SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO	
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ACT: 519 PRESENTACION	

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
DILIA MARIA RODRIGUEZ D ALEMAN

Asunto: Radicación: 13-109382- -1
Trámite: 11
Evento: 379
Actuación: 519
Oficio: 19879
Folios: 5

Patente de Invención	**X**			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	**X**	Capítulo I		Capítulo II	**X**
No. Solicitud Internacional	PCT/US2009/002248				
Fecha de Presentación Internacional	10/04/2009				

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	13-109382-00000-0000	30/abril/2013
Reivindicaciones:	13-109382-00000-0000	30/abril/2013
Dibujos:	13-109382-00000-0000	30/abril/2013
Secuencias:	13-109382-00000-0000	30/abril/2013
Prioridad:	US 61/124.021	11/abril/2008

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque ésta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Presentación de solicitudes Fraccionarias (Art 36 D 486)

Se aceptan para examen las solicitudes fraccionarias presentadas e identificadas como 13-109382-00000-0000, porque no implican ampliación de la materia contenida en la solicitud inicial 10-127271-00008-0000.

4. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del Título X de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada. En la presente solicitud se estudiaran las 33 reivindicaciones.

Título de la solicitud: “Polipéptidos del factor VIII que se modifican y usos de los mismos”

El problema planteado en esta solicitud consiste en la necesidad de proporcionar polipéptidos modificados de FVII que están diseñados para tener propiedades terapéuticas mejoradas.

Para resolver este problema técnico la solicitud en estudio proporciona polipéptidos modificados de FVII que exhiben actividades procoagulantes. Los polipeptidos de FVII son modificados en la secuencia primaria en comparación con un polipéptido de FVII no modificado, y puede incluir inserciones de aminoácidos, deleciones y sustituciones.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C12N 9/64

5. Reivindicaciones relativas a un uso o a un segundo uso (Art 14 y 21 D 486)

El uso de las reivindicaciones 30 a 33 no es patentable, de acuerdo con el Art 14.

6. De la solicitud de patente

Titulo

Se sugiere eliminar la palabra “usos” del título de la solicitud puesto que corresponde a materia no pantentable.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

La reivindicación 1 no es clara porque no define las siete sustituciones sobre las SEQ ID NO: 1-3 de manera que no es posible determinar la estructura de los péptidos reclamados. Por otra parte dicha reivindicación no tiene sustento puesto que la descripción hace referencia a ciertas sustituciones específicas, no a la sustitución de cualquier aminoácido en cualquier posición del péptido. Se recuerda a la solicitante que un péptido debe ser definido por su secuencia completa, en caso de presentarse variantes deben indicarse todas las posibles sustituciones por medio de su posición y posibles aminoácidos de remplazo. Las mismas observaciones de claridad se aplican a las reivindicaciones 7, 11, 20 a 22

El capítulo reivindicatorio no es conciso puesto que las reivindicaciones 3 a 5 reclaman la misma materia, además dichas reivindicación no son claras puesto que se refieren al “péptido no modificado” pero son dependientes de reivindicaciones relativas a un péptido modificado.

El capítulo reivindicatorio falla en definir las características esenciales de los péptidos reclamados puesto que las reivindicaciones 8 y 9 reclaman péptidos identificados por las sustituciones realizadas, y por otra parte la reivindicación 10 reclama péptidos por su SEQ ID NO, pero la solicitud no es clara en asociar que SEQ ID NO está relacionada a que sustituciones de las reclamadas, por lo cual se sugiere a la solicitante indicar claramente las modificaciones/sustituciones en el péptido de interés y asociarlo a su correspondiente SEQ ID NO.

La reivindicación 23 no es clara porque no proporciona la secuencia nucleotida correspondiente, se recuerda a la solicitante que un ácido nucleico debe caracterizarse por

su SEQ ID NO correspondiente. Las reivindicaciones dependientes 24 y 25 no son claras puesto que son dependientes de la reivindicación 23 que no define la estructura del ácido nucleico.

7. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: 11 de abril de 2008, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de publicación
D1	Bjelke et al.	A loop of coagulation factor Vila influencing macromolecular substrate specificity	11/12/2006
D2	WO 2007/031559	Human coagulation factor VII polypeptides	22/03/2007

El documento D1[1] divulga (resumen) el impacto de la mutación Met298(156) Lys en el factor de coagulación FVIIa, que permite a su vez encontrar otros sitios importantes de mutación en el loop de Gly283(140) a Met298(156)

El documento D2[2] divulga las sustituciones del factor de coagulación FVIIa en la posición 286 entre otras (Pág. 14, ln. 1-3; Pág. 38, ln. 21, Pág. 68, tabla).

8. Examen de Patentabilidad (Art 14 D486)

Nivel inventivo (Art18 D486)

La reivindicación 1 consiste en “Un polipeptido del factor VII (FVII) modificado, en el que... (Pág. 445 del radicado N° 13-109382-00000-0000

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1	D2
Un polipéptido del factor VII (FVII) modificado	Un polipéptido del factor VII (FVII) modificado	Un polipéptido del factor VII (FVII) modificado (Pág. 58 Ej. 1)
Sustituciones en hasta 7 posiciones posiciones incluyendo 286 (R).	Sustituciones en múltiples posiciones incluyendo Q286R (Pág.73, Col. izquierda)	Sustituciones en multiples posiciones incluyendo 286 (R) (Pág. 20 ln 21, Pág. 34 ln. 25-26)

Como se dijo anteriormente, el documento D1 es considerado el estado de la técnica cercano a la invención definida en la reivindicación 1 porque divulga el polipéptido modificado del factor de coagulación FVII con más de una modificación, donde la modificación principal presenta una o más sustituciones de aminoácido en el loop Gly283(140) a Met298(156) con el reemplazo Q286 (Pág.73, Col. izquierda). Dicho documento también divulga los ácidos nucleicos que codifican el polipéptido (Reivindicación 88), vectores que los contienen (Reivindicación 89), células hospederas (reivindicación 90) y composiciones (Reivindicación 99).

