

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO	
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DEP: 2020 DIRECCION DE NUEVAS CREACIONES	EVE: 378 FASE NACIONAL INCLUIDO CAPITULO I
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II	FOLIOS: 8
ACT: 519 PRESENTACION	

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
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Asunto: Radicación: 13-136450- -3
 Trámite: 11
 Evento: 378
 Actuación: 519
 Oficio: 7877
 Folios: 8

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I	X	Capítulo II	
No. Solicitud Internacional	PCT/US2011/059297				
Fecha de Presentación Internacional	04/11/2011				

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:	Radicado 13-136450-00000-0000	5/Junio/2013
Reivindicaciones:	Radicado 13-136450-00000-0000	5/Junio/2013
	Radicado 13-136450-00001-0000	16/Enero/2014
Dibujos:	Radicado 13-136450-00000-0000	5/Junio/2013
Prioridad:	US 61 410 437	5/Noviembre/2010

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. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del capítulo primero del Título 10 de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por la 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 el presente caso se estudiarán las reivindicaciones 1 a 23 del capítulo reivindicatorio (Radicado N°. 13-136450-00001-0000).

Título de la solicitud: “Una Nueva Variante del Factor VIII Antihemofílico con Actividad Específica Aumentada”.

El problema planteado en esta solicitud consiste en la necesidad de proporcionar una molécula de factor VIII más potente para el tratamiento de pacientes que sufren de hemofilia.

Para resolver este problema técnico la solicitud en estudio proporciona una variante del factor VIII antihemofílico que tiene actividad específica aumentada en comparación con productos conocidos del factor VIII.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K 038/037

4. Excepción a la Patentabilidad (Art 20 D 486 literal (d))

El objeto de las reivindicaciones 18 y 20 – 23 no es patentable de acuerdo con el Art citado.

5. Reivindicaciones relativas a un uso (Art 14 D 486)

El uso de la reivindicación 19 no es patentable, de acuerdo con el Art 14.

6. De la solicitud de patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

La reivindicación 1 no es clara porque:

La variante del factor VIII reclamada debe ser definida a partir de su estructura completa indicando el número de la secuencia de la misma. La alusión a que está desprovista de una secuencia de aminoácidos correspondiente a los aminoácidos 716 a 742 del factor VIII porcino como se representa en la Figura 2 o los aminoácidos 714 a 740 de la sec. con núm. de ident.: 7, no permite definirla. Se sugiere definirla correctamente.

Hace referencia a los dibujos, según la expresión “como se representa en la Figura 2”. Se sugiere eliminar la referencia a los dibujos.

Las reivindicaciones 2 - 4 no son claras porque definen la variante de FVIII a partir de porcentajes de homología lo que no permite establecer la secuencia nucleótida exacta a la que se refiere y además, no hay sustento que demuestre que todas las posibles variables tengan la actividad que se quiere demostrar. Se sugiere definirlo a partir de la secuencia nucleótida exacta indicando el número de la misma (SEQ ID NO).

La reivindicación 5 es inconsistente con la información divulgada en las reivindicaciones 2 – 5.

La reivindicación 6 no es clara porque:

La variante del factor VIII reclamada debe ser definida a partir de su estructura completa indicando el número de la secuencia de la misma. La alusión a que está desprovista de una secuencia de aminoácidos correspondiente a los aminoácidos 716 a 742 del factor VIII humano como se representa en la Figura 2 o los aminoácidos 714

a 740 de la sec. con núm. de ident.: 8, no permite definirla. Se sugiere definirla correctamente.

Hace referencia a los dibujos, según la expresión “como se representa en la Figura 2”. Se sugiere eliminar la referencia a los dibujos.

Las reivindicaciones 7 - 9 no son claras porque definen la variante de FVIII a partir de porcentajes de homología lo que no permite establecer la secuencia nucleótida exacta a la que se refiere y además, no hay sustento que demuestre que todas las posibles variables tengan la actividad que se quiere demostrar. Se sugiere definirlo a partir de la secuencia nucleótida exacta indicando el número de la misma (SEQ ID NO).

La reivindicación 10 es inconsistente con la información divulgada en las reivindicaciones 7 – 9.

Las reivindicaciones 1 – 10 no son claras porque no hay sustento que demuestre que la sec. con núm. de ident.: 1 de un FVIIIv3 porcino y la sec. con núm. de ident.: 2 de un FVIIIv3 humano corresponda a la variante de FVIIIv3 probada en los ejemplos presentados por la solicitante.

El polinucleótido debe ser caracterizado a partir de su secuencia nucleótida (reivindicación 12).

El vector reclamado en la reivindicación 13 debe ser descrito especificando por lo menos el tipo de vector y la secuencia recombinante que contiene.

La célula de mamífero reclamada en la reivindicación 14 debe ser definida como una línea celular e indicar su número de depósito.

El encabezado de la reivindicación 16 no es claro debido a que definen una composición farmacéutica que comprende la variante de FVIII junto con excipientes adecuados, se sugiere definir la composición por estas características y no caracterizar el método por las características de la composición, los métodos deben definirse mediante características como alguna etapa y alguna condición específica en que debiera realizarse. Además, se debe especificar los fármacos con los que se combina por medio de su denominación común internacional y tener pleno sustento en la descripción. Se sugiere modificar el encabezado de la reivindicación.

La composición farmacéutica reclamada en la reivindicación 17, debe ser definida por sus ingredientes, indicando las proporciones de cada uno de ellos dentro de la composición y tener pleno sustento en la descripción.

El capítulo reivindicatorio no es conciso porque contiene un excesivo número de reivindicaciones independientes, lo cual hace difícil definir cuál es el objeto que se busca proteger.

Descripción (Art 28 D 486)

Las reivindicaciones deben definir una razonable generalización de lo que se encuentra debidamente sustentado y para el presente caso las variantes del FVIII no cuentan con el debido soporte en la descripción.

La descripción no divulga la invención de manera suficientemente completa para que la persona del oficio normalmente versada en la materia pueda ejecutarla ó reproducirla.

Debido a que la solicitud se refiere a material biológico, es necesario presentar el Certificado de Depósito para el vector y célula de mamífero.

Se requiere a la solicitante hacer las aclaraciones necesarias.

7. Unidad de invención (Art 25 D 486)

La solicitud no cumple con el requisito de unidad de invención porque se refiere a 2, grupos inventivos.

Los grupos inventivos están definidos por las siguientes características esenciales:

- Grupo I: Reivindicaciones 1-5, 11 y 12-17 parcialmente. Variante del FVIII desprovista de una secuencia de aminoácidos correspondiente a los aminoácidos 716 a 742 del factor VIII porcino como se representa en la Figura 2 o los aminoácidos 714 a 740 de la sec. con núm. de ident.: 7.
- Grupo II: Reivindicaciones 6-10, 11 y 12-17 parcialmente. Variante del FVIII desprovista de una secuencia de aminoácidos correspondiente a los aminoácidos 716 a 742 del factor VIII humano como se representa en la Figura 2 o los aminoácidos 714 a 740 de la sec. con núm. de ident.: 8.

