherein a) the variant polypeptide comprises at least one N- or O-glycosylation site and the host cell is an eukaryotic host cell capable of in vivo glycosylation, and/or b) the variant polypeptide is subjected to conjugation to a non-polypeptide moiety in vitro.

[0211] It will be understood that the conjugation should be designed so as to produce the optimal molecule with respect to the number of non-polypeptide moieties attached, the size and form of such molecules (e.g. whether they are linear or branched), and the attachment site(s) in the polypeptide. The molecular weight of the non-polypeptide moiety to be used may, e.g., be chosen on the basis of the desired effect to be achieved. For instance, if the primary purpose of the conjugation is to achieve a conjugated variant having a high molecular weight (e.g. to reduce renal clearance) it is usually desirable to conjugate as few high molecular weight non-polypeptide moieties as possible to obtain the desired molecular weight. When a high degree of shielding is desirable this may be obtained by use of a sufficiently high number of low molecular weight non-polypeptide moieties (e.g. with a molecular weight of from about 300 Da to about 5 kDa, such as a molecular weight of from 300 Da to 2 kDa).

Conjugation to a Polymer Molecule

[0212] The polymer molecule to be coupled to the variant polypeptide may be any suitable polymer molecule, such as a natural or synthetic homo-polymer or hetero-polymer, typically with a molecular weight in the range of about 300-100,000 Da, such as about 500-20,000 Da, more preferably in the range of about 500-15,000 Da, even more preferably in the range of about 2-12 kDa, such as in the range of about 3-10 kDa. When the term "about" is used herein in connection with a certain molecular weight, the word "about" indicates an approximate average molecular weight and reflects the fact that there will normally be a certain molecular weight distribution in a given polymer preparation.

[0213] Examples of homo-polymers include a polyol (i.e. poly-OH), a polyamine (i.e. poly-NH_2) and a polycarboxylic acid (i.e. poly-COOH). A hetero-polymer is a polymer comprising different coupling groups, such as a hydroxyl group and an amine group.

[0214] Examples of suitable polymer molecules include polymer molecules selected from the group consisting of polyalkylene oxide (PAO), including polyalkylene glycol (PAG), such as polyethylene glycol (PEG) and polypropylene glycol (PPG), branched PEGs, poly-vinyl alcohol (PVA), poly-carboxylate, poly-(vinylpyrolidone), polyethylene-co-maleic acid anhydride, polystyrene-co-maleic acid anhydride, dextran, including carboxymethyl-dextran, or any other biopolymer suitable for reducing immunogenicity and/or increasing functional in vivo half-life and/or serum half-life. Another example of a polymer molecule is human albumin or another abundant plasma protein. Generally, polyalkylene glycol-derived polymers are biocompatible, non-toxic, non-antigenic, non-immunogenic, have various water solubility properties, and are easily excreted from living organisms.

[0215] PEG is the preferred polymer molecule, since it has only few reactive groups capable of cross-linking compared

to, e.g., polysaccharides such as dextran. In particular, mono-functional PEG, e.g. methoxypolyethylene glycol (mPEG), is of interest since its coupling chemistry is relatively simple (only one reactive group is available for conjugating with attachment groups on the polypeptide). Consequently, as the risk of cross-linking is eliminated, the resulting conjugated variants are more homogeneous and the reaction of the polymer molecules with the variant polypeptide is easier to control.

[0216] To effect covalent attachment of the polymer molecule(s) to the variant polypeptide, the hydroxyl end groups of the polymer molecule must be provided in activated form, i.e. with reactive functional groups (examples of which include primary amino groups, hydrazide (HZ), thiol, succinate (SUC), succinimidyl succinate (SS), succinimidyl succinamide (SSA), succinimidyl propionate (SPA), succinimidyl butyrate (SBA), succinimidy carboxymethylate (SCM), benzotriazole carbonate (BTC), N-hydroxysuccinimide (NHS), aldehyde, nitrophenylcarbonate (NPC), and tresylate (TRES)). Suitable activated polymer molecules are commercially available, e.g. from Shearwater Polymers, Inc., Huntsville, Ala., USA, or from PolyMASC Pharmaceuticals plc, UK.

[0217] Alternatively, the polymer molecules can be activated by conventional methods known in the art, e.g. as disclosed in WO 90/13540. Specific examples of activated linear or branched polymer molecules for use in the present invention are described in the Shearwater Polymers, Inc. 1997 and 2000 Catalogs (Functionalized Biocompatible Polymers for Research and pharmaceuticals, Polyethylene Glycol and Derivatives, incorporated herein by reference).

[0218] Specific examples of activated PEG polymers include the following linear PEGs: NHS-PEG (e.g. SPA-PEG, SSPA-PEG, SBA-PEG, SS-PEG, SSA-PEG, SC-PEG, SG-PEG, and SCM-PEG), and NOR-PEG, BTC-PEG, EPOX-PEG, NCO-PEG, NPC-PEG, CDI-PEG, ALD-PEG, TRES-PEG, VS-PEG, IODO-PEG, and MAL-PEG, and branched PEGs such as PEG2-NHS and those disclosed in U.S. Pat. No. 5,932,462 and U.S. Pat. No. 5,643,575, both of which are incorporated herein by reference. Furthermore, the following publications, incorporated herein by reference, disclose useful polymer molecules and/or PEGylation chemistries: U.S. Pat. No. 5,824,778, U.S. Pat. No. 5,476,653, WO 97/32607, EP 229,108, EP 402,378, U.S. Pat. No. 4,902,502, U.S. Pat. No. 5,281,698, U.S. Pat. No. 5,122,614, U.S. Pat. No. 5,219,564, WO 92/16555, WO 94/04193, WO 94/14758, WO 94/17039, WO 94/18247, WO 94/28024, WO 95/00162, WO 95/11924, WO95/13090, WO 95/33490, WO 96/00080, WO 97/18832, WO 98/41562, WO 98/48837, WO 99/32134, WO 99/32139, WO 99/32140, WO 96/40791, WO 98/32466, WO 95/06058, EP 439 508, WO 97/03106, WO 96/21469, WO 95/13312, EP 921 131, U.S. Pat. No. 5,736,625, WO 98/05363, EP 809 996, U.S. Pat. No. 5,629,384, WO 96/41813, WO 96/07670, U.S. Pat. No. 5,473,034, U.S. Pat. No. 5,516,673, EP 605 963, U.S. Pat. No. 5,382,657, EP 510 356, EP 400 472, EP 183 503 and EP 154 316.

[0219] Specific examples of activated PEG polymers particularly preferred for coupling to cysteine residues, include the following linear PEGs: vinylsulfone-PEG (VS-PEG), preferably vinylsulfone-mPEG (VS-mPEG); maleimide-PEG (MAL-PEG), preferably maleimide-mPEG (MAL-mPEG) and orthopyridyl-disulfide-PEG (OPSS-PEG), preferably orthopyridyl-disulfide-mPEG (OPSS-mPEG). Typically, such PEG or mPEG polymers will have a size of about 5 kDa, about 10 kD, about 12 kDa or about 20 kDa.

[0220] The conjugation of the polypeptide variant and the activated polymer molecules is conducted by use of any conventional method, e.g. as described in the following references (which also describe suitable methods for activation of polymer molecules): Harris and Zalipsky, eds., Poly(ethylene glycol) Chemistry and Biological Applications, AZC, Washington; R. F. Taylor, (1991), "Protein immobilisation. Fundamental and applications", Marcel Dekker, N.Y.; S. S. Wong, (1992), "Chemistry of Protein Conjugation and Crosslinking", CRC Press, Boca Raton; G. T. Hermanson et al., (1993), "Immobilized Affinity Ligand Techniques", Academic Press, N.Y.).

[0221] The skilled person will be aware that the activation method and/or conjugation chemistry to be used depends on the attachment group(s) of the variant polypeptide (examples of which are given further above), as well as the functional groups of the polymer (e.g. being amine, hydroxyl, carboxyl, aldehyde, sulfydryl, succinimidyl, maleimide, vinysulfone or haloacetate). The PEGylation may be directed towards conjugation to all available attachment groups on the variant polypeptide (i.e. such attachment groups that are exposed at the surface of the polypeptide) or may be directed towards one or more specific attachment groups, e.g. the N-terminal amino group as described in U.S. Pat. No. 5,985,265 or to cysteine residues. Furthermore, the conjugation may be achieved in one step or in a stepwise manner (e.g. as described in WO 99/55377).

[0222] For PEGylation to cysteine residues (see above) the FVII or FVIIa variant is usually treated with a reducing agent, such as dithiothreitol (DDT) prior to PEGylation. The reducing agent is subsequently removed by any conventional method, such as by desalting. Conjugation of PEG to a cysteine residue typically takes place in a suitable buffer at pH 6-9 at temperatures varying from 4° C. to 25° C. for periods up to 16 hours.

[0223] It will be understood that the PEGylation is designed so as to produce the optimal molecule with respect to the number of PEG molecules attached, the size and form of such molecules (e.g. whether they are linear or branched), and the attachment site(s) in the variant polypeptide. The molecular weight of the polymer to be used may e.g. be chosen on the basis of the desired effect to be achieved.

[0224] In connection with conjugation to only a single attachment group on the protein (e.g. the N-terminal amino group), it may be advantageous that the polymer molecule, which may be linear or branched, has a high molecular weight, preferably about 10-25 kDa, such as about 15-25 kDa, e.g. about 20 kDa.

[0225] Normally, the polymer conjugation is performed under conditions aimed at reacting as many of the available polymer attachment groups with polymer molecules. This is achieved by means of a suitable molar excess of the polymer relative to the polypeptide. Typically, the molar ratios of activated polymer molecules to polypeptide are up to about 1000-1, such as up to about 200-1, or up to about 100-1. In some cases the ration may be somewhat lower, however, such as up to about 50-1, 10-1, 5-1, 2-1 or 1-1 in order to obtain optimal reaction.

[0226] It is also contemplated according to the invention to couple the polymer molecules to the polypeptide through a linker. Suitable linkers are well known to the skilled person. A preferred example is cyanuric chloride (Abuchowski et al., (1977), J. Biol. Chem., 252, 3578-3581; U.S. Pat. No. 4,179,337; Shafer et al., (1986), J. Polym. Sci. Polym. Chem. Ed., 24, 375-378).

[0227] Subsequent to the conjugation, residual activated polymer molecules are blocked according to methods known in the art, e.g. by addition of primary amine to the reaction mixture, and the resulting inactivated polymer molecules are removed by a suitable method.