La diferencia entre la invención, definida en la reivindicación 1, y los compuestos del documento D1 consiste en que la solicitud hace referencia a hasta siete aminoácidos sustituidos.

El efecto que logra el incluir la sustitución de múltiples posiciones incluyendo la posición 286 (R) del polipéptido del FVII es que aumenta significativamente la actividad coagulante del

1URL: http://www.sciencedirect.eom/science/article/pii/S0014579306014293#

2URL:http://worldwide.espacenet.com/publicationDetails/biblio?DB=worldwide.espacenet.com&ll=0&ND=4&adjacent=true&locale=es_LP&FT=D&date=20070322&CC=WO&NR=2007031559A2&KC=A2

polipéptido (Ej. Tablas 14 y 15).

El problema técnico objetivo que pretende resolver esta invención se puede formular así: Cómo modificar el polipéptido del FVII para aumentar su actividad coagulante.

Sin embargo, la utilización de modificaciones en el loop Gly283(140) a Met298(156) ya había sido reportado como útil en el diseño de polipéptidos de del FVII para aumentar su actividad coagulante en el documento D2, específicamente en dicho documento se divulgan sustituciones en la posición 286 (R) (Pág. 20 ln 21, Pág. 34 ln. 25-26).

En consecuencia, los documentos proporcionan una motivación para que la persona normalmente versada en la materia diseñe y evalúe péptidos del factor VII con una o más sustituciones en el loop Gly283(140) a Met298(156), ya que dichos documentos específicamente señalan como posiciones de interés el loop y el aminoácido 286, por lo cual aplicando medios técnicos conocidos y divulgados en los documentos citados, habría llegado a las sustituciones reclamadas.

Puesto que las reivindicaciones dependientes 2 a 29 no mencionan características esenciales adicionales, se considera que tales reivindicaciones tampoco tienen nivel inventivo.

9. Conclusión

Los requisitos de Patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	Bjelke et al.	1 a 29	Nivel inventivo
D2	WO 2007/031559	1 a 29	Nivel inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir del día siguiente de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.

JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2013-10-29

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FEBS Letters 581 (2007) 71–76

A loop of coagulation factor VIIa influencing macromolecular substrate specificity

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Abstract **Coagulation factor VIIa (FVIIa) belongs to a family of proteases being part of the stepwise, self-amplifying blood coagulation cascade. To investigate the impact of the mutation Met$^{298\{156\}}$Lys in FVIIa, we replaced the Gly$^{283\{140\}}$–Met$^{298\{156\}}$ loop with the corresponding loop of factor Xa. The resulting variant exhibited increased intrinsic activity, concurrent with maturation of the active site, a less accessible N-terminus, and, interestingly, an altered macromolecular substrate specificity reflected in an increased ability to cleave factor IX (FIX) and a decreased rate of FX activation compared to that of wild-type FVIIa. In complex with tissue factor, activation of FIX, but not of FX, returned to normal. Deconvolution of the loop graft in order to identify important side chain substitutions resulted in the mutant Val$^{158\{21\}}$Asp/Leu$^{287\{144\}}$Thr/Ala$^{294\{152\}}$-Ser/Glu$^{296\{154\}}$ Ile/Met$^{298\{156\}}$Lys-FVIIa with almost the same activity and specificity profile. We conclude that a lysine residue in position 298{156} of FVIIa requires a hydrophilic environment to be fully accommodated. This position appears critical for substrate specificity among the proteases of the blood coagulation cascade due to its prominent position in the macromolecular exosite and possibly via its interaction with the corresponding position in the substrate (i.e. FIX or FX).**

Keywords: Allostery; Coagulation factor VIIa; Macromolecular substrate specificity; Met$^{298\{156\}}$

1. Introduction

Coagulation factor VIIa (FVIIa) is a serine protease that plays a central role in the initiation of the blood coagulation cascade of the vascular hemostatic system. FVIIa in complex with tissue factor (TF) at a site of vascular injury initiates coagulation by activation of factors IX (FIX) and X (FX). FIX and FX activation subsequently lead to thrombin generation, which in turn cleaves fibrinogen to fibrin. By cross-linking of fibrin by activated factor XIII, a strong clot is produced that restores vascular integrity and flow. All proteases of the coagulation cascade are secreted as zymogens and need proteolytic cleavage of the peptide bond preceding position {16} (trypsinogen numbering in brackets) and in some cases association with their cognate cofactor to be catalytically and biologically active. For FVIIa, association with TF facilitates insertion of the N-terminal Ile$^{\{16\}}$ into the activation pocket and establishment of a salt bridge with Asp$^{\{194\}}$ which is adjacent to the active site residue Ser$^{\{195\}}$ [1]. This is not only a common feature for the serine proteases in the coagulation cascade, but is also the canonical allosteric mechanism for all trypsin-like serine proteases [2,3]. FVIIa only gains full activity upon Ca^{2+}-dependent association with TF and binding of Ca^{2+} to the loop Glu$^{210\{70\}}$–Glu$^{220\{80\}}$ in the protease domain [4–7]. In the free form, FVIIa mainly remains in a zymogen-like state with low catalytic efficiency.

Macromolecular substrate interactions at exosites, sites within the FVIIa·TF complex distant from the active site of FVIIa, determine affinity and specificity in the productive recognition of FX [8,9]. At one of these sites, Met$^{298\{156\}}$ has been shown to be a key determinant for maintaining free FVIIa in the zymogen-like state [10,11]. Site-directed mutagenesis studies have demonstrated that replacement of Met$^{298\{156\}}$ with glutamine, which occupies the corresponding position in FIXa and thrombin, renders FVIIa intrinsically more active with enhanced turnover of FX [10–13]. Surprisingly, introduction of a lysine, as found in the homologous FXa, results in a protein with apparently diminished activity [11]. The Met$^{298\{156\}}$ residue is located within the loop Gly$^{283\{140\}}$–Met$^{298\{156\}}$, which acts as an allosteric modulator of FVIIa [10–13]. It is part of the activation domain which has been shown to undergo conformational changes in the trypsinogen to trypsin transition [3,14]. Accordingly, we have observed using a molecular dynamics simulation that upon extraction of the N-terminus from the activation pocket the loop becomes markedly disordered (O.H. Olsen, unpublished results).