Se le sugiere a la solicitante restringir la materia a patentar en un solo concepto común inventivo especificando el avance tecnológico sobre el estado de la técnica. Esto se logra entre otras formas definiendo las características esenciales de la invención y mostrando las otras características particulares como dependientes, pero sin perjuicio de la concisión de las nuevas reivindicaciones tanto independientes como dependientes.

La solicitante también puede realizar una o más de las divisionales de acuerdo a los grupos de conceptos técnicos inventivos presentados en esta solicitud, esto claro, sin ampliar el objeto inicial de la invención.

8. 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: noviembre 5 de 2010, se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	US2003068785	“Modified factor VIII”	10/Abril/2003
D2	Donath et al.	“Kinetics of factor VIII light-chain cleavage by thrombin and factor Xa A regulatory role of the factor VIII heavy-chain region Lys713-Arg740”	1996

El documento D1¹ divulga un loci de aminoácidos específico del factor humano VIII que interactúa con anticuerpos inhibidores de pacientes con hemofilia que han desarrollado tales anticuerpos después de ser tratados con el factor VIII. El factor VIII modificado es útil para el tratamiento de pacientes hemofílicos, ya sea para evitar o para prevenir la acción de los anticuerpos inhibidores.

El documento D2² divulga la cinética de la activación del factor VIII con una delección que incluye la secuencia Lys713 - Arg740 en un sistema que consiste en factor VIII humano, el factor IXa, el factor X, fosfolípidos, y la trombina o el factor Xa.

9. Examen de Patentabilidad (Art 14 D486)

Nivel inventivo Grupo I (Art18 D486)

1 http://worldwide.espacenet.com/advancedSearch?locale=en_EP ingresar US2003068785

2 Eur. J. Biochem. 240, 365-372 (1996)

La reivindicación 1 consiste en: *“Una variante del factor VIII (FVIII) aislado y recombinante, con el dominio B total o parcialmente eliminado, la variante del FVIII está desprovista de una secuencia de aminoácidos correspondiente a los aminoácidos 716 a 742 del factor VIII porcino como se representa en la Figura 2 o los aminoácidos 714 a 740 de la sec. con núm. de ident.: 7”* (Radicado N°. 13-136450-00001-0000).

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

Características esenciales	D1	D2
Variante del factor VIII aislado y recombinante	Factor VIII hibrido aislado y recombinante (Pág. 2 , Párr. 0017)	Variante del factor VIII purificado (Pág. 366, Col. 1)
Dominio B total o parcialmente eliminado	Dominio B total o parcialmente eliminado (Pág. 2 , Párr. 0022)	Carece de casi todo el Dominio B (Pág. 367, Col. 1)
Secuencia de aminoácidos correspondiente a los aminoácidos 716 a 742 del factor VIII porcino como se representa en la Figura 2 o los aminoácidos 714 a 740 de la sec. con núm. de ident.: 7	SEQ ID NO: 2 del factor VIII porcino (Pág. 3 , Párr. 0032)	Secuencia de aminoácidos Lys713-Arg740 del factor VIII humano (Resumen y Pág. 366, Col. 1)

El documento D1 se considera el estado de la técnica cercano a la invención definida en la reivindicación 1 porque divulga una variante del factor VIII porcino aislado y recombinante en la que la secuencia de aminoácidos se cambia por una sustitución en uno o más de los loci específicos, dominio B total o parcialmente eliminado, que comprende una secuencia aminoácida SEQ ID NO: 2 que es 99.7% idéntica a la SEQ ID NO: 1 de la presente invención. Además, enseña una composición farmacéutica que comprende la variante del factor VIII y un método para tratar un paciente que sufre de hemofilia que comprende administrar una cantidad terapéuticamente eficaz de la variante de FVIII.

La diferencia entre la invención, definida en la reivindicación 1 y la variante FVIII divulgada de D1 consiste en la secuencia de aminoácidos residuos 716 a 742 del factor VIII porcino.

El efecto que logra el incluir la secuencia de aminoácidos residuos 716 a 742 del factor VIII porcino es que aparentemente tiene una actividad específica aumentada en comparación con proteínas similares con dominio B parcialmente eliminado, sin embargo no es posible comprobar de acuerdo a la información divulgada en la descripción que la sec. con núm. de ident.: 1 de un FVIIIv3 porcino corresponda a la variante de FVIIIv3 probada en los ejemplos presentados por la solicitante.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: cómo obtener moléculas de factor VIII más potentes para el tratamiento de pacientes que sufren de hemofilia.

Sin embargo, la utilización de la secuencia de aminoácidos residuos 716 a 742 del factor VIII porcino para el aumento de la actividad específica, ya está divulgada en el documento D2 que se refiere a una variante del factor VIII humano recombinante, que tiene una delección de la secuencia de aminoácidos Lys713-Arg740 del dominio B que muestra una constante de velocidad de activación por trombina reducida diez veces en comparación con el factor VIII derivado de plasma, además sugiere, que este valor es similar al presentado por la activación del factor VIII porcino bajo condiciones ligeramente diferentes (Pág. 370, Col. 1).

En consecuencia, la persona del oficio normalmente versada en la materia, generaría variaciones del factor VIII recombinante en el dominio B empleando técnicas convencionales y métodos de biología molecular de acuerdo a las enseñanzas de los

documentos D1 y D2 para llegar así al objeto de la reivindicación 1 de la solicitud. Por lo cual se considera obvia.

Conclusión:

Las reivindicaciones 1-5, 11 y 12-17 parcialmente cumplen el requisito de novedad (Art. 16), pero no cumplen con el requisito de nivel inventivo (Art. 18).

Nivel inventivo Grupo II (Art18 D486)

La reivindicación 6 consiste en: *“Una variante del factor VIII (FVIII) aislado y recombinante, con el dominio B total o parcialmente eliminado, la variante del FVIII está desprovista de una secuencia de aminoácidos correspondiente a los aminoácidos 716 a 742 del factor VIII humano como se representa en la Figura 2 o los aminoácidos 714 a 740 de la sec. con núm. de ident.: 8”* (Radicado N°. 13-136450-00001-0000).

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D2
Variante del factor VIII aislado y recombinante	Factor VIII hibrido aislado y recombinante (Pág. 2 , Párr. 0017)	Variante del factor VIII purificado (Pág. 366, Col. 1)
Dominio B total o parcialmente eliminado	Dominio B total o parcialmente eliminado (Pág. 2 , Párr. 0022)	Carece de casi todo el Dominio B (Pág. 367, Col. 1)
Secuencia de aminoácidos correspondiente a los aminoácidos 716 a 742 del factor VIII humano como se representa en la Figura 2 o los aminoácidos 714 a 740 de la sec. con núm. de ident.: 8	SEQ ID NO: 2 del factor VIII porcino (Pág. 3 , Párr. 0032)	Secuencia de aminoácidos Lys713-Arg740 del factor VIII humano (Resumen y Pág. 366, Col. 1)

Los documentos D1 y D2 se consideran el estado de la técnica cercano porque divulgan variantes del factor VIII aislado y recombinante, sin embargo, el documento D1 divulga una variante del factor VIII humano que comprende una secuencia aminoácida SEQ ID NO: 39 que es 91.9% idéntica a la SEQ ID NO: 2 de la presente invención.