[0228] It will be understood that depending on the circumstances, e.g. the amino acid sequence of the variant polypeptide, the nature of the activated PEG compound being used and the specific PEGylation conditions, including the molar ratio of PEG to polypeptide, varying degrees of PEGylation may be obtained, with a higher degree of PEGylation generally being obtained with a higher ratio of PEG to variant polypeptide. The PEGylated variant polypeptides resulting from any given PEGylation process will, however, normally comprise a stochastic distribution of conjugated polypeptide variants having slightly different degrees of PEGylation.

Coupling to a Sugar Moiety

[0229] In order to achieve in vivo glycosylation of a FVII molecule comprising one or more glycosylation sites the nucleotide sequence encoding the variant polypeptide must be inserted in a glycosylating, eucaryotic expression host. The expression host cell may be selected from fungal (filamentous fungal or yeast), insect or animal cells or from transgenic plant cells. In one embodiment the host cell is a mammalian cell, such as a CHO cell, BHK or HEK, e.g. HEK 293, cell, or an insect cell, such as an SF9 cell, or a yeast cell, e.g. *S. cerevisiae* or *Pichia pastoris*, or any of the host cells mentioned hereinafter.

[0230] Covalent in vitro coupling of sugar moieties (such as dextran) to amino acid residues of the variant polypeptide may also be used, e.g. as described, for example in WO 87/05330 and in Aplin etl al., CRC Crit Rev. Biochem, pp. 259-306,1981. The in vitro coupling of sugar moieties or PEG to protein- and peptide-bound Gln-residues can be carried out by transglutaminases (TGases). Transglutaminases catalyse the transfer of donor amine-groups to protein- and peptide-bound Gln-residues in a so-called cross-linking reaction. The donor-amine groups can be protein- or peptide-bound, such as the s-amino-group in Lys-residues or it can be part of a small or large organic molecule. An example of a small organic molecule functioning as amino-donor in TGase-catalysed cross-linking is putrescine (1,4-diaminobutane). An example of a larger organic molecule functioning as amino-donor in TGase-catalysed cross-linking is an amine-containing PEG (Sato et al., 1996, Biochemistry 35, 13072-13080).

[0231] TGases, in general, are highly specific enzymes, and not every Gln-residues exposed on the surface of a protein is accessible to TGase-catalysed cross-linking to amino-containing substances. On the contrary, only few Gln-residues are naturally functioning as TGase substrates but the exact parameters governing which Gln-residues are good TGase substrates remain unknown. Thus, in order to render a protein susceptible to TGase-catalysed cross-linking reactions it is often a prerequisite at convenient positions to add stretches of amino acid sequence known to function very well as TGase substrates. Several amino acid sequences are known to be or to contain excellent natural TGase substrates e.g. substance P, elafin, fibrinogen, fibronectin, α_2-plasmin inhibitor, α-caseins, and β-caseins.

Conjugation to an Organic Derivatizing Agent

[0232] Covalent modification of the variant polypeptide may be performed by reacting one or more attachment groups of the variant polypeptide with an organic derivatizing agent. Suitable derivatizing agents and methods are well known in the art. For example, cysteinyl residues most commonly are reacted with α-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, α-bromo-β-(4-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole. Histidyl residues are derivatized by reaction with diethylpyrocarbonateat pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful. The reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0. Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing α-amino-containing residues include imidoesters such as methyl picolinimidate, pyridoxal phosphate, pyridoxal, chloroborohydride, trinitrobenzenesulfonic acid, O-methylisourea, 2,4-pentanedione and transaminase-catalyzed reaction with glyoxylate. Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pKa of the guanidine functional group.

[0233] Furthermore, these reagents may react with the groups of lysine as well as the arginine guanidino group. Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R—N═C═N—R'), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Conjugation to a Lipophilic Compound

[0234] The variant polypeptide and the lipophilic compound may be conjugated to each other, either directly or by use of a linker. The lipophilic compound may be a natural compound such as a saturated or unsaturated fatty acid, a fatty acid diketone, a terpene, a prostaglandin, a vitamine, a carotenoide or steroide, or a synthetic compound such as a carbon acid, an alcohol, an amine and sulphonic acid with one or more alkyl-, aryl-, alkenyl- or other multiple unsaturated compounds. The conjugation between the variant polypeptide and the lipophilic compound, optionally

through a linker may be done according to methods known in the art, e.g. as described by Bodanszky in Peptide Synthesis, John Wiley, New York, 1976 and in WO 96/12505.

Methods of Preparing a Polypeptide Variant of the Invention

[0235] The polypeptide variant of the present invention may be produced by any suitable method known in the art. Such methods include constructing a nucleotide sequence encoding the polypeptide variant and expressing the sequence in a suitable transformed or transfected host. Preferably, the host cell is a gammacarboxylating host cell such as a mammalian cell. However, polypeptide variants of the invention may be produced, albeit less efficiently, by chemical synthesis or a combination of chemical synthesis or a combination of chemical synthesis and recombinant DNA technology.

[0236] A nucleotide sequence encoding a polypeptide of the invention may be constructed by isolating or synthesizing a nucleotide sequence encoding the parent FVII, such as hFVII with the amino acid sequence shown in SEQ ID NO:2 and then changing the nucleotide sequence so as to effect introduction (i.e. insertion or substitution) or removal (i.e. deletion or substitution) of the relevant amino acid residue(s).

[0237] The nucleotide sequence is conveniently modified by site-directed mutagenesis in accordance with conventional methods. Alternatively, the nucleotide sequence is prepared by chemical synthesis, e.g. by using an oligonucleotide synthesizer, wherein oligonucleotides are designed based on the amino acid sequence of the desired polypeptide, and preferably selecting those codons that are favored in the host cell in which the recombinant polypeptide will be produced. For example, several small oligonucleotides coding for portions of the desired polypeptide may be synthesized and assembled by PCR, ligation or ligation chain reaction (LCR) (Barany, PNAS 88:189-193, 1991). The individual oligonucleotides typically contain 5' or 3' overhangs for complementary assembly.

[0238] Alternative nucleotide sequence modification methods are available for producing polypeptide variants for high throughput screening, for instance methods which involve homologous cross-over such as disclosed in U.S. Pat. No. 5,093,257, and methods which involve gene shuffling, i.e. recombination between two or more homologous nucleotide sequences resulting in new nucleotide sequences having a number of nucleotide alterations when compared to the starting nucleotide sequences. Gene shuffling (also known as DNA shuffling) involves one or more cycles of random fragmentation and reassembly of the nucleotide sequences, followed by screening to select nucleotide sequences encoding polypeptides with desired properties. In order for homology-based nucleic acid shuffling to take place, the relevant parts of the nucleotide sequences are preferably at least 50% identical, such as at least 60% identical, more preferably at least 70% identical, such as at least 80% identical. The recombination can be performed in vitro or in vivo.

[0239] Examples of suitable in vitro gene shuffling methods are disclosed by Stemmer et al. (1994), Proc. Natl. Acad. Sci. USA; vol. 91, pp. 10747-10751; Stemmer (1994), Nature, vol. 370, pp. 389-391; Smith (1994), Nature vol. 370, pp. 324-325; Zhao et al., Nat. Biotechnol. 1998, March; 16(3): 258-61; Zhao H. and Arnold, F B, Nucleic Acids Research, 1997, Vol. 25. No. 6 pp. 1307-1308; Shao et al., Nucleic Acids Research 1998, January 15; 26(2): pp. 681-83; and WO 95/17413.

[0240] An example of a suitable in vivo shuffling method is disclosed in WO 97/07205. Other techniques for mutagenesis of nucleic acid sequences by in vitro or in vivo recombination are disclosed e.g. in WO 97/20078 and U.S. Pat. No. 5,837,458. Examples of specific shuffling techniques include "family shuffling", "synthetic shuffling" and "in silico shuffling".

[0241] Family shuffling involves subjecting a family of homologous genes from different species to one or more cycles of shuffling and subsequent screening or selection. Family shuffling techniques are disclosed e.g. by Crameri et al. (1998), Nature, vol. 391, pp. 288-291; Christians et al. (1999), Nature Biotechnology, vol. 17, pp. 259-264; Chang et al. (1999), Nature Biotechnology, vol. 17, pp. 793-797; and Ness et al. (1999), Nature Biotechnology, vol. 17, 893-896.

[0242] Synthetic shuffling involves providing libraries of overlapping synthetic oligonucleotides based e.g. on a sequence alignment of homologous genes of interest. The synthetically generated oligonucleotides are recombined, and the resulting recombinant nucleic acid sequences are screened and if desired used for further shuffling cycles. Synthetic shuffling techniques are disclosed in WO 00/42561.

[0243] In silico shuffling refers to a DNA shuffling procedure, which is performed or modelled using a computer system, thereby partly or entirely avoiding the need for physically manipulating nucleic acids. Techniques for in silico shuffling are disclosed in WO 00/42560.

[0244] Once assembled (by synthesis, site-directed mutagenesis or another method), the nucleotide sequence encoding the polypeptide is inserted into a recombinant vector and operably linked to control sequences necessary for expression of the FVII in the desired transformed host cell.

[0245] It should of course be understood that not all vectors and expression control sequences function equally well to express the nucleotide sequence encoding the polypeptide variants described herein. Neither will all hosts function equally well with the same expression system. However, one of skill in the art may make a selection among these vectors, expression control sequences and hosts without undue experimentation. For example, in selecting a vector, the host must be considered because the vector must replicate in it or be able to integrate into the chromosome. The vector's copy number, the ability to control that copy number, and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered. In selecting an expression control sequence, a variety of factors should also be considered. These include, for example, the relative strength of the sequence, its controllability, and its compatibility with the nucleotide sequence encoding the polypeptide, particularly as regards potential secondary structures. Hosts should be selected by consideration of their compatibility with the chosen vector, the toxicity of the product coded for by the nucleotide

sequence, their secretion characteristics, their ability to fold the polypeptide variant correctly, their fermentation or culture requirements, and the ease of purification of the products coded for by the nucleotide sequence.

[0246] The recombinant vector may be an autonomously replicating vector, i.e. a vector, which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector is one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

[0247] The vector is preferably an expression vector, in which the nucleotide sequence encoding the polypeptide variant of the invention is operably linked to additional segments required for transcription of the nucleotide sequence. The vector is typically derived from plasmid or viral DNA. A number of suitable expression vectors for expression in the host cells mentioned herein are commercially available or described in the literature. Useful expression vectors for eukaryotic hosts, include, for example, vectors comprising expression control sequences from SV40, bovine papilloma virus, adenovirus and cytomegalovirus. Specific vectors are, e.g., pcDNA3.1(+)\Hyg (Invitrogen, Carlsbad, Calif., USA) and pCI-neo (Stratagene, La Jola, Calif., USA). Useful expression vectors for yeast cells include the 2μ plasmid and derivatives thereof, the POT1 vector (U.S. Pat. No. 4,931,373), the pJSO37 vector described in Okkels, Ann. New York Acad. Sci. 782, 202-207, 1996, and pPICZ A, B or C (Invitrogen). Useful vectors for insect cells include pVL941, pBG311 (Cate et al., "Isolation of the Bovine and Human Genes for Mullerian Inhibiting Substance And Expression of the Human Gene In Animal Cells", Cell, 45, pp. 685-98 (1986), pBluebac 4.5 and pMelbac (both available from Invitrogen). Useful expression vectors for bacterial hosts include known bacterial plasmids, such as plasmids from *E. coli*, including pBR322, pET3a and pET12a (both from Novagen Inc., WI, USA), wider host range plasmids, such as RP4, phage DNAs, e.g., the numerous derivatives of phage lambda, e.g., NM989, and other DNA phages, such as M13 and filamentous single stranded DNA phages.