Soejima and colleagues performed grafting of the loops Thr$^{233\{93\}}$–Asp$^{242\{102\}}$ and Cys$^{310\{168\}}$–Cys$^{329\{182\}}$, inserting the loops of trypsin, to obtain FVIIa molecules with significantly higher amidolytic activities and an impaired effect of TF binding, but with no effect on N-terminus burial [15,16]. In our laboratory, mutagenesis in the Cys$^{310\{168\}}$–Cys$^{329\{182\}}$ loop has also led to proteins with increased amidolytic activities [17].

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Abbreviations: AT, antithrombin; FIX(a), factor IX(a); FVIIa, factor VIIa; FVIIa$_{IIa}$, FVIIa variant Val$^{158\{21\}}$Asp/Glu$^{296\{154\}}$Val/Met$^{298\{156\}}$Gln; FVIIa$_{DIK}$, FVIIa variant Val$^{158\{21\}}$Asp/Glu$^{296\{154\}}$Ile/Met$^{298\{156\}}$Lys; FVIIa$_{DTSIK}$, FVIIa variant Val$^{158\{21\}}$Asp/Leu$^{287\{144\}}$-Thr/Ala$^{294\{152\}}$Ser/Glu$^{296\{154\}}$Ile/Met$^{298\{156\}}$Lys; FVIIa$_{FXLoop}$, FVIIa variant with residues 283{140}–298{156} replaced by the corresponding residues from FX together with mutations Val$^{158\{21\}}$Glu, Gln$^{176\{40\}}$Gly and Lys$^{341\{192\}}$Gln; FX(a), factor X(a); TF, tissue factor

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In this study, we strived not only for increased catalytic activity, but also for insights into macromolecular substrate specificity. Since lysine and glutamine are found in position {156} in a number of members of the trypsin family of serine proteases, we aimed at exploring the molecular environment in FVIIa required to accommodate a lysine in position 298{156} using rational loop grafting and site-directed mutagenesis. We obtained Met$^{298\{156\}}$Lys-FVIIa variants with increased intrinsic activity but at the same time with either an enhanced (with FIX) or diminished (with FX) proteolytic activity relative to wild-type FVIIa. Structural models of FVIIa/FXa complexes indicate that the close vicinity of the side chains of Lys$^{298\{156\}}$ in the FVIIa variants and the corresponding position in the substrate (FIX or FX) generates an electrostatic repulsion when the substrate is FX (Lys$^{378\{156\}}$), while a favourable interaction is seen with FIX (Gln$^{370\{156\}}$) [18, O. H. Olsen, unpublished data].

2. Materials and methods

2.1. Protein preparations

A restriction digest cassette system, DraIII and BstZ17I, was introduced by means of site-directed mutagenesis into the pZym219b vector harboring the full-length cDNA of FVII at position bp 1099{Aa272{129C}} and 1192{Aa303{161}}. This allowed for easy insertion of a loop graft by means of subcloning of annealed oligonucleotides. The following oligonucleotides were used for the construction of the loop graft, for introduction of site-specific mutations and for the cloning cassette (amino acid and nucleotide changes indicated with underline and italic, respectively):

Val$^{158\{21\}}$Asp: C CGA ATT GTG GGG GGC AAG *GAC* TGC CCC AAA GGG GAG TGT CC. Val$^{158\{21\}}$Glu: GA ATT GTG GGG GGC AAG *GA*G TGC CCC AAA GGG GAG TGT CC. Gln$^{176\{40\}}$Gly: G TTG TTG GTG AAT GGA GCT *GG*G TTG TGT GGG GGG ACC CTG ATC. Lys$^{341\{192\}}$Gln: GGC AGC AAG GAC TCC TGC *CA*G GGG GAC AGT GGA GGC CC. Leu$^{287\{144\}}$Thr/Ala$^{294\{152\}}$Ser/Glu$^{296\{154\}}$Ile/Met$^{298\{156\}}$Lys: G GTC AGC GGC TGG GGC CAG *ACG* CTG GAC CGT GGC GCC ACG *TCC* CTG *ATT* CTC A*AG* GTG CTC AAC GTG CCC CGG C. Glu$^{296\{154\}}$Ile/Met$^{298\{156\}}$Lys: GGC GCC ACG GCC CTG *ATT* CTC A*AG* GTC CTC AAC GTG CCC C. Dra*III*_cas: GG ACG TTC TCT GAG AGG AC*ACTG GGTG*TC GTG CGC TTC TCA TTG G. BstZ17*I*_cas: G CTC ATG GTC CTC A*GT ATA* CCC CGG CTG ATG ACC CAG GAC TGC. $_{FXLoop}$FVII: CC TTC GTC CGC TTC TCA TTG GTC AGC GGG TTT GGG CGT ACT CAT GAA AAA GGG CGT CAA TCT ACT CGT CTT AAA GTC CTC AAC G. $_{FXLoop}$FVII(COMP): C GTT GAG GAC TTT AAG ACG AGT AGA TTG ACG CCC TTT TTC ATG AGT ACG CCC AAA CCC GCT GAC CAA TGA GAA GCG CAC GAA GGC CA. FVII wild-type/variants were expressed using CHO K1 and BHK-producing cells, recombinant proteins purified and zymogens activated essentially as described [10,19]. Active site titration by means of irreversible inhibition using the PhePheArg chloromethylketone was employed to determine the active enzyme fraction after activation essentially as described [20].

FX and FIX were from Enzyme Research Laboratories. Soluble TF was produced as described [21]. Antithrombin (AT) was from Hematologic Technologies (Essex Junction, VT).