La variante reivindicada difiere del documento D1 en la secuencia de aminoácidos residuos 716 a 742 del factor VIII humano.

El efecto logrado por el hecho de incluir la secuencia de aminoácidos residuos 716 a 742 del factor VIII humano es que aparentemente tiene una actividad específica aumentada en comparación con proteínas similares con dominio B parcialmente eliminado, sin embargo no es posible comprobar de acuerdo a la información divulgada en la descripción que la sec. con núm. de ident.: 2 de un FVIIIv3 humano corresponda a la variante de FVIIIv3 probada en los ejemplos presentados por la solicitante.

Por lo tanto, el problema técnico objetivo a resolver consiste en: cómo obtener moléculas de factor VIII más potentes para el tratamiento de pacientes que sufren de hemofilia.

Sin embargo, la utilización de la secuencia de aminoácidos residuos 716 a 742 del factor VIII humano para el aumento de la actividad específica, ya está divulgada en el documento D2 que se refiere a una variante del factor VIII humano recombinante, que tiene una delección de la secuencia de aminoácidos Lys713-Arg740 del dominio B que muestra una constante de velocidad de activación por trombina reducida diez veces en comparación con el factor VIII derivado de plasma (Pág. 370, Col. 1).

De manera que el estado de la técnica sugiere a la persona del oficio normalmente versada en la materia la forma en que se resuelve el problema en esta reivindicación. Por lo cual no se considera obvia.

Conclusión:

Las reivindicaciones 6-10, 11 y 12-17 parcialmente cumplen el requisito de novedad (Art. 16), pero no cumplen con el requisito de nivel inventivo (Art. 18).

10. Conclusión

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

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	US2003068785	1 – 17	Nivel inventivo
D2	Donath et al.	1 – 17	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 2014-07-08

Elaboró: Sindy Johana Brochero Ballesteros
Revisó: Consuelo Leguizamon Leguizamon

Kinetics of factor VIII light-chain cleavage by thrombin and factor Xa A regulatory role of the factor VIII heavy-chain region Lys713–Arg740

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Activation and limited proteolysis of factor VIII have been investigated with respect to the role of the heavy-chain region Lys713–Arg740. The kinetics of factor VIII activation have been analyzed in a system consisting of human factor VIII, factor IXa, factor X, phospholipids, and thrombin or factor Xa. Plasma-derived factor VIII is activated by thrombin with a second-order rate constant of $3.3 \pm 0.3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, which proved to be slightly higher than for activation by factor Xa. The second-order rate constant of activation by thrombin of plasma-derived factor VIII in the presence of a monoclonal antibody against the sequence Lys713–Arg740 is markedly reduced. The same result was obtained for activation by thrombin and factor Xa of factor VIII with a deletion including the sequence Lys713–Arg740, des-(713–1637)-factor VIII. This suggests that the region Lys713–Arg740 promotes factor VIII activation by both thrombin and factor Xa. Since factor VIII activation is associated with proteolysis, cleavage of factor VIII heavy and light chains was analyzed quantitatively. These studies indicated that heavy-chain cleavage of des-(713–1637)-factor VIII is similar to that of plasma-derived factor VIII. In contrast, cleavage of the light chain of des-(713–1637)-factor VIII is clearly reduced. Furthermore, the second-order rate constant ($0.2 \pm 0.1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$) of des-(713–1637)-factor VIII light-chain cleavage by thrombin was reduced tenfold compared with that of plasma-derived factor VIII. Proteolysis by factor Xa yielded similar results. The rate of des-(713–1637)-factor VIII light-chain cleavage by thrombin is similar to that of isolated light-chain, but isolated light-chain is cleaved by factor Xa 20-fold more efficiently than the light chain in des-(713–1637)-factor VIII. We conclude that activation of factor VIII by both thrombin and factor Xa is closely associated with light-chain cleavage. Furthermore, within the factor VIII heterodimer, the heavy-chain sequence Lys713–Arg740 promotes both activation and light-chain proteolysis.

Keywords: blood coagulation; proteolysis; activation; factor VIII; acidic region.

Factor VIII serves an important role in blood coagulation since its absence or dysfunction is associated with the bleeding disorder haemophilia A. In the intrinsic pathway of factor X activation, factor VIII functions as cofactor for the serine protease factor IXa. The cofactor is synthesized as a large single-chain precursor protein of 2332 amino acids, which is arranged in a domain structure having the sequence A1-A2-B-A3-C1-C2 [1, 2]. In plasma, factor VIII circulates as a heterodimeric protein consisting of a light chain (A3-C1-C2) and a heavy chain (A1-A2-B). The mass of the light chain is 80 kDa whereas the size of the heavy chain is in the range 90–220 kDa due to proteolysis within the B-domain [3–5]. The B-domain is dispensable for factor VIII function which has been assessed *in vitro* and *in*

vivo [3, 4, 6–9]. Heterodimeric plasma factor VIII does not exert cofactor activity unless it is converted into an active species by limited proteolysis. Both thrombin and factor Xa are capable of activating factor VIII by cleavage in the heavy chain at amino acid positions Arg372 and Arg740 and in the light chain at Arg1689 [4, 10]. Recently, cleavage by factor Xa at position Arg1721 in factor VIII light chain has also been associated with factor VIII activation [11]. The significance of two cleavages at Arg372 and Arg1689 during factor VIII activation is apparent from the occurrence of point mutations at these positions in factor VIII in several haemophilia A patients [12]. Furthermore, their role in factor VIII activation has been established by *in vitro* mutagenesis studies [10].

A striking observation with regard to factor VIII structure is that all thrombin cleavage sites are adjacent to a region particularly rich in acidic amino acids and containing sulphated tyrosine residues that are at amino acid positions 346, 718, 719, 723, 1664, and 1680 [13–15]. These acidic regions are proposed to represent high-affinity thrombin-binding sites as they are homologous with binding sites for thrombin in proteins like hirudin [16], and heparin cofactor II [17]. The acidic heavy-chain region Ser337–Arg372 is involved in the stabilization of the heterotrimeric structure of activated factor VIII [18]. Another acidic region comprising the sequence Glu1649–Arg1689 of the light

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Abbreviations. PtdSer, 3-*sn*-phosphatidyl-L-serine; PtdCho, 3-*sn*-phosphatidylcholine; Glu-Gly-Arg-CH₂Cl, glutamylglycylarginine-chloromethane; Phe-Pro-Arg-CH₂Cl, phenylalanylprolylargininechloromethane.

Enzymes. Thrombin (EC 3.4.21.5); coagulation factor Xa (EC 3.4.21.6); coagulation factor IXa (EC 3.4.21.22).

chain apparently has a dual role in factor VIII function: in particular, sulphation of the tyrosine residue at position 1680 in the acidic region of the light chain is required for the high-affinity interaction of factor VIII with von Willebrand factor [6, 19] and cleavage of this region from factor VIII light chain is associated with development of factor VIII cofactor activity [11, 19]. The heavy-chain acidic region Lys713–Arg740 was previously assessed to be important in factor VIII function as factor VIII activity was inhibited by monoclonal antibodies against this sequence [20]. More specifically, residues 721–729 have been reported to contribute to full factor VIII activity *in vitro* [21]. In addition, a heterodimeric factor VIII variant in which the heavy-chain lacks residues 713–740 has been found to display a reduced haemostatic activity *in vivo* [7].