[0248] Other vectors for use in this invention include those that allow the nucleotide sequence encoding the polypeptide variant to be amplified in copy number. Such amplifiable vectors are well known in the art. They include, for example, vectors able to be amplified by DHFR amplification (see, e.g., Kaufman, U.S. Pat. No. 4,470,461, Kaufman and Sharp, "Construction Of A Modular Dihydrafolate Reductase cDNA Gene: Analysis Of Signals Utilized For Efficient Expression", Mol. Cell. Biol., 2, pp. 1304-19 (1982)) and glutamine synthetase ("GS") amplification (see, e.g., U.S. Pat. No. 5,122,464 and EP 338,841).

[0249] The recombinant vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. An example of such a sequence (when the host cell is a mammalian cell) is the SV40 origin of replication. When the host cell is a yeast cell, suitable sequences enabling the vector to replicate are the yeast plasmid 2μ replication genes REP 1-3 and origin of replication.

[0250] The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or the *Schizosaccharomyces pombe* TPI gene (described by P. R. Russell, Gene 40, 1985, pp. 125-130), or one which confers resistance to a drug, e.g. ampicillin, kanamycin, tetracyclin, chloramphenicol, neomycin, hygromycin or methotrexate. For *Saccharomyces cerevisiae*, selectable markers include ura3 and leu2. For filamentous fungi, selectable markers include amdS, pyrG, arcB, niaD and sC.

[0251] The term "control sequences" is defined herein to include all components, which are necessary or advantageous for the expression of the polypeptide variant of the invention. Each control sequence may be native or foreign to the nucleic acid sequence encoding the polypeptide variant. Such control sequences include, but are not limited to, a leader sequence, polyadenylation sequence, propeptide sequence, promoter, enhancer or upstream activating sequence, signal peptide sequence, and transcription terminator. At a minimum, the control sequences include a promoter.

[0252] A wide variety of expression control sequences may be used in the present invention. Such useful expression control sequences include the expression control sequences associated with structural genes of the foregoing expression vectors as well as any sequence known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof.

[0253] Examples of suitable control sequences for directing transcription in mammalian cells include the early and late promoters of SV40 and adenovirus, e.g. the adenovirus 2 major late promoter, the MT-1 (metallothionein gene) promoter, the human cytomegalovirus immediate-early gene promoter (CMV), the human elongation factor 1α (EF-1α) promoter, the *Drosophila* minimal heat shock protein 70 promoter, the Rous Sarcoma Virus (RSV) promoter, the human ubiquitin C (UbC) promoter, the human growth hormone terminator, SV40 or adenovirus E1b region polyadenylation signals and the Kozak consensus sequence (Kozak, M. *J Mol Biol* 1987 August 20;196(4):947-50).

[0254] In order to improve expression in mammalian cells a synthetic intron may be inserted in the 5' untranslated region of the nucleotide sequence encoding the polypeptide. An example of a synthetic intron is the synthetic intron from the plasmid pCI-Neo (available from Promega Corporation, Wisconsin, USA).

[0255] Examples of suitable control sequences for directing transcription in insect cells include the polyhedrin promoter, the P10 promoter, the *Autographa californica* polyhedrosis virus basic protein promoter, the baculovirus immediate early gene 1 promoter and the baculovirus 39K delayed-early gene promoter, and the SV40 polyadenylation sequence. Examples of suitable control sequences for use in yeast host cells include the promoters of the yeast α-mating system, the yeast triose phosphate isomerase (TPI) promoter, promoters from yeast glycolytic genes or alcohol dehydrogenase genes, the ADH2-4c promoter, and the inducible GAL promoter. Examples of suitable control sequences for use in filamentous fungal host cells include the ADH3 promoter and terminator, a promoter derived from the genes encoding *Aspergillus oryzae* TAKA amylase triose phosphate isomerase or alkaline protease, an *A. niger* α-amylase, *A. niger* or *A. nidulans* glucoamylase, *A. nidu-*

lans acetamidase, *Rhizomucor miehei* aspartic proteinase or lipase, the TPI1 terminator and the ADH3 terminator. Examples of suitable control sequences for use in bacterial host cells include promoters of the lac system, the trp system, the TAC or TRC system, and the major promoter regions of phage lambda.

[0256] The presence or absence of a signal peptide will, e.g., depend on the expression host cell used for the production of the polypeptide variant to be expressed (whether it is an intracellular or extracellular polypeptide) and whether it is desirable to obtain secretion. For use in filamentous fungi, the signal peptide may conveniently be derived from a gene encoding an *Aspergillus* sp. amylase or glucoamylase, a gene encoding a *Rhizomucor miehei* lipase or protease or a *Humicola lanuginosa* lipase. The signal peptide is preferably derived from a gene encoding *A. oryzae* TAKA amylase, *A. niger* neutral α-amylase, *A. niger* acid-stable amylase, or *A. niger* glucoamylase. For use in insect cells, the signal peptide may conveniently be derived from an insect gene (cf. WO 90/05783), such as the Lepidopteran *manduca sexta* adipokinetic hormone precursor, (cf. U.S. Pat. No. 5,023,328), the honeybee melittin (Invitrogen), ecdysteroid UDPglucosyltransferase (egt) (Murphy et al., Protein Expression and Purification 4, 349-357 (1993) or human pancreatic lipase (hpl) (Methods in Enzymology 284, pp. 262-272, 1997). A preferred signal peptide for use in mammalian cells is that of hFVII or the murine Ig kappa light chain signal peptide (Coloma, M (1992) J. 1 mm. Methods 152:89-104). For use in yeast cells suitable signal peptides have been found to be the α-factor signal peptide from *S. cereviciae* (cf. U.S. Pat. No. 4,870,008), a modified carboxypeptidase signal peptide (cf. L. A. Valls et al., Cell 48, 1987, pp. 887-897), the yeast BAR1 signal peptide (cf. WO 87/02670), the yeast aspartic protease 3 (YAP3) signal peptide (cf. M. Egel-Mitani et al., Yeast 6, 1990, pp. 127-137), and the synthetic leader sequence TA57 (WO98/32867). For use in *E. coli* cells a suitable signal peptide have been found to be the signal peptide ompA (EP581821).

[0257] The nucleotide sequence of the invention encoding a polypeptide variant, whether prepared by site-directed mutagenesis, synthesis, PCR or other methods, may optionally include a nucleotide sequence that encode a signal peptide. The signal peptide is present when the polypeptide variant is to be secreted from the cells in which it is expressed. Such signal peptide, if present, should be one recognized by the cell chosen for expression of the polypeptide variant. The signal peptide may, e.g. be that normally associated with hFVII) or, alternatively, the signal peptide may be from another source than hFVII, such as any of those normally associated with other human wild-type vitamin K-dependent polypeptides. Furthermore, the signal peptide may be a signal peptide normally expressed from the host cell or one which is not normally expressed from the host cell. Accordingly, the signal peptide may be prokaryotic, e.g. derived from a bacterium such as *E. coli*, or eukaryotic, e.g. derived from a mammalian, or insect or yeast cell.

[0258] Any suitable host may be used to produce the polypeptide variant, including bacteria (although not particularly preferred), fungi (including yeasts), plant, insect, mammal, or other appropriate animal cells or cell lines, as well as transgenic animals or plants. Examples of bacterial host cells include grampositive bacteria such as strains of *Bacillus*, e.g. *B. brevis* or *B. subtilis, Pseudomonas* or *Streptomyces*, or gramnegative bacteria, such as strains of *E. coli*. The introduction of a vector into a bacterial host cell may, for instance, be effected by protoplast transformation (see, e.g., Chang and Cohen, 1979, *Molecular General Genetics* 168: 111-115), using competent cells (see, e.g., Young and Spizizin, 1961, *Journal of Bacteriology* 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, *Journal of Molecular Biology* 56: 209-221), electroporation (see, e.g., Shigekawa and Dower, 1988, *Biotechniques* 6: 742-751), or conjugation (see, e.g., Koehler and Thorne, 1987, *Journal of Bacteriology* 169: 5771-5278). Examples of suitable filamentous fungal host cells include strains of *Aspergillus*, e.g. *A. oryzae, A. niger*, or *A. nidulans, Fusarium* or *Trichoderma*. Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known per se. Suitable procedures for transformation of *Aspergillus* host cells are described in EP 238 023 and U.S. Pat. No. 5,679,543. Suitable methods for transforming *Fusarium* species are described by Malardier et al., 1989, *Gene* 78: 147-156 and WO 96/00787. Examples of suitable yeast host cells include strains of *Saccharomyces*, e.g. *S. cerevisiae, Schizosaccharomyces, Klyveroinyces, Pichia*, such as *P. pastoris* or *P. methzanolica, Hansenula*, such as *H. Polymorpha* or *Yarrowia*. Yeast may be transformed using the procedures described by Becker and Guarente, In Abelson, J. N. and Simon, M. I., editors, *Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology*, Volume 194, pp 182-187, Academic Press, Inc., New York; Ito et al., 1983, *Journal of Bacteriology* 153: 163; Hinnen et al., 1978, *Proceedings of the National Academy of Sciences USA* 75: 1920: and as disclosed by Clontech Laboratories, Inc, Palo Alto, Calif., USA (in the product protocol for the Yeastmaker™ Yeast Transformation System Kit). Examples of suitable insect host cells include a *Lepidoptora* cell line, such as *Spodoptera frugiperda* (Sf9 or Sf21) or *Trichoplusioa ni* cells (High Five) (U.S. Pat. No. 5,077,214). Transformation of insect cells and production of heterologous polypeptides therein may be performed as described by Invitrogen. Examples of suitable mammalian host cells include Chinese hamster ovary (CHO) cell lines, (e.g. CHO-K1; ATCC CCL-61), Green Monkey cell lines (COS) (e.g. COS 1 (ATCC CRL-1650), COS 7 (ATCC CRL-1651)); mouse cells (e.g. NS/O), Baby Hamster Kidney (BHK) cell lines (e.g. ATCC CRL-1632 or ATCC CCL-10), and human cells (e.g. HEK 293 (ATCC CRL-1573)), as well as plant cells in tissue culture. Additional suitable cell lines are known in the art and available from public depositories such as the American Type Culture Collection, Rockville, Md. Also, the mammalian cell, such as a CHO cell, may be modified to express sialyltransferase, e.g. 1,6-sialyltransferase, e.g. as described in U.S. Pat. No. 5,047,335, in order to provide improved glycosylation of the polypeptide variant.