2.2. Amidolytic activity assay

The amidolytic activity of variant/wild-type FVIIa (100 nM) with or without 1 μM soluble TF were measured at room temperature in microtiter plates. The assay buffer used was 50 mM Hepes, pH 7.4, containing 0.1 M NaCl, 5 mM $CaCl_2$, and 1 mg/ml BSA. Initial activity rates were determined from absorbance (405 nm) development using the chromogenic substrates S-2288 (1 mM) (H-D-Ile-Pro-Arg-pNA, Chromogenix) and S-2238 (1 mM) (H-D-Phe-Pip-Arg-pNA, Chromogenix), and monitored for 5–30 min with readings every 30 s in a kinetic SpectraMax 340 microplate reader (Global Medical Instrumentation Inc., Minnesota, USA).

2.3. FX activation assay

FX activation was assayed by incubating 100 nM variant/wild-type FVIIa with or without 1 μM soluble TF with 0–0.5 μM FX in 50 μl of assay buffer (see Amidolytic activity assay) for 20 min at room temperature. 150 μl assay buffer containing 20 mM EDTA instead of $CaCl_2$ was added to quench the FX activation process, followed by the addition of 50 μl of 2.5 mM FX substrate S-2765 (Z-D-Arg-Gly-Arg-pNA, Chromogenix). The rate of S-2765 hydrolysis was monitored for 2–5 min during which the absorbance increase was linear.

2.4. FIX activation assay

FIX activation was assayed by incubating 0.2–1 μM variant/wild-type FVIIa with or without 5 μM soluble TF with 0–0.5 μM FIX in 100 μl of assay buffer (see Amidolytic activity assay) for 1–2 h at room temperature. One hundred microliter assay buffer containing 20 mM EDTA, 60% ethylene glycol, 1 mM substrate CBS31.39 (CH_3SO_2-D-Leu-Gly-Arg-pNA, Diagnostica Stago) was added and mixed thoroughly to quench the FIX activation process and to initiate FIXa measurement. The rate of CBS31.39 hydrolysis was monitored for 2–5 min during which the absorbance increase was linear.

2.5. Carbamylation assay

The carbamylation of Ile153{16} was performed by incubation of 2 μM to 100 nM variant/wild-type FVIIa with 0.2 M potassium cyanate in assay buffer (see Amidolytic activity assay) without BSA for 0–120 min. Samples were redrawn at different time points and diluted 10-fold in assay buffer containing 5 mM S-2288 (H-D-Ile-Pro-Arg-pNA, Chromogenix) and hydrolysis was monitored for 2–5 min (in a similar fashion as described for the amidolytic assay).

2.6. AT inhibition assay

AT inhibition was performed by incubation of 100 nM variant/wild-type FVIIa with 100 μg/ml AT and 1 unit/ml heparin in assay buffer for 0–45 min. Samples were redrawn at different time points and diluted 2-fold in assay buffer containing 5 mM S-2288 (H-D-Ile-Pro-Arg-pNA, Chromogenix) and hydrolysis was monitored for 2–5 min.

3. Results and discussion

3.1. Molecular design

Based on structure and sequence homologies among the serine proteases of the blood coagulation cascade, we designed different FVIIa constructs with the Met$^{298\{156\}}$Lys substitution to address both activity and macromolecular substrate selectivity. We focused on the 298{156} position within the Gly$^{283\{140\}}$–Met$^{298\{156\}}$ loop (see Fig. 1), since previous studies have shown that residues within this region, including Glu$^{296\{154\}}$, constitute part of the macromolecular substrate exosite and are determinants for the zymogen-like conformation involved in the allosteric activation of FVIIa via insertion of the N-terminus [11,12]. Rational mutagenesis of specific residues surrounding the N-terminus has led to FVIIa variants, which all included the Met$^{298\{156\}}$Gln substitution, with higher catalytic turnover of FX and of small peptidyl substrates [10–13]. The explanation for this is presumably that the Met$^{298\{156\}}$Gln mutation tethers the N-terminus via an expanded hydrogen bonding network.

In analogy to the Met$^{298\{156\}}$Gln mutation in FVIIa$_{IIa}$ (Val$^{158\{21\}}$Asp/Glu$^{296\{154\}}$Val/Met$^{298\{156\}}$Gln), the corresponding lysine mutation is believed to be able to support a similar hydrogen bond network. However, apparently it is not in itself sufficient as the single mutant shows diminished activity, at least in complex with TF [11]. To try to design a FVIIa variant with higher activity containing the mutation Met$^{298\{156\}}$Lys, we designed a FXa loop graft construct. From structural superimposition of the crystal structures of FVIIa and FXa,