The aim of the present study was to examine the role of the acidic region Lys713–Arg740 in factor VIII activation and proteolysis by both thrombin and factor Xa. For both factor-VIII-activating enzymes, rate constants of activation and limited proteolysis of a factor VIII heterodimer lacking the heavy-chain sequence 713–740 have been determined. In this way, a relation has been established between the role of the region Lys713–Arg740 in activation of factor VIII and proteolysis at specific cleavage sites.

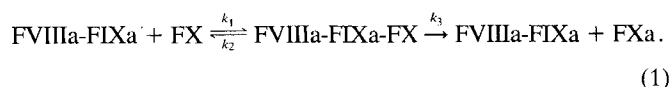
MATERIALS AND METHODS

Materials. The synthetic substrate S2337 was purchased from Chromogenix AB. Hirudin (from leeches), 3-*sn*-phosphatidyl-L-serine (PtdSer), and 3-*sn*-phosphatidylcholine, type I-EH (PtdCho) were from Sigma. Glutamylglycylargininechloromethane (Glu-Gly-Arg-CH₂Cl) and phenylalanylprolylargininechloromethane (Phe-Pro-Arg-CH₂Cl) were obtained from Calbiochem. All other reagents were analytical grade.

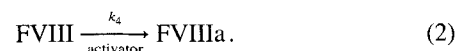
Antibodies and purified proteins. Anti-(factor VIII light chain) monoclonal antibodies CLB-Cag 12, CLB-Cag 69, and CLB-Cag 117, and anti-(factor VIII heavy chain) monoclonal antibody CLB-Cag 9 have been described previously [20, 22]. The polyclonal anti-(factor VIII) antibody p53 (raised against a peptide comprising the factor VIII sequence 329–345) was generously donated by Dr D. S. Pepper (Scottish National Blood Transfusion Service, Edinburgh). A polyclonal antibody against factor VIII light chain was obtained by immunizing rabbits with thrombin-cleaved purified factor VIII light chain [11, 23] employing standard procedures. Human factor VIII and its light-chain subunit were purified according to a previously described method [23]. The construction, production and purification of des-(713–1637)-factor VIII have been described in detail elsewhere [7]. Plasma factor VIII differs at the protein level from heterodimeric des-(713–1637)-factor VIII in that the latter lacks the heavy-chain sequence Lys713–Arg740. Concentrations of factor IXa and factor Xa [24], factor X [25], and α -thrombin [26] were determined by active site titration [25]. Proteins were homogenous as assessed by SDS/PAGE.

Factor Xa formation. Activation of factor VIII was determined using factor Xa formation experiments [26]. Briefly, phospholipid vesicles (100 μ M) were prepared from PtdSer and PtdCho (1:1 molar ratio) and incubated in a Ca²⁺-containing buffer. Then factor IXa (0.3 nM), factor VIII (0.4 nM), factor X (0.2 μ M), and various concentrations of thrombin or factor Xa were added in the denoted order. Subsamples were removed and analyzed for factor Xa concentration employing the synthetic substrate S2337. The conversion of substrate was monitored at 405 nm and active-site-titrated factor Xa was used to relate substrate hydrolysis to factor Xa concentrations. Thrombin was inhibited by the addition of hirudin (1 U/pmol thrombin).

Kinetics of factor VIII activation. We have analyzed the activation of factor VIII kinetically via its contribution as cofactor for factor IXa in factor Xa formation. Factor X activation is represented by the following scheme:



Factor VIII is present in its non-activated form at the start of factor Xa formation. Therefore, the initial factor Xa formation is coupled to the activation of factor VIII:



Factor Xa formation in the presence of an increasing concentration of cofactor results in increasing reaction velocities with time. A general solution for a system of two coupled reactions has previously been given [27, 28], and the same model is used to analyze the exponentially increasing factor Xa formation (compare Fig. 1):

$$[\text{FXa}]_t - [\text{FXa}]_0 = ck_4t^2. \quad (3)$$

Eqn (3) is derived after integration between $t = 0$ and $t = t$ of the following equation:

$$\frac{d[\text{FXa}]}{dt} = \frac{k_4[\text{FVIII}]_0 tk_3[\text{FX}]_0}{K_m + [\text{FX}]_0} \quad (4)$$

where $k_4[\text{FVIII}]_0t$ represents the initial rate of factor VIII activation (see page 129 in reference 29). The constant parameters $[\text{FVIII}]_0$, $[\text{FX}]_0$, K_m and k_3 yield the constant c of Eqn (3) by the expression $c = k_3[\text{FVIII}]_0[\text{FX}]_0/2(K_m + [\text{FX}]_0)$. The factor X activation rate constant k_3 ($10.3 \pm 3.3 \text{ min}^{-1}$) has been experimentally determined from the steady state of factor Xa formation (see the Results section). The K_m value of 200 nM [30] and protein concentrations are as described above. Taken together, these values yield a c value of $1.03 \times 10^{-9} \text{ M min}^{-1}$. We used saturating concentrations of phospholipids to eliminate this component as a potentially rate-limiting step in factor Xa formation. Apparent first-order rate constants of factor VIII activation (k_4) were obtained by fitting data into Eqn (3) using Enzfitter software (Elsevier, The Netherlands).

Analysis of proteolysis fragments by quantitative immunoblotting. Factor VIII or des-(713–1637)-factor VIII was activated by 2 nM thrombin in assays for the measurement of factor Xa. At various times, subsamples were collected in sample buffer [2% (mass/vol.) SDS, 10% (by vol.) glycerol, 0.01% (mass/vol.) bromophenol blue, 62.5 mM Tris, pH 6.0] for SDS/PAGE containing 2 M urea. After electrophoresis, proteins were immunoblotted using antibody p53 to detect the A1 domain of the factor VIII heavy chain and with monoclonal antibody CLB-Cag 69 to detect intact factor VIII light chain. After incubation with horseradish-peroxidase-labeled antibodies, immunoblot fragments with the cleavage products of interest (50-kDa A1 domain or 80-kDa light chain) were excised. The amounts of the cleavage products were quantified spectrophotometrically by incubation of the immunoblot fragments with 3,3',5,5'-tetramethylbenzidine in 0.11 M sodium acetate/citric acid, pH 5.5. The reaction was stopped with H₂SO₄ and the absorbance was measured at 450 nm.