[0259] In order to increase secretion it may be of particular interest to produce the polypeptide variant of the invention together with an endoprotease, in particular a PACE (paired basic amino acid converting enzyme) (e.g. as described in U.S. Pat. No. 5,986,079), such as a Kex2 endoprotease (e.g. as described in WO 00/28065).

[0260] Methods for introducing exogeneous DNA into mammalian host cells include calcium phosphate-mediated transfection, electroporation, DEAE-dextran mediated transfection, liposome-mediated transfection, viral vectors

and the transfection method described by Life Technologies Ltd, Paisley, UK using Lipofectamin 2000. These methods are well known in the art and e.g. described by Ausbel et al. (eds.), 1996, Current Protocols in Molecular Biology, John Wiley & Sons, New York, USA. The cultivation of mammalian cells are conducted according to established methods, e.g. as disclosed in (Animal Cell Biotechnology, Methods and Protocols, Edited by Nigel Jenkins, 1999, Human Press Inc, Totowa, N.J., USA and Harrison M A and Rae I F, General Techniques of Cell Culture, Cambridge University Press 1997).

[0261] In the production methods of the present invention, the cells are cultivated in a nutrient medium suitable for production of the polypeptide variant using methods known in the art. For example, the cell may be cultivated by shake flask cultivation, small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermenters performed in a suitable medium and under conditions allowing the polypeptide to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or may be prepared according to published compositions (e.g., in catalogues of the American Type Culture Collection). If the polypeptide variant is secreted into the nutrient medium, the polypeptide can be recovered directly from the medium. If the polypeptide variant is not secreted, it can be recovered from cell lysates.

[0262] The resulting polypeptide variant may be recovered by methods known in the art. For example, the polypeptide variant may be recovered from the nutrient medium by conventional procedures including, but not limited to, centrifugation, filtration, extraction, spray drying, evaporation, or precipitation.

[0263] The polypeptides may be purified by a variety of procedures known in the art including, but not limited to, chromatography (e.g., ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (e.g., preparative isoelectric focusing), differential solubility (e.g., ammonium sulfate precipitation), HPLC, or extraction (see, e.g., *Protein Purification*, J.-C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989).

[0264] Single chain polypeptide variants of the invention can be purified and activated to two-chain polypeptide variants by a number of methods as described in the literature (Broze and Majerus, 1980, J. Biol. Chem. 255:1242-47 and Hedner and Kisiel, 1983, J. Clin. Invest. 71:1836-41). Another method whereby single chain polypeptide variant can be purified is by incorporation of Zn ions during purification as described in U.S. Pat. No. 5,700,914. In a preferred embodiment the polypeptide variant is purified as a single chain polypeptide variant. The single chain polypeptide variant is activated by either use of an immobilized enzyme (e.g. factors IIa, IXa, Xa and XIIa) or by autoactivation using a positively charged ion exchange matrix or the like.

[0265] It is advantageous to first purify the polypeptide variant in its single chain form, then PEGylate (if desired) and last activate by one of the methods described above or by autoactivation as described by Pedersen et al, 1989, Biochemistry 28: 9331-36. The advantage of carrying out PEGylation before activation is that PEGylation of the new aminoterminal formed by cleavage of R152-I153 is avoided. PEGylation of this new amino terminal would render the molecule inactive since the formation of a hydrogen bond between D242 and the amino terminal of I153 is necessary for activity.

Pharmaceutical Composition of the Invention and Its Use

[0266] In a further aspect, the present invention relates to a composition, in particular to a pharmaceutical composition, comprising a polypeptide variant of the invention and a pharmaceutically acceptable carrier or excipient.

[0267] The polypeptide variant or the pharmaceutical composition according to the invention may be used as a medicament.

[0268] Due to the improved properties mentioned hereinbefore, the polypeptide variants of the invention, or the pharmaceutical composition of the invention, are particular useful for the treatment of uncontrollable bleeding events in trauma patients, thrombocytopenic patients, patients in anticoagulant treatment, and cirrhosis patients with variceal bleeds, or other upper gastrointestinal bleedings, and in patients undergoing orthotopic liver transplantation, or liver resection (allowing for transfusion free surgery).

[0269] Trauma is defined as an injury to living tissue caused by an extrinsic agent. It is the 4th leading cause of death in the US and places a large financial burden on the economy.

[0270] Trauma is classified as either blunt or penetrative. Blunt trauma results in internal compression, organ damage and internal haemorrhage whereas penetrative trauma (as the consequence of an agent penetrating the body and destroying tissue, vessels and organs) results in external haemorrhage.

[0271] Haemorrhage, as a result of trauma, can start a cascade of problems. For example physiological compensation mechanisms are initiated with initial peripheral and mesenteric vasoconstriction to shunt blood to the central circulation. If circulation is not restored, hypovolemia shock (multiple organ failure due to inadequate perfusion) ensues. Since tissues throughout the body become starved for oxygen, anaerobic metabolism begins. However, the concomitant lactic acid leads the blood pH to drop and metabolic acidosis develops. If acidosis is severe and uncorrected, the patient may develop multisystem failure and die.

[0272] Although the majority of trauma patients are hypothermic on arrival in the emergency room due to the environmental conditions at the scene, inadequate protection, intravenous fluid administration and ongoing blood loss worsen the hypothermic state. Deficiencies in coagulation factors can result from blood loss or transfusions. Meanwhile, acidosis and hypothermia interfere with blood clotting mechanisms. Thus coagulopathy develops, which in turn, may mask surgical bleeding sites and hamper the control of mechanical bleeding. Hypothermia, coagulopathy and acidosis are often characterised as the "trauma triad of death"

[0273] Trauma may be caused by several events. For example, road traffic accidents result in many different types of trauma. Whilst some road traffic accidents are likely to result in penetrative trauma, many road traffic accidents are

likely to inflict blunt trauma to both head and body. However, these various types of trauma can all result in coagulopathy in the patient. Road traffic accidents are the leading cause of accidental death in the US. There are over 42,000 deaths from them in the US each year. Many trauma patients die at the location of the accident either whilst being treated by the paramedics, before they arrive or in transit to the ER.

[0274] Another example includes gunshot wounds. Gunshot wounds are traumas that can result in massive bleeding. They are penetrative and destroy tissue as the bullet passes through the body, whether it be in the torso or a limb. In the US about 40,000 people a year die from gunshot wounds.

[0275] A further example includes falls. Falls result in a similar profile of trauma type to road traffic accidents. By falling onto a solid object or the ground from height can cause both penetrative and decelerative blunt trauma. In the US, falls are a common cause of accidental death, numbering about 13,000.

[0276] A still further example includes machinery accidents. A smaller number of people die in the US from machinery accident related deaths, whether struck by, or entangled in machinery. The figures are small but significant—around 2,000.

[0277] A still further example includes stab wounds. Stab wounds are penetrative injuries that can also cause massive bleeding. The organs most likely to be damaged in a stab wound are the liver, small intestine and the colon.

[0278] Cirrhosis of the liver is the terminal sequel of prolonged repeated injury to the hepatic parenchyma. The end result is the formation of broad bands of fibrous tissue separating regenerative nodules that do not maintain the normal organization of liver lobules and thus cause deteriorated liver function. Patients have prolonged prothrombin times as a result of the depletion of vitamin K-dependent coagulation factors. Pathogenetically, liver cirrhosis should be regarded as the final common pathway of chronic liver injury, which can result from any form of intense repeated prolonged liver cell injury. Cirrhosis of the liver may be caused by direct liver injury, including chronic alcoholism, chronic viral hepatitis (types B, C, and D), and auto immune hepatitis as well as by indirect injury by way of bile duct damage, including primary biliary cirrhosis, primary sclerosing cholangitis and biliary atresia. Less common causes of cirrhosis include direct liver injury from inherited disease such as cystic fibrosis, alpha-1-antitrypsin deficiency, hemochromatosis, Wilson's disease, galactosemia, and glycogen storage disease.

[0279] Transplantation is primarily reserved for late stage cirrhotic patients, where it is the key intervention for treating the disease. To be eligible for transplantation, a patient must be classified as Child's B or C, as well as meet additional criteria for selection. Last year, in the US alone, 4,954 transplants were performed.

[0280] It has been estimated that there are 6,000 bleeding episodes associated with patients undergoing resection each year. This correlates with the reserved position of this procedure although seems slightly high in comparison with transplantation numbers.

[0281] Accurate data on the incidence of variceal bleeding is hard to obtain. The key facts known are that at the time of diagnosis, varices are present in about 60% of decompensated and 30% of compensated patients and that about 30% of these patients with varices will experience a bleed and that each episode of variceal bleeding is associated with a 30% risk of mortality.

[0282] Thus, in a further aspect the present invention relates to a polypeptide variant of the invention for the manufacture of a medicament for the treatment of diseases or disorder wherein clot formation is desirable. A still further aspect of the present invention relates to a method for treating a mammal having a disease or disorder wherein clot formation is desirable, comprising administering to a mammal in need thereof an effective amount of the polypeptide variant or the pharmaceutical composition of the invention.

[0283] Thrombocytopenia is caused by one of three mechanisms-decreased bone marrow production, increased splenic sequestration, or accelerated destruction of platelets. Thrombocytopenia is a risk factor for hemorrhage, and platelet transfusion reduces the incidence of bleeding. The threshold for prophylactic platelet transfusion is 10,000/μl. In patients without fever or infections, a threshold of 5000/μl may be sufficient to prevent spontaneous hemorrhage. For invasive procedures, 50,000/μl platelets is the usual target level. In patients who develop antibodies to platelets following repeated transfusions, bleeding can be extremely difficult to control.

[0284] Examples of diseases/disorders wherein increased clot formation is desirable include, but is not limited to, hemorrhages, including brain hemorrhages, as well as patient with severe uncontrolled bleedings, such as trauma. Further examples include patients undergoing living transplantations, patients undergoing resection and patients with variceal bleedings.