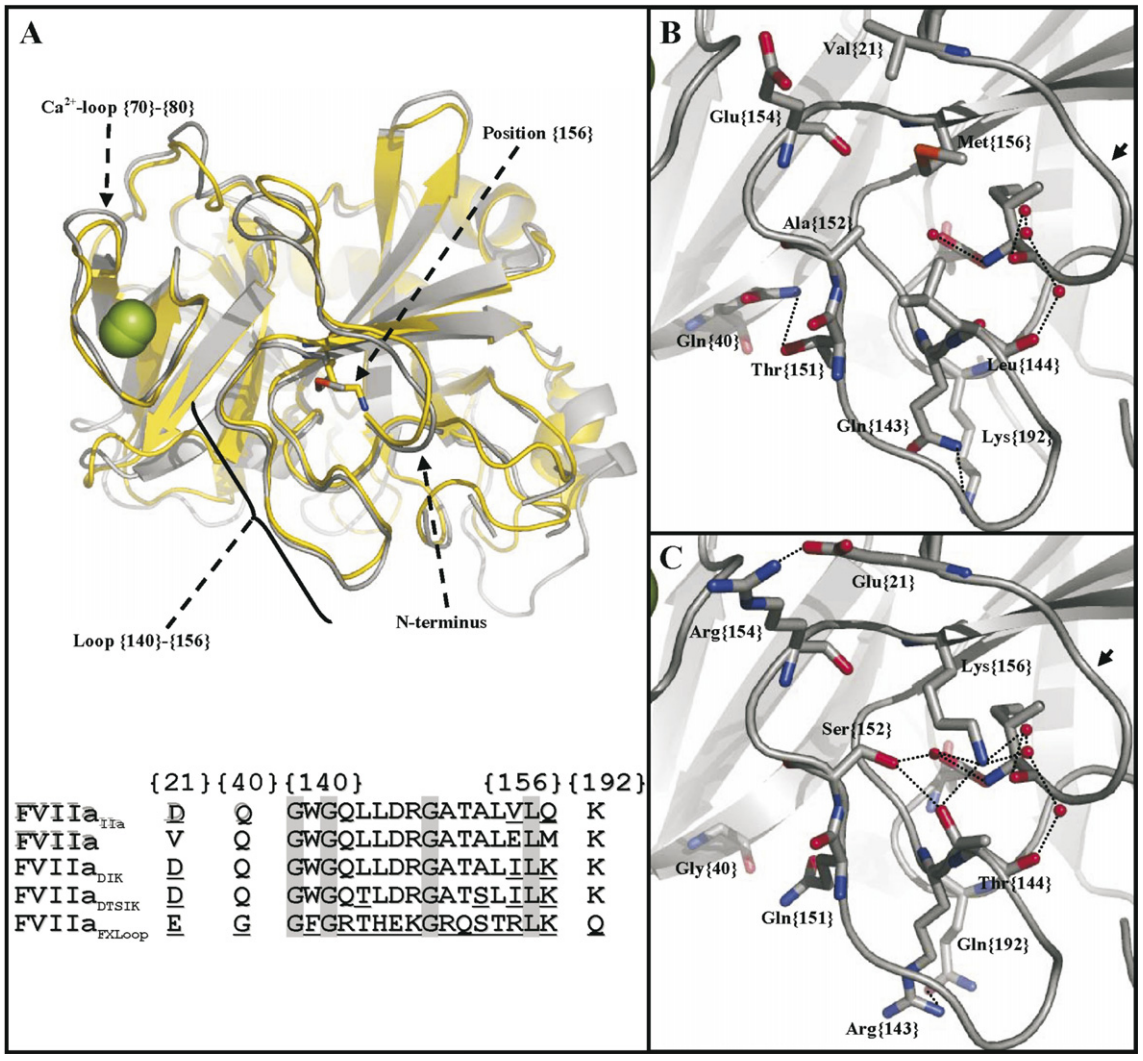

Fig. 1. Structure and sequence alignment of FVIIa and FXa. (A) Top: Superimposition of the protease domains of FVIIa (grey; pdb id. 1DAN) and FXa (yellow; pdb id. 1XKA). The grafted loop 283{140}–298{156} is indicated together with the key allosteric switch residue 298{156} (FVIIa, methionine; FXa, lysine), the Ca^{2+}-binding loop 210{70}–220{80} and the N-terminus with the salt bridge between $Ile^{153\{16\}}$ and $Asp^{343\{194\}}$ (residues not shown). The Ca^{2+} ion in the protease domain is shown as a green sphere. Note that the disordered parts (high crystallographic temperature factors) of loops $Ala^{330\{183\}}$–$Ser^{339\{190\}}$ and $Gln^{366\{217\}}$–$Phe^{374\{225\}}$ adjacent to the N-terminus of FVIIa, are not present in the analogous loops of FXa. Bottom: sequence alignment of the grafted loop 283{140}–298{156}. Shaded boxes indicate full identity, while underlines indicate performed mutations. (B) and (C) highlight important substitutions surrounding the 298{156} position. Wild-type FVIIa (B, pdb id. 1DAN) and a model of $FVIIa_{FXLoop}$ with optimized rotamer conformations (C) are shown. Arrows indicate the N-terminal tail. In B, the loop of FVIIa is shown with the centrally positioned $Met^{298\{156\}}$ engaged in a solvent exposed, hydrophobic patch composed of $Val^{158\{21\}}$, $Leu^{287\{144\}}$ and $Ala^{294\{152\}}$ with no energetically beneficial interaction with the outmost part of the N-terminal tail. In (C), the 298{156} Lys engages in a hydrogen bond network with the substituted polar $Leu^{287\{144\}}$Thr and $Ala^{294\{152\}}$Ser, which have direct impact on the outmost part of the N-terminal tail (water molecules identified in the FVIIa structure that could engage in hydrogen bonding network are shown). Thus, $Met^{298\{156\}}$Lys is packed in a hydrophilic environment, which was utilized in the design of $FVIIa_{DTSIK}$. The mutations $Val^{158\{21\}}$Glu, $Gln^{176\{40\}}$Gly and $Lys^{341\{192\}}$Gln were introduced into $FVIIa_{FXLoop}$ because of unfavourable interactions with $Glu^{296\{154\}}$Arg, $Thr^{293\{151\}}$Gln and $Gln^{286\{143\}}$Arg, respectively. Interestingly, the acidic residue $Glu^{296\{154\}}$ of FVIIa has a basic counterpart ($Arg^{376\{154\}}$) in FXa, which in FVIIa, from an activity point of view, is most optimally substituted with a hydrophobic residue as found in thrombin [10,12]. The same is the case for the mutation $Val^{158\{21\}}$Glu. In $FVIIa_{DTSIK}$, the $Val^{158\{21\}}$Asp mutation was utilized in analogy to FVIIa variant $Val^{158\{21\}}$Asp/$Glu^{296\{154\}}$Val/$Met^{298\{156\}}$Gln providing the same functionality as $Val^{158\{21\}}$Glu in $FVIIa_{FXLoop}$.

we identified potentially unfavourable interactions between residues of the inserted loop and the surrounding activation pocket of FVIIa which required optimization (Fig. 1). Because of unfavourable interactions with $Glu^{296\{154\}}$Arg, $Thr^{293\{151\}}$Gln and $Gln^{286\{143\}}$Arg, three repair mutations ($Val^{158\{21\}}$Glu, $Gln^{176\{40\}}$Gly and $Lys^{341\{192\}}$Gln) were introduced in addition to loop graft (Fig. 1). The resulting FVIIa variant, $FVIIa_{FXLoop}$, possessed increased intrinsic activity and changed macromolecular substrate specificity (see below). Two of the compensatory mutations were introduced at positions reported to influence substrate processing ($Gln^{176\{40\}}$ [8] and $Lys^{341\{192\}}$ [22]). However, we believe that the Arg side chain in position 286{143} of $FVIIa_{FXLoop}$ replaces that of $Lys^{341\{192\}}$, and that the amide of $Gln^{293\{151\}}$ replaces that of $Gln^{176\{40\}}$, thereby silencing the two compensatory mutations. The indistinguishable activities of $FVIIa_{FXLoop}$ and $FVIIa_{DTSIK}$ (FVIIa variant $Val^{158\{21\}}$Asp/$Leu^{287\{144\}}$Thr/$Ala^{294\{152\}}$-Ser/$Glu^{296\{154\}}$Ile/$Met^{298\{156\}}$Lys), with the latter having the

wild-type residues in these four positions, suggests that this is indeed the situation and that the repair strategy was successful.