Kinetics of factor VIII light chain cleavage. Factor VIII light-chain cleavage rates were determined either in subsamples from experiments for the measurement of factor Xa formation or in incubations of factor VIII with a selection of components used in factor Xa formation experiments. The specific conditions are explained in the Results section. Light-chain cleavage was

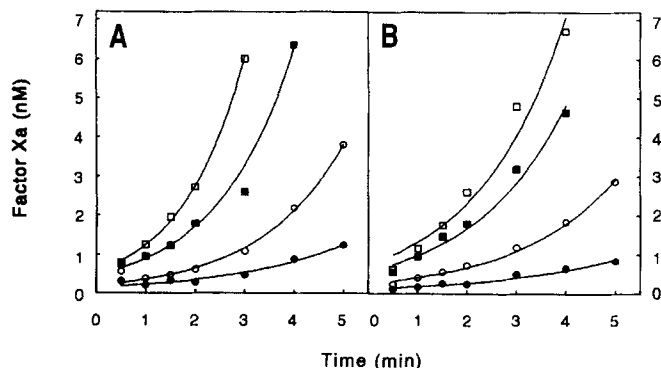


Fig. 1. Activation of factor VIII by thrombin as determined by factor Xa formation. Factor X (0.2 μ M) was activated by factor IXa (0.3 nM) in the presence of phospholipids (50 μ M), and factor VIII (0.4 nM) in 100 mM NaCl, 10 mM CaCl_2 , 50 mM Tris, pH 7.5, at 37°C. Factor X activation was initiated by the addition of various concentrations of thrombin to mixtures containing plasma-derived factor VIII (A) or des-(713–1637)-factor VIII (B). The thrombin concentrations used to activate plasma-derived factor VIII were 0.1 (●), 0.3 (○), 1 (■), and 2 nM (□), whereas concentrations of 2 (●), 5 (○), 10 (■), and 20 nM (□) were used to activate des-(713–1637)-factor VIII. Curves were obtained by fitting the data to Eqn(3) (see the Materials and Methods section).

assessed in an assay employing immobilized monoclonal antibody CLB-Cag 12 and the detection antibody was CLB-Cag 69. Samples were incubated with immobilized antibody (0.5 μ g/well) for 2 h at 37°C. Dilutions were made in 1 M NaCl, 10 mM EDTA, 1% (mass/vol.) human serum albumin, 50 mM Tris, pH 7.5. Thrombin and factor Xa were inhibited by a twofold molar excess of Phe-Pro-Arg- CH_2Cl and Glu-Gly-Arg- CH_2Cl over enzyme, respectively. Bound, intact light chain was detected by incubation with peroxidase-conjugated CLB-Cag 69 (15 ng/well) in 150 mM NaCl, 0.1% (by vol.) Tween-20, 50 mM Tris, pH 7.5, for 1 h at 37°C. The decay in intact factor VIII light-chain antigen was calculated relative to the concentration of factor VIII light chain at zero time. Cleavage rates were determined employing the following equation, which describes a single exponential decay of intact factor VIII light-chain antigen:

$$[\text{FVIII-LC}]_t = [\text{FVIII-LC}]_0 \exp(-kt) \quad (5)$$

$[\text{FVIII-LC}]_0$ is the initial factor VIII light-chain concentration and k is the first-order rate constant of cleavage at a specific activator concentration. Data were fitted into Eqn (5) to obtain factor VIII light-chain cleavage rate constants employing Enzfitter software. Second-order rate constants ($\text{M}^{-1} \text{s}^{-1}$) were obtained from the slopes of plots of first-order rate constant versus activator concentration.

RESULTS

Activation of factor VIII. Previously, we described that activation of the deletion mutant des-(713–1637)-factor VIII requires more thrombin than plasma-derived factor VIII or a mutant lacking nearly the complete B-domain; i.e. des-(868–1562)-factor VIII [7]. In the present study, we investigated the kinetics of factor VIII activation in detail. Factor VIII activation was determined by factor Xa formation in mixtures containing factor VIII, factor IXa, factor X, phospholipids, and varying concentrations of thrombin or factor Xa. An exponential increase in factor Xa formation was observed when plasma-derived factor VIII was activated with thrombin (Fig. 1A). Increasing thrombin concentrations resulted in acceleration of factor Xa formation. The curves in Fig. 1 show that the experimental data of factor Xa

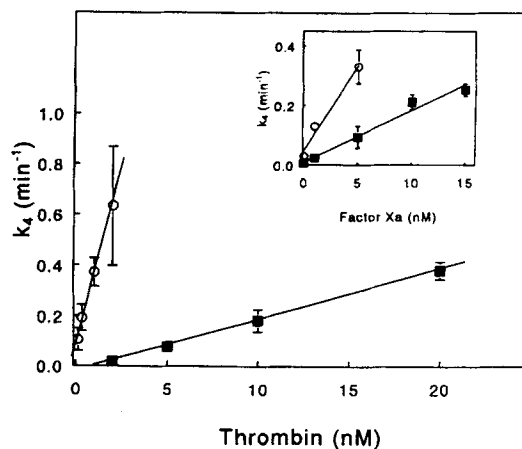


Fig. 2. Rate constants of plasma-derived factor VIII and des-(713–1637)-factor VIII activation. Factor VIII activation was analyzed by factor Xa formation kinetics, and apparent first-order rate constants were obtained by fitting the data as described in the legend of Fig. 1. Apparent second-order rate constants were calculated from the slopes of the lines derived by plotting first-order rate constants against thrombin concentrations. The rate constants ($\pm$ SE) were determined for plasma-derived factor VIII (○) and des-(713–1637)-factor VIII (■) in at least three independent experiments. The inset shows a similar plot for the activation of plasma-derived factor VIII (○) or des-(713–1637)-factor VIII (■) by factor Xa. Apparent second-order rate constants are listed in Table 1.

formation fit well in the exponential model for factor VIII activation as described in the Materials and Methods section. Des-(713–1637)-factor VIII was activated under similar experimental conditions (Fig. 1B). At first glance, exponential factor Xa formation curves for des-(713–1637)-factor VIII display the same pattern as plasma-derived factor VIII (compare Fig. 1B with Fig. 1A). However, it is important to note that approximately tenfold higher concentrations of thrombin are used to activate des-(713–1637)-factor VIII to the same extent. These results indicate that the region that is deleted in des-(713–1637)-factor VIII contributes to efficient factor VIII activation by thrombin.

Factor VIII activation kinetics. To assess quantitatively the activation of factor VIII and the contribution of the sequence 713–1637, data for factor Xa formation were fitted to the exponential model of two coupled enzymatic reactions (see the Materials and Methods section). The rate constant k_3 describing the conversion of factor X into its activated form was derived from that part of the progress curves where the maximal rate of factor Xa formation has become independent of the thrombin concentration (i.e. at later timer points than those shown in Fig. 1). Our approach allows for the calculation of apparent first-order rate constants of factor VIII activation [k_4 in Eqn (3)] at various thrombin concentrations. The rate constants obtained were plotted against the thrombin concentrations used to activate plasma-derived factor VIII and des-(713–1637)-factor VIII (Fig. 2). From the slopes of the resulting lines, apparent second-order rate constants of factor VIII activation by thrombin can be derived. Deletion of the heavy-chain region 713–1637 and in particular 713–740 results in a tenfold reduction of the second-order rate constant to $0.3 \pm 0.1 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$ for thrombin-mediated activation (Table 1). This reduction should not be attributed to the deleted B-domain sequence as activation of a mutant factor VIII lacking the majority of the B-domain is similar to that of plasma factor VIII [18]. We also tested the effect of a monoclonal antibody directed against the sequence Lys713–Arg740 which is

Table 1. Apparent second-order rate constants of activation. Rate constants of activation were determined as described in the legend of Fig. 2. Plasma factor VIII was preincubated with a 15-fold molar excess of monoclonal antibody CLB-CAG 9 for 2 h at 37°C prior to factor X activation. The calculated values each represent the average $\pm$ SD of at least three experiments. n.d., not determined.