[0285] The polypeptide variants of the invention is administered to patients in a therapeutically effective dose, normally one approximately paralleling that employed in therapy with rFVII such as NovoSeven®, or at lower dosage. By "therapeutically effective dose" herein is meant a dose that is sufficient to produce the desired effects in relation to the condition for which it is administered. The exact dose will depend on the circumstances, and will be ascertainable by one skilled in the art using known techniques. Normally, the dose should be capable of preventing or lessening the severity or spread of the condition or indication being treated. It will be apparent to those of skill in the art that an effective amount of a polypeptide variant or composition of the invention depends, inter alia, upon the disease, the dose, the administration schedule, whether the polypeptide variant or composition is administered alone or in conjunction with other therapeutic agents, the plasma half-life of the compositions, and the general health of the patient. Preferably, the polypeptide variant or composition of the invention is administered in an effective dose, in particular a dose which is sufficient to normalize the coagulation disorder.

[0286] The polypeptide variant of the invention is preferably administered in a composition including a pharmaceutically acceptable carrier or excipient. "Pharmaceutically acceptable" means a carrier or excipient that does not cause any untoward effects in patients to whom it is administered. Such pharmaceutically acceptable carriers and excipients are well known in the art (see, for example, Remington's

Pharmaceutical Sciences, 18th edition, A. R. Gennaro, Ed., Mack Publishing Company [1990]; Pharmaceutical Formulation Development of Peptides and Proteins, S. Frokjaer and L. Hovgaard, Eds., Taylor & Francis [2000]; and Handbook of Pharmaceutical Excipients, 3rd edition, A. Kibbe, Ed., Pharmaceutical Press [2000]).

[0287] The polypeptide variant of the invention can be formulated into pharmaceutical compositions by well-known methods. Suitable formulations are described by Remington's Pharmaceutical Sciences by E. W. Martin (Mark Publ. Co., 16th Ed., 1980).

[0288] The polypeptide variant of the invention can be used "as is" and/or in a salt form thereof. Suitable salts include, but are not limited to, salts with alkali metals or alkaline earth metals, such as sodium, potassium, calcium and magnesium, as well as e.g. zinc salts. These salts or complexes may by present as a crystalline and/or amorphous structure.

[0289] The pharmaceutical composition of the invention may be administered alone or in conjunction with other therapeutic agents. These agents may be incorporated as part of the same pharmaceutical composition or may be administered separately from the polypeptide variant of the invention, either concurrently or in accordance with another treatment schedule. In addition, the polypeptide variant or pharmaceutical composition of the invention may be used as an adjuvant to other therapies.

[0290] A "patient" for the purposes of the present invention includes both humans and other mammals. Thus, the methods are applicable to both human therapy and veterinary applications. The pharmaceutical composition comprising the polypeptide variant of the invention may be formulated in a variety of forms, e.g. as a liquid, gel, lyophilized, or as a compressed solid. The preferred form will depend upon the particular indication being treated and will be apparent to one skilled in the art.

[0291] In particular, the pharmaceutical composition comprising the polypeptide variant of the invention may be formulated in lyophilised or stable soluble form. The polypeptide variant may be lyophilised by a variety of procedures known in the art. The polypeptide variant may be in a stable soluble form by the removal or shielding of proteolytic degradation sites as described herein. The advantage of obtaining a stable soluble preparation lies in easier handling for the patient and, in the case of emergencies, quicker action, which potentially can become life saving. The preferred form will depend upon the particular indication being treated and will be apparent to one of skill in the art.

[0292] The administration of the formulations of the present invention can be performed in a variety of ways, including, but not limited to, orally, subcutaneously, intravenously, intracerebrally, intranasally, transdermally, intraperitoneally, intramuscularly, intrapulmonary, vaginally, rectally, intraocularly, or in any other acceptable manner. The formulations can be administered continuously by infusion, although bolus injection is acceptable, using techniques well known in the art, such as pumps or implantation. In some instances the formulations may be directly applied as a solution or spray.

Parentals

[0293] A preferred example of a pharmaceutical composition is a solution designed for parenteral administration. Although in many cases pharmaceutical solution formulations are provided in liquid form, appropriate for immediate use, such parenteral formulations may also be provided in frozen or in lyophilized form. In the former case, the composition must be thawed prior to use. The latter form is often used to enhance the stability of the active compound contained in the composition under a wider variety of storage conditions, as it is recognized by those skilled in the art that lyophilized preparations are generally more stable than their liquid counterparts. Such lyophilized preparations are reconstituted prior to use by the addition of one or more suitable pharmaceutically acceptable diluents such as sterile water for injection or sterile physiological saline solution.

[0294] In case of parenterals, they are prepared for storage as lyophilized formulations or aqueous solutions by mixing, as appropriate, the polypeptide variant having the desired degree of purity with one or more pharmaceutically acceptable carriers, excipients or stabilizers typically employed in the art (all of which are termed "excipients"), for example buffering agents, stabilizing agents, preservatives, isotonifiers, non-ionic surfactants or detergents, antioxidants and/or other miscellaneous additives.

[0295] Buffering agents help to maintain the pH in the range which approximates physiological conditions. They are typically present at a concentration ranging from about 2 mM to about 50 mM. Suitable buffering agents for use in the present invention include both organic and inorganic acids and salts thereof such as citrate buffers (e.g., monosodium citrate-disodium citrate mixture, citric acid-trisodium citrate mixture, citric acid-monosodium citrate mixture, etc.), succinate buffers (e.g., succinic acid-monosodium succinate mixture, succinic acid-sodium hydroxide mixture, succinic acid-disodium succinate mixture, etc.), tartrate buffers (e.g., tartaric acid-sodium tartrate mixture, tartaric acid-potassium tartrate mixture, tartaric acid-sodium hydroxide mixture, etc.), fumarate buffers (e.g., fumaric acid-monosodium fumarate mixture, fumaric acid-disodium fumarate mixture, monosodium fumarate-disodium fumarate mixture, etc.), gluconate buffers (e.g., gluconic acid-sodium glyconate mixture, gluconic acid-sodium hydroxide mixture, gluconic acid-potassium glyuconate mixture, etc.), oxalate buffer (e.g., oxalic acid-sodium oxalate mixture, oxalic acid-sodium hydroxide mixture, oxalic acid-potassium oxalate mixture, etc.), lactate buffers (e.g., lactic acid-sodium lactate mixture, lactic acid-sodium hydroxide mixture, lactic acid-potassium lactate mixture, etc.) and acetate buffers (e.g., acetic acid-sodium acetate mixture, acetic acid-sodium hydroxide mixture, etc.). Additional possibilities are phosphate buffers, histidine buffers and trimethylamine salts such as Tris.

[0296] Stabilizers refer to a broad category of excipients, which can range in function from a bulking agent to an additive which solubilizes the therapeutic agent or helps to prevent denaturation or adherence to the container wall. Typical stabilizers can be polyhydric sugar alcohols (enumerated above); amino acids such as arginine, lysine, glycine, glutamine, asparagine, histidine, alanine, ornithine, L-leucine, 2-phenylalanine, glutamic acid, threonine, etc., organic sugars or sugar alcohols, such as lactose, trehalose,

stachyose, mannitol, sorbitol, xylitol, ribitol, myoinisitol, galactitol, glycerol and the like, including cyclitols such as inositol; polyethylene glycol; amino acid polymers; sulfur-containing reducing agents, such as urea, glutathione, thioctic acid, sodium thioglycolate, thioglycerol, α-monothioglycerol and sodium thiosulfate; low molecular weight polypeptides (i.e. <10 residues); proteins such as human serum albumin, bovine serum albumin, gelatin or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; monosaccharides such as xylose, mannose, fructose and glucose; disaccharides such as lactose, maltose and sucrose; trisaccharides such as raffinose, and polysaccharides such as dextran. Stabilizers are typically present in the range of from 0.1 to 10,000 parts by weight based on the active protein weight.

[0297] Preservatives are added to retard microbial growth, and are typically added in amounts of about 0.2%-1% (w/v). Suitable preservatives for use with the present invention include phenol, benzyl alcohol, meta-cresol, methyl paraben, propyl paraben, octadecyldimethylbenzyl ammonium chloride, benzalkonium halides (e.g. benzalkonium chloride, bromide or iodide), hexamethonium chloride, alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol and 3-pentanol.

[0298] Isotonicifiers are added to ensure isotonicity of liquid compositions and include polyhydric sugar alcohols, preferably trihydric or higher sugar alcohols, such as glycerin, erythritol, arabitol, xylitol, sorbitol and mannitol. Polyhydric alcohols can be present in an amount between 0.1% and 25% by weight, typically 1% to 5%, taking into account the relative amounts of the other ingredients.

[0299] Non-ionic surfactants or detergents (also known as "wetting agents") may be present to help solubilizing the therapeutic agent as well as to protect the therapeutic polypeptide against agitation-induced aggregation, which also permits the formulation to be exposed to shear surface stress without causing denaturation of the polypeptide. Suitable non-ionic surfactants include polysorbates (20, 80, etc.), polyoxamers (184, 188 etc.), Pluronic® polyols, polyoxyethylene sorbitan monoethers (Tween®-20, Tween®-80, etc.).

[0300] Additional miscellaneous excipients include bulking agents or fillers (e.g. starch), chelating agents (e.g. EDTA), antioxidants (e.g., ascorbic acid, methionine, vitamin E) and cosolvents.

[0301] The active ingredient may also be entrapped in microcapsules prepared, for example, by coascervation techniques or by interfacial polymerization, for example hydroxymethylcellulose, gelatin or poly-(methylmethacylate) microcapsules, in colloidal drug delivery systems (for example liposomes, albumin micro spheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, supra.

[0302] Parenteral formulations to be used for in vivo administration must be sterile. This is readily accomplished, for example, by filtration through sterile filtration membranes.

Sustained Release Preparations

[0303] Examples of sustained-release preparations include semi-permeable matrices of solid hydrophobic polymers containing the polypeptide variant, the matrices having a suitable form such as a film or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate) or poly(vinylalcohol)), polylactides, copolymers of L-glutamic acid and ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the ProLease® technology or Lupron Depot® (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(−)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for long periods such as up to or over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated polypeptides remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37° C., resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization is depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S—S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

[0304] The invention is further described in the following non-limiting examples.

Materials and Methods

Accessible Surface Area (ASA)

[0305] The computer program Access (B. Lee and F. M. Richards, J. Mol. Biol. 55: 379-400 (1971)) version 2 (© 1983 Yale University) is used to compute the accessible surface area (ASA) of the individual atoms in the structure. This method typically uses a probe-size of 1.4 Å and defines the Accessible Surface Area (ASA) as the area formed by the center of the probe. Prior to this calculation all water molecules and all hydrogen atoms should be removed from the coordinate set, as should other atoms not directly related to the protein.