In addition, a triple mutant (Val$^{158\{21\}}$Asp/Glu$^{296\{154\}}$-Ile/Met$^{298\{156\}}$Lys-FVIIa (FVIIa$_{DIK}$)) and a penta mutant (Val$^{158\{21\}}$Asp/Leu$^{287\{144\}}$Thr/Ala$^{294\{152\}}$Ser/Glu$^{296\{154\}}$Ile/Met$^{298\{156\}}$Lys-FVIIa (FVIIa$_{DTSIK}$)) were constructed to identify the (most) important substitutions in FVIIa$_{FXLoop}$. Both variants resemble the superactive mutant FVIIa variant Val$^{158\{21\}}$Asp/Glu$^{296\{154\}}$Val/Met$^{298\{156\}}$Gln previously characterized in our laboratory [10,13] in that they address amino acid changes of and in the vicinity of residue 298{156}.

3.2. Production of FVIIa variants

FVIIa$_{FXLoop}$, FVIIa variant Val$^{158\{21\}}$Asp/Leu$^{287\{144\}}$Thr/Ala$^{294\{152\}}$Ser/Glu$^{296\{154\}}$Ile/Met$^{298\{156\}}$Lys and FVIIa variant Val$^{158\{21\}}$Asp/Glu$^{296\{154\}}$Ile/Met$^{298\{156\}}$Lys were expressed and purified to homogeneity, giving distinct bands at 50 kDa on SDS–PAGE stained with Coomassie Blue (not shown). FVIIa variant Val$^{158\{21\}}$Asp/Leu$^{287\{144\}}$Thr/Ala$^{294\{152\}}$Ser/Glu$^{296\{154\}}$Ile/Met$^{298\{156\}}$Lys and FVIIa$_{FXLoop}$ were fully activated by means of autoactivation at a concentration of more than 0.5 mg/ml as judged from reduced SDS–PAGE analysis and were almost completely converted to the two-chain forms during purification. FVIIa variant Val$^{158\{21\}}$Asp/Glu$^{296\{154\}}$Ile/Met$^{298\{156\}}$Lys behaved more like wild-type FVIIa and was fully activated only upon incubation with Sepharose-coupled FXa after purification.

3.3. Amidolytic activity

Amidolytic activities were measured using two different *p*-nitroanilide peptidyl substrates, S-2288 (H-D-Ile-Pro-Arg-pNA) and S-2238 (H-D-Phe-Pip-Arg-pNA). FVIIa$_{FXLoop}$ and FVIIa$_{DTSIK}$ displayed 4–8-fold higher amidolytic activities compared to wild-type FVIIa depending on substrate (Table 1). This is similar to FVIIa variant Val$^{158\{21\}}$Asp/Glu$^{296\{154\}}$-Val/Met$^{298\{156\}}$Gln, which showed 5–7-fold higher activity as previously published [10]. Thus, FVIIa variant Val$^{158\{21\}}$Asp/Leu$^{287\{144\}}$Thr/Ala$^{294\{152\}}$Ser/Glu$^{296\{154\}}$Ile/Met$^{298\{156\}}$Lys retained the high level of activity observed for the loop graft mutant FVIIa$_{FXLoop}$. Neither of the mutants showed significantly higher activity when in complex with soluble TF compared to wild-type FVIIa, indicating that the effects of the mutations are part of, or overruled by, the effects induced by cofactor binding (data not shown). FVIIa variant Val$^{158\{21\}}$Asp/Glu$^{296\{154\}}$Ile/Met$^{298\{156\}}$Lys showed a reduced amidolytic activity of approx. 70% of wild-type and thus was not analyzed further. The results with FVIIa variant Val$^{158\{21\}}$Asp/Glu$^{296\{154\}}$Ile/Met$^{298\{156\}}$Lys demonstrates that a Lys residue in position 298{156} of FVIIa per se does not induce an activity enhancement, which was theoretically conceivable based on the role of the corresponding residue in the zymogen activity of tissue-type plasminogen activator [23]. Moreover, there is no reason to assume that this Lys residue contributes to the increased activities of FVIIa$_{FXLoop}$ and FVIIa variant Val$^{158\{21\}}$Asp/Leu$^{287\{144\}}$Thr/Ala$^{294\{152\}}$Ser/Glu$^{296\{154\}}$-Ile/Met$^{298\{156\}}$Lys by a direct interaction with Asp343{194} especially considering that these two FVIIa variants have a Thr residue in position 287{144} as in FX [23].

Table 1
Enzymatic activities without TF relative to wild-type FVIIa (set to 1)

	Amidolytic activity (ΔA_{405}/min)		Proteolytic activity (k_{cat}/K_m)	
	S-2288	S-2238	Factor IX	Factor X
Wild-type	1.0	1.0	1.0	1.0
FVIIa$_{FXLoop}$	8.1±0.1	3.8±1.2	4.3±1.3	0.5±0.1
FVIIa$_{DTSIK}$	8.7±0.6	6.1±1.0	5.7±1.0	0.5±0.3
FVIIa$_{DIK}$	0.7±0.1	n.d.	n.d.	n.d.