Incubation	$10^{-6} \times$ Apparent second-order rate constant	
	thrombin	factor Xa
	$M^{-1} s^{-1}$	
Plasma-derived factor VIII	3.3 ± 0.3	1.1 ± 0.1
Des-(713–1637)-factor VIII	0.3 ± 0.1	0.3 ± 0.1
Plasma-derived factor VIII + CLB-CAG 9	1.3 ± 0.1	n.d.

known to inhibit factor VIII activity. A reduction was determined although to a lesser extent than for activation of des-(713–1637)-factor VIII (Table 1). Thus, binding of an antibody to the region Lys713–Arg740 interferes with factor VIII activation.

Plasma-derived factor VIII and des-(713–1637)-factor VIII were also activated by factor Xa instead of by thrombin. Various factor Xa concentrations yielded apparent first-order rate constants (k_a) of factor VIII activation which were plotted against factor Xa concentration (Fig. 2). The second-order rate constants were obtained from the slopes of these plots and are listed in Table 1. The rate constant for activation of plasma-derived factor VIII by factor Xa was threefold lower than for activation by thrombin. The second-order rate constant of des-(713–1637)-factor VIII activation by factor Xa displayed the same value of $0.3 \pm 0.1 \times 10^6 M^{-1} s^{-1}$ compared with activation by thrombin. These results suggest that the heavy-chain region Lys713–Arg740 is of importance in activation of factor VIII by both thrombin and factor Xa.

Proteolysis of factor VIII. Activation of factor VIII is associated with limited proteolysis and activation of des-(713–1637)-factor VIII is reduced compared with plasma-derived factor VIII. Thus, it is likely that the rate of cleavage at one or more positions in des-(713–1637)-factor VIII is reduced. Des-(713–1637)-factor VIII and plasma-derived factor VIII were activated by thrombin and proteolysis of heavy and light chains was analyzed. The appropriate cleavage products on immunoblot fragments were quantified spectrophotometrically (see the Materials and Methods section). Heavy-chain cleavage was monitored by generation of the heavy-chain A1 domain employing an antibody against the amino acid sequence 329–346. The cleavage of the heavy chain of des-(713–1637)-factor VIII was slightly delayed compared with heavy-chain cleavage of plasma-derived factor VIII (Fig. 3A). Exclusively, intact light chain was detected employing a monoclonal antibody directed against the amino acid sequence 1673–1689. By this method, we determined that cleavage of the des-(713–1637)-factor VIII light chain is much slower than of plasma-derived factor VIII light chain (Fig. 3B). Thus, the heavy-chain region Lys713–Arg740 predominantly affects factor VIII light-chain cleavage and not heavy-chain cleavage at position Arg372. This suggests that in the factor VIII heterodimer association of the heavy-chain sequence 1–740 with the light chain is required for efficient proteolysis of the factor VIII light chain.

Kinetics of factor VIII light-chain cleavage. The finding that the heavy-chain region Lys713–Arg740 contributes to proteoly-

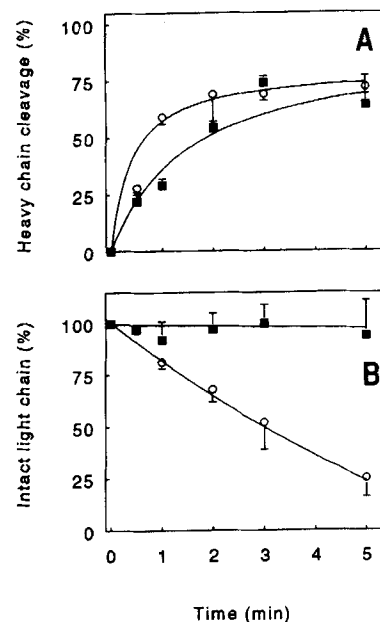


Fig. 3. Proteolysis of plasma-derived factor VIII and des-(713–1637)-factor VIII. Factor VIII (10 nM) was incubated with 2 nM thrombin in a buffer containing 50 μ M phospholipids in 100 mM NaCl, 10 mM $CaCl_2$, 50 mM Tris, pH 7.5, at 37°C. Subsamples were subjected to SDS/PAGE and immunoblotting. The A1 heavy-chain fragment of factor VIII was detected employing a polyclonal anti-(factor VIII) antibody (p53) (A). Intact light chain was detected employing monoclonal antibody CLB-CAG 69 (B). Quantification of cleaved fragments was as described in the Materials and Methods section. Proteolysis is shown for plasma-derived factor VIII (○) and des-(713–1637)-factor VIII (■). The average $\pm$ SE of 2–3 experiments is shown.

sis of the factor VIII light chain (Fig. 3) prompted us to analyze the kinetics of this cleavage more extensively. We developed an assay to determine proteolysis of factor VIII light chain by thrombin and factor Xa. This assay is based on a specific combination of two monoclonal antibodies to discriminate between intact and cleaved light chain. By virtue of this selectivity, cleavage of intact light chain can be determined. The extent of cleavage is calculated for each time point with the initial concentration as a reference. The time courses of proteolysis of the light chain of various factor VIII species by 2 nM thrombin and factor Xa are shown in Fig. 4A and B, respectively. Compared with plasma-derived factor VIII, the light chain of des-(713–1637)-factor VIII is cleaved at a lower rate by thrombin and factor Xa. A similar result was obtained for cleavage of plasma-derived factor VIII after preincubation with an antibody directed against the Lys713–Arg740 sequence. Thus, the sequence Lys713–Arg740 contributes to the rate of factor VIII light-chain cleavage by thrombin and factor Xa. Cleavage rates were obtained by fitting the light-chain cleavage data into a model describing a single exponential decay Eqn (5) in the Materials and Methods section). This analysis yields the first-order rate constant of factor VIII light-chain cleavage. At various enzyme concentrations, the first-order rate constants were determined and subsequently plotted against thrombin or factor Xa concentration employing a method similar to that shown in Fig. 2 for the activation of factor VIII. Second-order rate constants were derived from the slopes of those plots and the values are listed in Table 2. The second-order rate constant of des-(713–1637)-factor VIII light-chain cleavage was approximately sevenfold lower than for plasma-derived factor VIII light-chain cleavage. Furthermore, incubation of factor VIII with an antibody directed against the sequence Lys713–Arg740 reduced the light-chain cleavage rate