Fractional ASA of Side Chain

[0306] The fractional ASA of the side chain atoms is computed by division of the sum of the ASA of the atoms in the side chain with a value representing the ASA of the side chain atoms of that residue type in an extended Ala-x-Ala tripeptide (See Hubbard, Campbell & Thornton (1991) J. Mol. Biol. 220,507-530). For this example the CA atom is regarded as a part of the side chain of Glycine residues but not for the remaining residues. The following table is used as standard 100% ASA for the side chain:

Ala	69.23 Å^2
Arg	200.35 Å^2
Asn	106.25 Å^2
Asp	102.06 Å^2
Cys	96.69 Å^2
Gln	140.58 Å^2
Glu	134.61 Å^2
Gly	32.28 Å^2
His	147.00 Å^2

-continued

Ile	137.91 Å^2
Leu	140.76 Å^2
Lys	162.50 Å^2
Met	156.08 Å^2
Phe	163.90 Å^2
Pro	119.65 Å^2
Ser	78.16 Å^2
Thr	101.67 Å^2
Trp	210.89 Å^2
Tyr	176.61 Å^2
Val	114.14 Å^2

[0307] Residues not detected in the structure are defined as having 100% exposure as they are thought to reside in flexible regions. The gamma-carboxy glutamic acids at positions 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35 are all defined as being 100% exposed.

Determining Distances Between Atoms

[0308] The distance between atoms is most easily determined using molecular graphics software e.g. InsightII® 98.0, MSI INC.

Active Site Region

[0309] The active site region is defined as any residues having at least one atom within 10 Å of any atom in the catalytic triad (residues H193, D242, S344).

Determination of Tissue Factor Binding Site

[0310] The TF binding site is defined as comprising of all residues having their accessible surface area changed upon TF binding. This is determined by at least two ASA calculations; one on the isolated ligand(s) in the ligand(s)/receptor(s) complex and one on the complete ligand(s)/receptor(s) complex.

Measurement of Reduced Sensitivity to Proteolytic Degradation

[0311] Proteolytic degradation can be measured using the assay described in U.S. Pat. No. 5,580,560, Example 5, where proteolysis is autoproteolysis.

[0312] Furthermore, reduced proteolysis can be tested in an in vivo model using radiolabelled samples and comparing proteolysis of rhFVIIa and the polypeptide variant of the invention by withdrawing blood samples and subjecting these to SDS-PAGE and autoradiography.

[0313] Irrespectively of the assay used for determining proteolytic degradation, "reduced proteolytic degradation" is intended to mean a measurable reduction in cleavage compared to is that obtained by rhFVIIa as measured by gel scanning of Coomassie stained SDS-PAGE gels, HPLC or as measured by conserved catalytic activity in comparison to wild type using the tissue factor independent activity assay decribed below.

Determination of the Molecular Weight of Polypeptide Variants

[0314] The molecular weight of polypeptide variants is determined by either SDS-PAGE, get filtration, Western Blots, matrix assisted laser desorption mass spectrometry or equilibrium centrifugation, e.g. SDS-PAGE according to Laemmli, U.K., Nature Vol 227 (1970), pp. 680-85.

TF-Independent Factor X Activation Assay

[0315] This assay has been described in detail on page 39826 in Nelsestuen et al., J Biol Chem, 2001; 276:39825-39831.

[0316] Briefly, the molecule to be assayed (either hFVIIa, rhFVIIa or the polypeptide variant of the invention in its activated form) is mixed with a source of phospholipid (phosphatidylcholine and phosphatidylserine in a ratio of 8:2 or phosphatidyleholine, phosphatidylserine and phosphatidylethanol in a ratio of 4:2:4) and Factor X in Tris buffer containing BSA. After a specified incubation time the reaction is stopped by addition of excess EDTA. The concentration of factor Xa is then measured from absorbance change at 405 nm after addition of a chromogenic substrate (S-2222, Chromogenix). After correction from background the tissue factor independent activity of rhFVIIa (a_{wt}) is determined as the absorbance change after 10 minutes and the tissue factor independent activity of the polypeptide variant of the invention ($a_{variant}$) is also determined as the absorbance change after 10 minutes. The ratio between the activity of the polypeptide variant, in its activated form, and the activity of rhFVIIa is defined as $a_{variant}/a_{wt}$.

Clotting Assay

[0317] Clotting activity is measured in one-stage assays and clotting times are recorded on a Thrombotrack IV coagulometer (MEDINOR). FVII depleted human plasma (American Diagnostica) is reconstituted and equilibrated at room temperature for 15-20 minutes. 50 μl of plasma is then transferred to the coagulometer cups.

[0318] hFVIIa, rhFVIIa or variants are diluted in Glyoxaline Buffer (5.7 mM barbiturate, 4.3 mM sodium citrate, 117 mM NaCl, 1 mg/mL BSA, pH 7.35). The samples are added to the cup in 50 μl and incubated at 37° C. for 2 minutes.

[0319] Thromboplastin (MEDINOR) is reconstituted with water and $CaCl_2$ is added. The reaction is initiated by adding 0.1 ml thromboplastin containing 4.5 mM $CaCl_2$.

[0320] Data are analysed using PRISM software.

TF-Independent Clotting Assay

[0321] This assay is performed as described above under "Clotting Assay" but without addition of thromboplastin.

Amidolytic Assay

[0322] The ability of the variants to cleave small peptide substrates can be measured using the chromogenic substrate S-2288 (D-Ile-Pro-Arg-p-nitroanilide). The amidolytic activity may be measured both in the presence and absence of antithrombin III (ATIII).

[0323] HFVIIa, rhFVIIa or variant is diluted to 90 nM in assay buffer (50 mM Na-Hepes pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 0.1% BSA, IU/ml Heparin). Furthermore, soluble TF (sTF) is diluted to 450 nM in assay buffer. ATIII is diluted to 900 nM in assay buffer. 120 μl of assay buffer is mixed with 20 μl of the FVIIa sample, 20 μl sTF and 2041 ATIII or assay buffer. The final concentrations of FVIIa, sTF and ATIII are 10, 50 and 100 nM, respectively. After 5 min incubation at room temperature with gentle shaking, followed by 10 min incubation at 37° C., the reaction is started by addition of the S-2288 substrate to 1 mM and the absorption at 405 nm is determined at several time points.

Thrombogram Assay

[0324] The effect of hFVIIa, rhFVIIa or variant on thrombin generation in human plasma is tested in a modified version of the assay described on page 589 in Hemker et al. in Thromb Haemost 2000; 83: 589-91. Briefly, the molecule to be assayed (either hFVIIa, rhFVIIa or variant) is mixed with normal platelet rich plasma (PRP), normal platelet poor plasma (PPP) or FVII depleted PPP with or without the addition of recombinant human tissue factor (rTF), relipidated rTF or another source of TF (such as thromboplastin). A source of phospholipid (phosphatidylcholine and phosphatidylserine in a ratio of 8:2 or phosphatidylcholine, phosphatidylserine and phosphatidylethanol in a ratio of 4:2:4) can be added.

[0325] The reaction is started by addition of a fluoregenic thrombin substrate and calcium chloride. The fluorescence is measured continuously and the thrombin amidolytic activity is calculated by calculating the slope of the fluorescence curve (the increase in fluorescence over time). In this way the time until maximum thrombin amidolytic activity is obtained (T_{max}) and total thrombin work (area under the curve (AUC)) can be calculated.

[0326] The following procedure is used: PRP is obtained by centrifuging freshly drawn blood at 250 g, 15° C. for 10 min. Blood coagulation is inhibited either by using citrate (13 mM tri-sodium citrate), corn trypsin inhibitor (50-100 μg/ml blood) or a combination of citrate and corn trypsin inhibitor. The platelet count is adjusted to 3×10^8/mL using buffer or autologous platelet poor plasma (PPP). PPP is obtained by double centrifugation of PRP at 1000 g, 15° C. for 10 min. FVII depletion is done by incubating PPP with a FVII specific monoclonal antibody coupled to a solid phase.

[0327] Per well of a 96-well microtiter plate 80 μl PRP is added and 20 μL buffer containing rhFVII or variant to be tested in final concentrations between 0.1 and 100 nM. rTF is added in 5 μL assay buffer to a final concentration of 1 pM. The assay buffer consists of 20 mM Hepes, 150 mM NaCl and 60 mg/ml BSA in distilled water. The reaction is started by adding 20 μL of the substrate solution containing 0.1 M calcium chloride. The assay plate and reagents are prewarmed to 37° C. and the reaction takes place at this temperature. The fluorimeter used is a BMG Fluormeter with a excitation filter at 390 nm and an emission filter at 460 nm. The fluorescence is measured in each well of 96-well clear bottom plates in 20-40 second interval over 30-180 minutes. Data are analyzed using PRISM Software.

ELISA Assay

[0328] FVII/FVIIa (or variant) concentrations are determined by ELISA. Wells of a microtiter late are coated with an antibody directed against the protease domain using a solution of 2 μg/ml in PBS (100 μl per well). After 2 hours coating at R.T., the wells are washed 4 times with THT buffer (100 mM NaCl, 50 mM Tris-HCl pH 7.2 0.05% Tween-20). Subsequently, 200 μl of 1% Casein (diluted from 2.5% stock using 100 mM NaCl, 50 mM Tris-HCl pH 7.2) is added per well for blocking. After 1 hr incubation at R.T., the wells are emptied, and 100 μl of sample (optionally diluted in dilution buffer (THT+0.1% Casein)) is added. After another incubation of 1 hr at room temperature, the wells are washed 4 times with THT buffer, and 100 μl of a biotin-labelled antibody directed against the EGF-like domain (1 μg/ml) is added. After another 1 hr incubation at R.T., followed by 4 more washes with THT buffer, 100 μl of streptavidin-horse radish peroxidase (DAKO A/S, Glostrup, Denmark, 1/10000 diluted) is added. After another 1 hr incubation at R.T., followed by 4 more washes with THT buffer, 100 μl of TMB (3,3',5,5'-tetramethylbenzidine, Kem-en-Tech A/S, Denmark) is added. After 30 min incubation at R.T. in the dark, 100 μl of 1 M H_2SO_4 is added and OD_{450nm} is determined. A standard curve is prepared using rhFVIIa (NovoSeven®).

[0329] Alternatively, FVII/FVIIa or variants may be quantified through the Gla domain rather than through the protease domain. In this ELISA set-up, wells are coated overnight with an antibody directed against the EGF-like domain and for detection, a calcium-dependent biotin-labelled monoclonal anti-Gla domain antibody is used (2 μg/ml, 100 μl per well). In this set-up, mM $CaCl_2$ is added to the THT and dilution buffers.

Whole Blood Assay

[0330] The whole blood clotting assay is performed as described by Elg et al. Thrombosis Res. 2001, 101(3):159-170.