3.4. Active site maturation and N-terminus accessibility as judged from AT inhibition and carbamylation of Ile$^{153\{16\}}$

The susceptibility of TF-bound FVIIa to inhibition by AT has been shown to be 33-fold enhanced compared to free FVIIa as a result of maturation of the active site [4,24]. Thus, changes in AT inhibition kinetics can be used to assess the state of the active site. Previous studies of FVIIa variant Val$^{158\{21\}}$Asp/Glu$^{296\{154\}}$Val/Met$^{298\{156\}}$Gln and other variants with higher intrinsic activities have shown a correlation between a faster rate of inhibition by AT and a higher level of intrinsic amidolytic activity of the enzyme [10]. We observed 3.5-fold and 3-fold faster inhibition of FVIIa$_{FXLoop}$ and FVIIa variant Val$^{158\{21\}}$Asp/Leu$^{287\{144\}}$Thr/Ala$^{294\{152\}}$Ser/Glu$^{296\{154\}}$Ile/Met$^{298\{156\}}$Lys, respectively, compared to wild-type FVIIa (Fig. 2), supporting a maturation of the active site with improved substrate accommodation. FVIIa variant Val$^{158\{21\}}$Asp/Glu$^{296\{154\}}$Val/Met$^{298\{156\}}$Gln was included for comparison and was inhibited 4.5 times faster (data not shown).

Chemical modification of the N-terminal Ile$^{153\{16\}}$ of FVIIa by means of carbamylation is a way of assessing the extent of burial in the activation pocket. In essence, the more buried an N-terminus, the less susceptibility to modification and resulting reduction of enzymatic activity. This has been shown to correlate with activity and TF binding in the sense that the active conformation of FVIIa has a more buried N-terminus [25,26], and enhanced burial of the N-terminus induced by mutagenesis has been shown to be accompanied by a shift in the equilibrium of FVIIa from a zymogen-like towards a catalytically competent conformation [10]. Carbamylation was

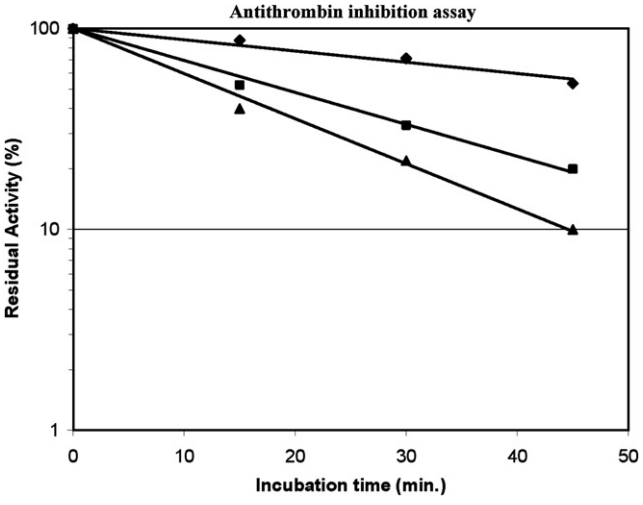

Fig. 2. Antithrombin inhibition assay. The residual activity of wild-type FVIIa (♦), FVIIa$_{DTSIK}$ (■) and FVIIa$_{FXLoop}$ (▲) after indicated incubation times with antithrombin is shown. The curves show the result of a representative experiment (n = 3).

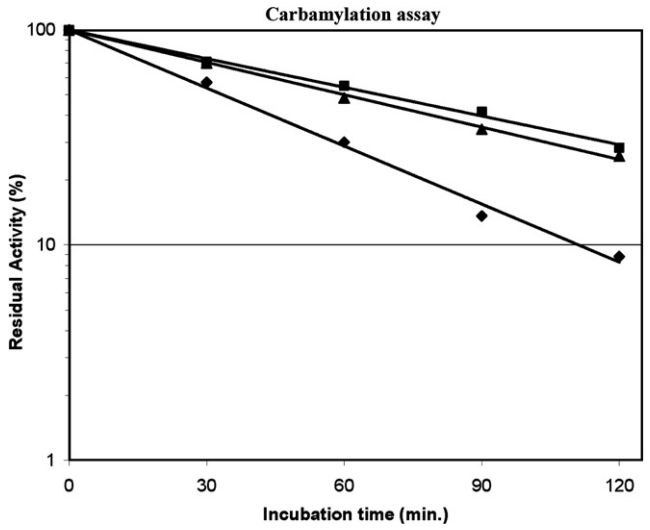

Fig. 3. Carbamylation assay. The residual activity of wild-type FVIIa (◆), $FVIIa_{DTSIK}$ (■) and $FVIIa_{FXLoop}$ (▲) after indicated incubation times with KOCN is shown. The curves show the result of a representative experiment ($n = 3$).

used here as a tool to assess the accessibilities of the N-termini in $FVIIa_{FXLoop}$ and FVIIa variant $Val^{158\{21\}}Asp/Leu^{287\{144\}}Thr/Ala^{294\{152\}}Ser/Glu^{296\{154\}}Ile/Met^{298\{156\}}Lys$. The carbamylation rate was decreased about 2-fold for both variants compared to that of wild-type (Fig. 3), indicating a more inserted N-terminus. Thus, the higher intrinsic activities of the two FVIIa variants concur with a higher degree of burial of the N-terminus as seen previously for FVIIa variant $Val^{158\{21\}}Asp/Glu^{296\{154\}}Val/Met^{298\{156\}}Gln$. Hence, the hydrogen bonding network surrounding the N-terminus in the activation pocket appears to be stabilized also after introduction of a lysine in position 298{156}.