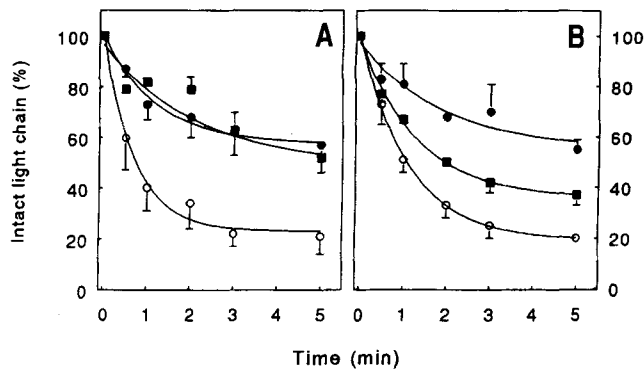


Fig. 4. Light-chain cleavage of various factor VIII species. Light-chain cleavage by thrombin (A) and factor Xa (B) was determined in subsamples of factor Xa formation experiments that contained 0.4 nM factor VIII, 0.3 nM factor IXa, 0.2 μ M factor X, 50 μ M phospholipids, and 2 nM thrombin or factor Xa. Plasma-derived factor VIII ($\circ$), plasma-derived factor VIII preincubated with 6 nM of antibody CLB-Ca9 ($\bullet$), and des-(713–1637)-factor VIII ($\blacksquare$) were activated and proteolysis of intact light chain was assessed in a specific immunological assay (see the Materials and Methods section).

Table 2. Second-order rate constants of proteolysis. Rate constants of factor VIII light-chain cleavage were determined from the slopes of plots of first-order rate constants against activator concentration as described in the Materials and Methods section. Incubation of plasma-derived factor VIII with a 15-fold molar excess of monoclonal antibody CLB-Ca9 was for 2 h at 37°C prior to proteolysis experiments. The calculated values each represent the average $\pm$ SD of at least three experiments.

Incubation	$10^{-6} \times$ Second-order rate constant	
	thrombin	factor Xa
	$M^{-1} s^{-1}$	
Plasma-derived factor VIII	2.1 ± 0.2	2.2 ± 0.3
Des-(713–1637)-factor VIII	0.2 ± 0.1	0.5 ± 0.2
Plasma-derived factor VIII + CLB-Ca9	0.3 ± 0.1	0.4 ± 0.1
Isolated light-chain	0.2 ± 0.1	11.3 ± 5.1

constant to the same extent as observed for des-(713–1637)-factor VIII. We conclude that the reduction in the light-chain cleavage rate of des-(713–1637)-factor VIII is associated with a role of the sequence Lys713–Arg740 that is lacking in the heterodimeric mutant compared with wild-type factor VIII.

Contribution of the heavy chain that lacks the Lys713–Arg740 sequence to light-chain cleavage. The heavy-chain region Ala1–Asp712, in addition to the region Lys713–Arg740, may contribute to the efficiency of factor VIII light-chain cleavage. To test this hypothesis, cleavage of the light chain of des-(713–1637)-factor VIII was compared with cleavage of isolated light chain. The second-order rate constant of cleavage by thrombin was identical for des-(713–1637)-factor VIII light chain and isolated light chain (Table 2). This suggests that the heavy chain comprising Ala1–Asp712 does not contribute to factor VIII light-chain cleavage by thrombin. Remarkably, cleavage of isolated light chain by factor Xa appeared to be a very efficient process. Fig. 5 shows that the conversion of isolated light chain by 2 nM factor Xa proceeds more rapidly than conversion of the light chain within the factor VIII heterodimer. The light chain of des-(713–1637)-factor VIII is cleaved by

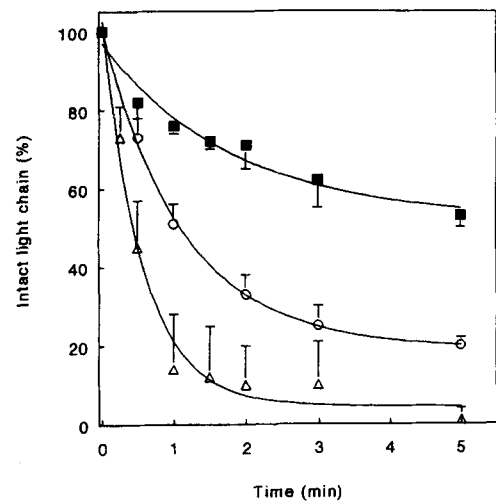


Fig. 5. Contribution of heavy chain to factor VIII light-chain cleavage. Plasma-derived factor VIII (0.4 nM) ($\circ$), des-(713–1637)-factor VIII (0.4 nM) ($\blacksquare$), and isolated factor VIII light chain (2.8 nM) ($\triangle$), were incubated with 2 nM factor Xa in the presence of 50 μ M phospholipids in 100 mM NaCl, 10 mM $CaCl_2$, 50 mM Tris, pH 7.5. Light-chain cleavage was assessed as described in the Materials and Methods section and the decay of intact light chain was calculated with $t = 0$ as reference. Data represent the average $\pm$ SE of 2–4 experiments.

factor Xa at a lower rate than the light chain in plasma factor VIII. Moreover, the second-order rate constant of cleavage of des-(713–1637)-factor VIII light chain by factor Xa is similar to cleavage by thrombin (Table 2). Apparently, the heavy-chain region Ala1–Asp712 inhibits cleavage of the factor VIII light chain by factor Xa. However, factor VIII light-chain cleavage by both thrombin and factor Xa is promoted by the heavy-chain region Lys713–Arg740.

DISCUSSION

It has been well established that proteolysis of factor VIII at positions Arg372 and Arg1689 is required for factor VIII activation [12, 31–33]. Although cleavage at position Arg740 does not contribute to factor VIII activation [10, 34], we recently obtained data suggesting that the heavy-chain region Lys713–Arg740 adjacent to this cleavage site is involved in factor VIII activation by thrombin [7]. In the present study, we have explored the role of this acidic region in proteolytic activation of factor VIII in more detail. For this purpose, we analyzed the kinetics of factor VIII activation and proteolysis employing the variant des-(713–1637)-factor VIII which lacks the heavy-chain region Lys713–Arg740. It may be relevant to note that this variant also lacks nearly the entire B-domain. Therefore, our results with regard to the reduced proteolytic activation of the deletion variant may be explained by a role of the heavy-chain region 713–740 or alternatively by a role of the B-domain sequence 741–1637. Several possibilities may be considered in assessing which region is most likely involved in activation of factor VIII. First, the specific coagulant activity of factor VIII heterodimers consisting of the light chain and heavy chain of the 1–740 sequence is similar to that of heterodimers containing light chain and variably sized heavy chain comprising remnant B-domain portions [35]. Secondly, a factor VIII variant lacking the sequence 771–1666 activated at a normal rate compared with plasma factor VIII [36]. Thirdly, we have previously described that activation of the variant des-(741–1668)-factor VIII lacking the entire B-domain is similar to that of plasma factor VIII [9].

Thus, it is very unlikely that B-domain sequences contribute to activation of factor VIII. Moreover, our experiments employing the anti-(713–740) monoclonal antibody (Fig. 4, Tables 1 and 2) as well as the recent observation that the region 721–729 is required for full procoagulant activity of factor VIII [21] indicate that the C-terminal acidic region of the heavy chain contributes to factor VIII activation and cleavage. Collectively, these data provide strong support to our conclusion that it is the lack of the region Lys713–Arg740 that endows the variant des-(713–1637)-factor VIII with its specific activation characteristics.