Reconstituted Coagulation Assay

[0331] The reconstituted coagulation assay is performed as described by Van't Veer et al. Blood 2000, 95(4), 1330-1335.

EXAMPLES

Example 1

[0332] The X-ray structure of hFVIIa in complex with soluble tissue factor by Banner et. al., J Mol Biol, 1996; 285:2089 is used for this example. It is noted that the numbering of residues in the reference does not follow the sequence. Here we have used the sequential numbering according to SEQ ID NO:2. The gamma-carboxy glutamic acids at positions 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35 are all here named GLU (three letter abbreviation) or E (one letter abbreviation). Residues 143-152 are not present in the structure.

Surface Exposure

[0333] Performing fractional ASA calculations on FVII fragments alone combined with the definition of accessibilities of non standard and/or missing residues described in the methods resulted in the following residues having more than 25% of their side chain exposed to the surface: A1, N2, A3, F4, L5, E6, E7, L8, R9, P10, S12, L13, E14, E16, K18, E19, E20, Q21, S23, F24, E25, E26, R8, E29, F31, K32, D33, A34, E35, R36, K38, L39, W41, I42, S43, S45, G47, D48, Q49, A51, S52, S53, Q56, G58, S60, K62, D63, Q64, L65, Q66, S67, I69, F71, L73, P74, A75, E77, G78, R79, E82, T83, H84, K85, D86, D87, Q88, L89, I90, V92, N93, E94, G97, E99, S103, D104, H105, T106, G107, T108, K109, S111, R113, E116, G117, S119, L120, L121, A122, D123, G124, V125, S126, T128, P129, T130, V131, E132, I140, L141, E142, K143, R144, N145, A146, S147, K148, P149, Q150, G151, R152, G155, K157, V158, P160, K161, E163, L171, N173, G174, A175, N184, T185, I186, H193, K197, K199, N200, R202, N203, I205, S214, E215, H216, D217,

G218, D219, S222, R224, S232, T233, V235, P236, G237, T238, T239, N240, H249, Q250, P251, V253, T255, D256, E265, R266, T267, E270, R271, F275, V276, R277, F278, L280, L287, L288, D289, R290, G291, A292, T293, L295, E296, N301, M306, T307, Q308, D309, L311, Q312, Q313, R315, K316, V317, G318, D319, S320, P321, N322, T324, E325, Y326, Y332, S333, D334, S336, K337, K341, G342, H351, R353, G354, Q366, G367, T370, V371, G372, R379, E385, Q388, K389, R392, S393, E394, P395, R396, P397, G398, V399, L400, L401, R402, P404 and P406 (A1-S45 are located in the Gla domain, the remaining positions are located outside the Gla domain).

[0334] The following residues had more than 50% of their side chain exposed to the surface: A1, A3, F4, L5, E6, E7, L8, R9, P10, E14, E16, K18, E19, E20, Q21, S23, E25, E26, E29, K32, A34, E35, R36, K38, L39, I42, S43, G47, D48, A51, S52, S53, Q56, G58, S60, K62, L65, Q66, S67, I69, F71, L73, P74, A75, E77, G78, R79, E82, H84, K85, D86, D87, Q88, L89, I90, V92, N93, E94, G97, T106, G107, T108, K109, S111, E116, S119, L121, A122, D123, G124, V131, E132, L141, E142, K143, R144, N145, A146, S147, K148, P149, Q150, G151, R152, G155, K157, P160, N173, G174, A175, K197, K199, N200, R202, S214, E215, H216, G218, R224, V235, P236, G237, T238, H249, Q250, V253, D256, T267, F275, R277, F278, L288, D289, R290, G291, A292, T293, L295, N301, M306, Q308, D309, L311, Q312, Q313, R315, K316, G318, D319, N322, E325, D334, K341, G354, G367, V371, E385, K389, R392, E394, R396, P397, G398, R402, P404 and P406 (A1-S43 are located in the Gla domain, the remaining positions are located outside the Gla domain).

Tissue Factor Binding Site

[0335] Performing ASA calculations the following residues in human FVII change their ASA in the complex. These residues were defined as constituting the receptor binding site: L13, K18, F31, E35, R36, L39, F40, I42, S43, S60, K62, D63, Q64, L65, I69, C70, F71, C72, L73, P74, F76, E77, G78, R79, E82, K85, Q88, I90, V92, N93, E94, R271, A274, F275, V276, R277, F278, R304, L305, M306, T307, Q308, D309, Q312, Q313, E325 and R379.

Active Site Region

[0336] The active site region is defined as any residue having at least one atom within a distance of 10 Å from any atom in the catalytic triad (residues H193, D242, S344): I153, Q167, V168, L169, L170, L171, Q176, L177, C178, G179, G180, T181, V188, V189, S190, A191, A192, H193, C194, F195, D196, K197, I198, W201, V228, I229, I230, P231, S232, T233, Y234, V235, P236, G237, T238, T239, N240, H241, D242, I243, A244, L245, L246, V281, S282, G283, W284, G285, Q286, T293, T324, E325, Y326, M327, F328, D338, S339, C340, K41, G342, D343, S344, G345, (3346, P347, H348, L358, T359, G360, I361, V362, S363, W364, G365, C368, V376, Y377, T378, R379, V380, Q382, Y383, W386, L387, L400 and F405.

The Ridge of the Active Site Binding Cleft

[0337] The ridge of the active site binding cleft region was defined by visual inspection of the FVIIa structure 1FAK-.pdb as: N173, A175, K199, N200, N203, D289, R290, G291, A292, P321 and T370.

Example 2

Design of an Expression Cassette for Expression of rhFVII in Mammalian Cells

[0338] The DNA sequence shown in SEQ ID NO:1, encompassing the short form of the full length cDNA encoding hFVII with its native short signal peptide (Hagen et al., 1986. PNAS 83:2412), was synthesized in order to facilitate high expression in mammalian cells. First the ATG start codon context was modified according to the Kozak consensus sequence (Kozak, M. *J Mol Biol* 1987 Aug. 20;196(4):947-50), so that there is a perfect match to the consensus sequence upstream of the ATG start codon. Secondly the open reading frame of the native cDNA was modified by making a bias in the codon usage towards the codons frequently used in highly expressed human genes. Further, two translational stop codons were inserted at the end of the open reading frame in order to facilitate efficient translational stop. The fully synthetic and expression optimized hFVII gene was assembled from 70-mer DNA oligonucleotides and finally amplified using end primers inserting BamHI and HindIII sites at the 5' and 3, ends respectively using standard PCR techniques, which resulted in the following sequence:

```
ggatcccgccaccatggtcagccaggccctccgcctcctgtgcctgctcc
tggggctgcagggctgcctggctgccgtcttcgtcacccaggaggaagcc
catggcgtcctgcatcgccggcgccgggccaatgcctttctggaagagct
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ggcgtctacacgcgcgtcagccagtacattgagtggctgcagaagctcat
gcggagcgaaccccggcccggggtgctcctgcgggccccttttccttgat
aaaagctt
```

[0339] A vector for the cloning of the generated PCR product encompassing the expression cassette for hFVII was prepared by cloning the inkron from pCINeo (Promega). The synthetic intron from pCI-Neo was amplified using standard PCR conditions and the primers:

```
CBProFpr174: 5'-AGCTGGCTAGCCACTGGGCAGGTAAGTATCA-3'
and

CBProFpr175: 5'-TGGCGGGATCCTTAAGAGCTGTAATTGAACT-3'
```

resulting in a 332 bp PCR fragment. The fragment was cut with NheI and BamHI before cloning into pcDNA3.1/HygR (obtained from Invitrogen) resulting in PF#34.

[0340] The expression cassette for hFVII was cloned between the BamHI and HindIII sites of PF#34, resulting in plasmid PF#226.

Example 3

Expression of Polypeptide Variants in CHO K1 Cells

[0341] The cell line CHO K1 (ATCC # CCL-61) is seeded at 50% confluence in T-25 flasks using MEMO, 10% FCS (Gibco/BRL Cat # 10091), P/S and 5 μg/ml phylloquinone and allowed to grow until confluent. The confluent mono cell layer is transfected with 5 μg of the relevant plasmid described above using the Lipofectamine 2000 transfection agent (Life technologies) according to the manufacturer's instructions. Twenty four hours post transfection a sample is drawn and quantified using e.g. an ELISA recognizing the EGF1 domain of hFVII. At this time point relevant selection (e.g. Hygromycin B) may be applied to the cells with the purpose of generating a pool of stable transfectants. When using CHO K1 cells and the Hygromycin B resistance gene as selectable marker on the plasmid, this is usually achieved within one week.

Example 4

Generation of CHO K1 Cells Stably Expressing Polypeptide Variants.

[0342] A vial of CHO-K1 transfectant pool is thawed and the cells seeded in a 175 cm^2 tissue flask containing 25 ml of MEMα, 10% FCS, phylloquinone (5 μg/ml), 100 U/l penicillin, 100 μg/l streptomycin and grown for 24 hours. The cells are harvested, diluted and plated in 96 well microtiter plates at a cell density of ½-1 cell/well. After a week of growth, colonies of 20-100 cells are present in the wells and those wells containing only one colony are labelled. After a further two weeks, the media in all wells containing only one colony is substituted with 200 μl fresh medium. After 24 hours, a medium sample is withdrawn and analysed by e.g. ELISA. High producing clones are selected and used to produce FVII or variant on large scale.

Example 5

Purification of Polypeptide Variants and Subsequent Activation

[0343] FVII and FVII variants are purified as follows: The procedure is performed at 4° C. The harvested culture media from large-scale production is ultrafiltered using a Millipore TFF system with 30 KDa cut-off Pellicon membranes. After concentration of the medium, citrate is added to 5 mM and the pH is adjusted to 8.6. If necessary, the conductivity is lowered to below 10 mS/cm. Subsequently, the sample is applied to a Q-sepharose FF column, equilibrated with 50 mM NaCl, 10 mM Tris pH 8.6. After washing the column with 100 mM NaCl, 10 mM Tris pH 8.6, followed by 150 mM NaCl, 10 mM Tris pH 8.6, FVII is eluted using 10 mM Tris, 25 mM NaCl, 35 mM $CaCl_2$, pH 8.6.

[0344] For the second chromatographic step, an affinity column is prepared by coupling of a monoclonal Calcium-dependent antiGla-domain antibody to CNBr-activated Sepharose FF. About 5.5 mg antibody is coupled per ml resin. The column is equilibrated with 10 mM Tris, 100 mM NaCl, 35 mM $CaCl_2$, pH 7.5. NaCl is added to the sample to a concentration of 100 mM NaCl and the pH is adjusted to 7.4-7.6. After O/N application of the sample, the column is washed with 100 mM NaCl, 35 mM $CaCl_2$, 10 mM Tris pH 7.5, and the FVII protein is eluted with 100 mM NaCl, 50 mM citrate, 75 mM Tris pH 7.5.