3.5. Proteolytic activity

The ability to proteolytically activate the zymogens FIX and FX was quantified by determining k_{cat}/K_m [27]. In the free form, $FVIIa_{FXLoop}$ showed a 4.3-fold higher cleavage rate of FIX than FVIIa (Table 1), whereas in complex with soluble TF, $FVIIa_{FXLoop}$ and FVIIa cleaved FIX at equal rates. Interestingly, a 2-fold slower activation rate of FX compared to that of FVIIa was observed both in the presence and absence of TF, indicating that activation was compromised upon loop grafting. FVIIa variant $Val^{158\{21\}}Asp/Leu^{287\{144\}}Thr/Ala^{294\{152\}}Ser/Glu^{296\{154\}}Ile/Met^{298\{156\}}Lys$ exhibited the same profile, with a 5.7-fold faster activation of FIX (also only in the free form) and a 2-fold slower activation of FX both in the absence and presence of soluble TF (Table 1). Free FVIIa variant $Val^{158\{21\}}Asp/Glu^{296\{154\}}Val/Met^{298\{156\}}Gln$ appeared to cleave both macromolecular substrates at increased rates compared to wild-type FVIIa, whereas FVIIa variant $Val^{158\{21\}}Asp/Glu^{296\{154\}}Val/Met^{298\{156\}}Gln$ bound to soluble TF was indistinguishable from FVIIa [10]. The increased ability to activate FIX compared to FX observed for $FVIIa_{FXLoop}$ and FVIIa variant $Val^{158\{21\}}Asp/Leu^{287\{144\}}Thr/Ala^{294\{152\}}Ser/Glu^{296\{154\}}Ile/Met^{298\{156\}}Lys$ is interesting and shows that the $Gly^{283\{140\}}$–$Met^{298\{156\}}$ loop helps to define the macromolecular substrate exosite specificity in agreement with previous data [8,10,28]. Inspection of the differences between $FVIIa_{FXLoop}$ or FVIIa variant $Val^{158\{21\}}Asp/Leu^{287\{144\}}Thr/Ala^{294\{152\}}Ser/Glu^{296\{154\}}Ile/Met^{298\{156\}}Lys$ and FVIIa variant $Val^{158\{21\}}Asp/Glu^{296\{154\}}Val/Met^{298\{156\}}Gln$ reveals residues that determine the specificity among the coagulation factors. The $Met^{298\{156\}}Lys$ mutation appears to have a central role, while the mutations $Val^{158\{21\}}Asp$ and $Glu^{296\{154\}}Ile$ presumably act as in FVIIa variant $Val^{158\{21\}}Asp/Glu^{296\{154\}}Val/Met^{298\{156\}}Gln$ and increase the catalytic efficiency by stabilizing the catalytically competent state of FVIIa via burial of the N-terminus in the activation pocket. A hydrophilic glutamine residue in the 298{156} position, as in factor IXa and in the majority of chymotrypsin-like serine proteases, results in an improved ability to activate both FX as previously reported [10–13] and FIX. Whereas the same effect on the two macromolecular substrates is achieved with glutamine, the introduction of a positive charge via the $Met^{298\{156\}}Lys$ mutation apparently hampers FX activation. The two hydrophilic substitutions $Leu^{287\{144\}}Thr$ and $Ala^{294\{152\}}Ser$ cannot be ruled out as being important for the differential effect as well. However, these two residues appear to be necessary for optimal accommodation of lysine in the 298{156} position.

3.6. Speculation

We show in this study, that by replacing $Met^{298\{156\}}$ in FVIIa with Lys and providing the new side chain with an optimal environment, a positive effect on catalytic turnover and an altered macromolecular substrate specificity is obtained. Since only $FVIIa_{FXLoop}$ and FVIIa variant $Val^{158\{21\}}Asp/Leu^{287\{144\}}Thr/Ala^{294\{152\}}Ser/Glu^{296\{154\}}Ile/Met^{298\{156\}}Lys$ (not $FVIIa_{DIK}$) turned out to have increased amidolytic activity compared to wild-type, we believe that a hydrophilic environment, accomplished by the mutations $Leu^{287\{144\}}Thr$ and $Ala^{294\{152\}}Ser$, surrounding the Lys side chain in position 298{156} is mandatory for enhanced activity (Fig. 1). Besides an increased amidolytic activity, FVIIa variant $Val^{158\{21\}}Asp/Leu^{287\{144\}}Thr/Ala^{294\{152\}}Ser/Glu^{296\{154\}}Ile/Met^{298\{156\}}Lys$ showed specificity almost identical to that of $FVIIa_{FXLoop}$.

Residue 298{156} is a part of the interface between macromolecular substrates and FVIIa, which is extensive and involves several residues as shown by alanine scanning mutagenesis [28]. Based on models of the ternary complex of FVIIa·TF·FXa, the $Gly^{\{140\}}$–$Met^{\{156\}}$ loops of FVIIa and FX(a) can be pinpointed as spatially centered in the interface between the two protease domains [18, O. H. Olsen, unpublished data]. Since the models suggest that the loop is part of both the exosite of the enzyme and the binding site of the macromolecular substrate, it apparently acts as a specificity anchor in the enzyme-substrate interaction and the Lys in position 298{156} in the FVIIa variants might be incompatible with the corresponding $Lys^{378\{156\}}$ of zymogen FX due to charge repulsion. This could explain the decreased activation of zymogen FX. Hence, the side chain in this position appears to reflect the sequential order in which these enzymes work in the coagulation cascade. FVIIa (Met298 {156}) activates both FIX (Gln{156}) and FX (Lys{156}). FIXa activates FX, whereas FXa activates prothrombin (Gln{156}) to thrombin. Other specificity determinants do obviously exist, because otherwise thrombin would for instance readily activate FX, FVII and prothrombin. Moreover, the fact that the $FVIIa_{FXLoop}$ and FVIIa variant $Val^{158\{21\}}Asp/Leu^{287\{144\}}Thr/Ala^{294\{152\}}Ser/Glu^{296\{154\}}Ile/Met^{298\{156\}}Lys$ variants readily autoactivate also

points in this direction. It should also be kept in mind that the three substrates for FVIIa (FVII, FIX and FX) may bind to slightly different exosites. Many residues are involved in the binding of macromolecular substrates and they are not restricted to the protease domain [29,30] and even extend to interactions between FIX/FX and TF [31]. A subgroup of residues apparently confer specificity, such as that in position {156} identified here in two super active FVIIa variants mimicking the $Gly^{\{140\}}$–$Met^{\{156\}}$ loop of FX.

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