Our kinetic analysis of factor VIII activation is based on a model of two coupled enzymatic reactions, which employs the lag phase in factor Xa formation in the presence of varying concentrations of factor VIII activator to derive rate constants of factor VIII activation (Figs 1 and 2). Our kinetic analysis of this complex system generates calculated data for factor Xa formation curves that are in excellent agreement with the experimental observations (see Fig. 1). For plasma-derived factor VIII, the apparent second-order rate constant of activation by thrombin was $3.3 \pm 0.3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (Table 1). This value is similar to that reported for the activation of porcine factor VIII under slightly different conditions [37]. For des-(713–1637)-factor VIII, the rate constant of activation by thrombin is tenfold reduced compared with plasma-derived factor VIII (Table 1). Incubation of des-(713–1637)-factor VIII with thrombin concentrations exceeding 30 nM yields a fully activated species that functions similar to activated plasma factor VIII [7]. Thus, the mutant has no defect in the interaction with the other components of the factor Xa formation assay. In addition, preincubation of plasma-derived factor VIII with a monoclonal antibody against the region Lys713–Arg740 also resulted in a substantial reduction of the activation rate constant (Table 1). These kinetic data indicate that the heavy-chain region Lys713–Arg740 promotes factor VIII activation by thrombin.

The regulatory function of the region Lys713–Arg740 might seem to be in agreement with the concept that factor VIII activation by thrombin involves the acidic regions that provide binding sites for thrombin, thereby facilitating proteolysis at the adjacent cleavage sites. However, several data imply that this particular region of the heavy chain serves a more complicated role. First, the region Lys713–Arg740 also contributes to factor VIII activation by factor Xa (Fig. 2, Table 1), while acidic, tyrosine-sulphate-containing regions so far have exclusively been associated with thrombin binding [13, 38]. Secondly, cleavage at position Arg740 is thought to be dispensable for factor VIII activation [10, 34]. Thirdly, Kjalke et al. concluded that absence of the region 721–729 affects the rate of factor VIII proteolysis but not the affinity of thrombin for factor VIII [21]. It is further of interest to note that the mutant des-(713–1637)-factor VIII contains two residual sites at which cleavage may be considered as potentially rate-limiting in factor VIII activation, although Arg372 and Arg1689 are distant from the region Lys713–Arg740 in the amino acid sequence of factor VIII.

To assess the relation between factor VIII activation and individual cleavages within factor VIII, we employed an approach based on kinetic analysis of factor VIII cleavage and activation under identical conditions. Our method has the advantage over previous studies that it does not require radiolabeled factor VIII, while still allowing the quantification of factor VIII cleavage within the complete factor X activating mixture [compare 8, 34, 37]. Quantitative immunoblotting of reaction mixtures containing plasma-derived factor VIII or des-(713–1637)-factor VIII revealed that in the mutant lacking the heavy-chain Lys713–Arg740 region virtually no light-chain cleavage occurred (Fig. 3). This unexpected finding was corroborated by our rigor-

ous kinetic analysis of factor VIII light-chain cleavage. Comparison of cleavage and activation kinetics (Fig. 2, Tables 1 and 2) leads to the following conclusions: (a) second-order rate constants of des-(713–1637)-factor VIII activation and light-chain cleavage by thrombin are identical; (b) the same parallel between activation and light-chain cleavage exists for proteolysis of des-(713–1637)-factor VIII by factor Xa; (c) for plasma-derived factor VIII, the kinetics of activation and light-chain cleavage by factor Xa and thrombin display the same parallel behaviour, but the rate constants are 5–10-fold higher than for the mutant lacking the region Lys713–Arg740. These findings lead us to propose that light-chain cleavage is rate limiting in factor VIII activation, and that the region Lys713–Arg740 serves as a regulator for thrombin and factor Xa in the proteolytic activation of heterodimeric factor VIII.

A remarkable difference between thrombin and factor Xa in factor VIII proteolysis is apparent from the cleavage kinetics of the isolated light chain (Table 2). Cleavage of heterodimeric factor VIII by thrombin is more efficient than of the isolated light chain, which supports our concept that the heavy chain, and in particular its region Lys713–Arg740, stimulates the interaction of factor VIII with thrombin. For factor Xa, however, the situation seems considerably more complex. First, the association between light chain and the truncated heavy chain of des-(713–1637)-factor VIII inhibits light-chain cleavage by factor Xa (Fig. 5). Although this apparent inhibition is partially overcome in heterodimeric factor VIII that contains the complete heavy chain including Lys713–Arg740, cleavage of the isolated light chain by factor Xa still remains more favourable (Table 2). These data imply that the heavy-chain residues Ala1–Asp712 comprise a region that interferes with light-chain cleavage by factor Xa, whereas the same cleavage is promoted by the region Lys713–Arg740. Although the precise mechanism of light-chain proteolysis remains unclear, it seems evident that the heterodimeric state of intact factor VIII greatly contributes to the susceptibility of the light chain to proteolysis. In terms of the three-dimensional structure, this would imply that sites where thrombin and factor Xa cleave the light chain (at positions Arg1689 and Arg1721, respectively, compare [11]) are located in close proximity to the region Lys713–Arg740 on the complementary subunit. Other examples of the role of association of heavy and light chain in the proteolysis of factor VIII concern the interaction with the enzymes factor IXa and activated protein C. Activated protein C binds to the A3 domain [39], whereas this enzyme cleaves factor VIII within the A2 domain [4, 40]. Compared to cleavage in the absence of the light chain, factor IXa has been shown to cleave the factor VIII heavy chain at position Arg336 at a higher rate when the factor VIII light chain is associated with the heavy chain [41]. Together, these data imply a close interrelation between the heavy chain and the light chain of factor VIII at specific positions.

If the acidic region Lys713–Arg740 serves to regulate cleavage of the factor VIII light chain at position Arg1689 or Arg1721, a similar role might be attributed to the adjacent region Glu1649–Arg1689. In this respect, it should be noted that sulphation at Tyr1664 has been shown to affect factor VIII activity only to a minor extent compared with sulphation at other tyrosine residues in factor VIII, e.g. at positions 718, 719, and 723 [38]. In addition, Tyr1664 seems completely dispensable for factor VIII activity [9, 36]. Considering that the sulphated tyrosine at position 1680 is required for high-affinity binding of factor VIII to the large multimeric von Willebrand factor [6, 20], it seems difficult to conceive that the region Glu1649–Arg1689 also comprises a binding site for thrombin or factor Xa. Our finding that subunit interaction regulates proteolysis by thrombin and factor Xa (Table 2) suggests that subunit interaction may be

of more significance than the acidic region Glu1649–Arg1689 in proteolysis of the factor VIII light chain. At present, we cannot distinguish between the possibility that acidic regions serve as primary binding sites for enzymes or that these regions regulate the exposure of specific binding sites for thrombin and factor Xa that are located elsewhere on the factor VIII protein.

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