[0345] For the third chromatographic, the conductivity of the sample is lowered to below 10 mS/cm, if necessary, and the pH is adjusted to 8.6. The sample is then applied to a Q-sepharose column (equilibrated with 50 mM NaCl, 10 mM Tris pH 8.6) at a density around 3-5 mg protein per ml gel to obtain efficient activation. After application, the column is washed with 50 mM NaCl, 10 mM Tris pH 8.6 for about 4 hours with a flow of 3-4 column volumes (cv) per hour. The FVII protein is eluted using a gradient of 0-100% of 500 mM NaCl, 10 mM Tris pH 8.6 over 40 cv. FVII containing fractions are pooled.

[0346] For the final chromatographic step, the conductivity is lowered to below 10 mS/cm. Subsequently, the sample is applied to a Q-sepharose column (equilibrated with 140 mM NaCl, 10 mM glycylglycine pH 8.6) at a concentration of 3-5 mg protein per ml gel. The column is then washed with 140 mM NaCl, 10 mM glycylglycine pH 8.6 and FVII is eluted with 140 mM NaCl, 15 mM $CaCl_2$, 10 mM glycylglycine pH 8.6. The eluate is diluted to 10 mM $CaCl_2$ and the pH is adjusted 6.8-7.2. Finally, Tween-80 is added to 0.01% and the pH is adjusted to 5.5 for storage at −80° C.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 4

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<211> LENGTH: 1338
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
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<222> LOCATION: (115)..(1335)

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```
gccgtcttcg tcacccagga ggaagcccat ggcgtcctgc atcgccggcg ccgg gcc      117
                                                          Ala
                                                           1

aat gcc ttt ctg gaa gag ctc cgc cct ggc tcc ctg gaa cgc gaa tgc      165
Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu Cys
             5                  10                  15

aaa gag gaa cag tgc agc ttt gag gaa gcc cgg gag att ttc aaa gac      213
Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys Asp
         20                  25                  30

gct gag cgg acc aaa ctg ttt tgg att agc tat agc gat ggc gat cag      261
Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp Gln
     35                  40                  45

tgc gcc tcc agc cct tgc cag aac ggg ggc tcc tgc aaa gac cag ctg      309
Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln Leu
 50                  55                  60                  65

cag agc tat atc tgc ttc tgc ctg cct gcc ttt gag ggg cgc aat tgc      357
Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn Cys
                 70                  75                  80

gaa acc cat aag gat gac cag ctg att tgc gtc aac gaa aac ggg ggc      405
Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly Gly
             85                  90                  95

tgc gag cag tac tgc agc gat cac acg ggc acg aag cgg agc tgc cgc      453
Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys Arg
        100                 105                 110

tgc cac gaa ggc tat agc ctc ctg gct gac ggg gtg tcc tgc acg ccc      501
Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr Pro
    115                 120                 125

acg gtg gaa tac cct tgc ggg aag att ccc att cta gaa aag cgg aac      549
Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg Asn
130                 135                 140                 145

gct agc aaa ccc cag ggc cgg atc gtc ggc ggg aag gtc tgc cct aag      597
Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro Lys
                150                 155                 160

ggg gag tgc ccc tgg cag gtc ctg ctc ctg gtc aac ggg gcc cag ctg      645
Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln Leu
            165                 170                 175

tgc ggc ggg acc ctc atc aat acc att tgg gtc gtg tcc gcc gct cac      693
Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala His
        180                 185                 190

tgc ttc gat aag att aag aat tgg cgg aac ctc atc gct gtg ctc ggc      741
Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu Gly
    195                 200                 205

gaa cac gat ctg tcc gag cat gac ggg gac gaa cag tcc cgc cgg gtg      789
Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg Val
210                 215                 220                 225

gct cag gtc atc att ccc tcc acc tat gtg cct ggc acg acc aat cac      837
Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn His
                230                 235                 240

gat atc gct ctg ctc cgc ctc cac cag ccc gtc gtg ctc acc gat cac      885
Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp His
            245                 250                 255

gtc gtg cct ctg tgc ctg cct gag cgg acc ttt agc gaa cgc acg ctg      933
Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr Leu
        260                 265                 270

gct ttc gtc cgc ttt agc ctc gtg tcc ggc tgg ggc cag ctg ctc gac      981
Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu Asp
    275                 280                 285
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cgg ggc gct acc gct ctc gag ctg atg gtg ctc aac gtc ccc cgg ctg     1029
Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg Leu
290                 295                 300                 305

atg acc cag gac tgc ctg cag cag tcc cgc aaa gtg ggg gac tcc ccc     1077
Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser Pro
                310                 315                 320

aat atc acg gag tat atg ttt tgc gct ggc tat agc gat ggc tcc aag     1125
Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser Lys
            325                 330                 335

gat agc tgc aag ggg gac tcc ggc ggg ccc cat gcc acg cac tat cgc     1173
Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr Arg
        340                 345                 350

ggg acc tgg tac ctc acc ggg atc gtc agc tgg ggc cag ggc tgc gcc     1221
Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys Ala
    355                 360                 365

acg gtg ggg cac ttt ggc gtc tac acg cgc gtc agc cag tac att gag     1269
Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile Glu
370                 375                 380                 385

tgg ctg cag aag ctc atg cgg agc gaa ccc cgg ccc ggg gtg ctc ctg     1317
Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu Leu
                390                 395                 400

cgg gcc cct ttc cct tga taa                                         1338
Arg Ala Pro Phe Pro
            405


<210> SEQ ID NO 2
<211> LENGTH: 406
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 2

Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu
  1               5                  10                  15

Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys
             20                  25                  30

Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp
         35                  40                  45

Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln
     50                  55                  60

Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn
 65                  70                  75                  80

Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly
                 85                  90                  95

Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys
            100                 105                 110

Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr
        115                 120                 125

Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg
    130                 135                 140

Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro
145                 150                 155                 160

Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln
                165                 170                 175

Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala
            180                 185                 190
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-continued

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His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu
        195                 200                 205

Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg
    210                 215                 220

Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn
225                 230                 235                 240

His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp
                245                 250                 255

His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr
            260                 265                 270

Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu
        275                 280                 285

Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg
    290                 295                 300

Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser
305                 310                 315                 320

Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser
                325                 330                 335

Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr
            340                 345                 350

Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys
        355                 360                 365

Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile
    370                 375                 380

Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu
385                 390                 395                 400

Leu Arg Ala Pro Phe Pro
                405


<210> SEQ ID NO 3
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Primer
      CBProFpr174

<400> SEQUENCE: 3

agctggctag ccactgggca ggtaagtatc a                                    31


<210> SEQ ID NO 4
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Primer
      CBProFpr175

<400> SEQUENCE: 4

tggcgggatc cttaagagct gtaattgaac t                                    31
```

1. A Factor VII (FVII) or Factor VIIa (FVIIa) polypeptide variant having an amino acid sequence comprising 3-15 amino acid modifications relative to human Factor VII (hFVII) or human Factor VIIa (hFVIIa) having the amino acid sequence shown in SEQ ID NO:2, wherein said amino acid sequence of the variant comprises an amino acid substitution in position 10 and 32 and wherein a sugar moiety is covalently attached to an introduced in vivo N-glycosylation site located outside the Gla domain.

2. The variant according to claim 1, wherein said substitution in position 10 is P10Q.

3. The variant according to claim 1, wherein said substitution in position 32 is K32E.

4. The variant according to claim 1, wherein said substitution in position 10 is P10Q and said substitution in position 32 is K32E.

5. The variant according to claim 1, wherein said variant comprises at least one further amino acid modification in the Gla domain.

6-15. (canceled)

16. The variant according to claim 5, wherein said further modification in the Gla domain comprises an amino acid substitution in position 34.

17. The variant according to claim 16, wherein a negatively charged amino acid residue is introduced by substitution in position 34.

18. The variant according to claim 17, wherein said substitution is A34E.

19. The variant according to claim 18, wherein said variant comprises the substitutions P10Q+K32E+A34E.

20-25. (canceled)

26. The variant according to claim 1, wherein said in vivo N-glycosylation site is introduced by substitution.

27-28. (canceled)

29. The variant according to claim 26, wherein said in vivo N-glycosylation site is introduced by a substitution selected from the group consisting of A51N, G58N, T106N, K109N, G124N, K143N+N145T, A175T, I205S, I205T, V253N, T267N, T267N+S269T, S314N+K316S, S314N+K316T, R315N+V317S, R315N+V317T, K316N+G318S, K316N+G318T, G318N, D334N and combinations thereof.

30. The variant according to claim 29, wherein said in vivo N-glycosylation site is introduced by a substitution selected from the group consisting of A51N, G58N, T106N, K109N, G124N, K143N, N145T, A175T, I205T, V253N, T267N+S269T, S314N+K316T, R315N+V317T, K316N+G318T, G318N, D334N and combinations thereof.

31. The variant according to claim 30, wherein said in vivo N-glycosylation site is introduced by a substitution selected from the group consisting of T106N, A175T, I205T, V253N, T267N+S269T and combinations thereof.

32. The variant according to claim 16, wherein one in vivo N-glycosylation site has been introduced by substitution.

33. The variant according to claim 16, wherein two or more in vivo N-glycosylation sites have been introduced by substitution.

34-40. (canceled)

41. The variant according to claim 1, wherein said variant is in its activated form.

42. The variant according to claim 1, wherein said variant, in its activated form, has at least 10% of the amidolytic activity of rhFVIIa when assayed in the "Amidolytic Assay" described herein.

43. The variant according to claim 1, wherein said variant, in its activated form, has at least 10% of the clotting activity of rhFVIIa when assayed in the "Clotting Assay" described herein.

44. A nucleotide sequence encoding the variant of claim 1.

45. An expression vector comprising the nucleotide sequence of claim 44.

46. A host cell comprising the nucleotide sequences of claim 44.

47. The host cell according to claim 46, wherein said host cell is a gammacarboxylating cell capable of in vivo glycosylation.

48. A pharmaceutical composition comprising the variants of claim 1, and a pharmaceutical acceptable carrier or excipient.

49-54. (canceled)

55. A method for treating a mammal having a disease or a disorder wherein clot formation is desirable, comprising administering to a mammal in need thereof an effective amount of the pharmaceutical composition of claim 48.

56. The method according to claim 55, wherein said disease or disorder is selected from the group consisting of hemorrhages, including brain hemorrhages, severe uncontrolled bleedings, such as trauma, bleedings in patients undergoing living transplantations, bleeding in patients undergoing resection and variceal bleedings.

57-59. (canceled)

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