

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO	
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DEP: 2020 DIRECCION DE NUEVAS CREACIONES	EVE: 378 FASE NACIONAL INCLUIDO CAPITULO I
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ACT: 519 PRESENTACION	

DIRECCIÓN DE NUEVAS CREACIONES

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Abogado(a)/Señor(a)
CARLOS REINALDO OLARTE GARCIA

Asunto: Radicación: 12-123109- -4
 Trámite: 11
 Evento: 378
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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/EP2011/050749				
Fecha de Presentación Internacional	20/01/2011				

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 No. 12-123109-0000000014	23/Julio/2012
Reivindicaciones:	Radicado No. 12-123109-0000000014	23/Julio/2012
Dibujos:	Radicado No. 12-123109-0000000014	23/Julio/2012
Secuencias:	Radicado No. 12-123109-0000000014	23/Julio/2012
Prioridad:	EP 10151239.0 US 61 383 914	20/Enero/2010 17/Septiembre/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 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. Para la presente solicitud, si bien se estudió el capítulo reivindicatorio completo (Radicado No. 12-123109-0000000014) es necesario que cancele la tasa correspondiente a la reivindicación 11 en adelante.

Título de la solicitud: “Antídotos anticoagulantes”

El problema planteado en esta solicitud consiste en la necesidad de proporcionar un antídoto para poder detener acontecimientos hemorrágicos relacionados con anticoagulantes.

Para resolver este problema técnico la solicitud en estudio proporciona una molécula de anticuerpo capaz de neutralizar la actividad de un anticoagulante.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K 39/0, C7K 16/44.

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

Teniendo en cuenta el artículo citado, los métodos para el tratamiento humano o animal hacen parte de la materia no patentable en este país. Por lo tanto, el objeto de las reivindicaciones 21, 34 - 38, por tratarse de métodos de tratamiento en un paciente, no es patentable.

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

No obstante las reivindicaciones 18 - 20 encabezan como “molécula de anticuerpo”, hacen referencia a un uso, por lo tanto el objeto de dichas reivindicaciones no es patentable, de acuerdo con el Art 14.

6. De la solicitud de patente

Título

El título no corresponde con el objeto de la solicitud porque de manera muy general se refiere a “antídotos anticoagulantes” sin especificar la materia reclamada, por lo cual se sugiere al solicitante hacer la corrección necesaria.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

1) La reivindicación 1 carece de claridad y concisión ya que no incluye las características técnicas esenciales de la invención. Se sugiere al solicitante tener en cuenta que la reivindicación independiente debe ser autosuficiente y no debe declarar las características esenciales en las reivindicaciones dependientes. Así mismo, la expresión “*capaz de neutralizar la actividad de un anticoagulante*” se considera un resultado a alcanzar, que no permite tener claridad sobre la materia reclamada puesto

que la reivindicación incluiría no sólo la solución propuesta por la solicitante, sino todas las alternativas presentes o futuras que lleguen a ese mismo resultado.

De igual manera, las reivindicaciones 2 - 8 están definidas por un resultado a alcanzar, puesto que hacen referencia al antígeno al cual se une la molécula de anticuerpo reclamada en la reivindicación 1, sin indicar las características esenciales, estructurales o funcionales, que contribuyen a la obtención de ese resultado.

Se recuerda a la solicitante que la forma correcta de reclamar un anticuerpo es definirlo a partir de su estructura completa, esto es, de las secuencias de las regiones variables de la cadena liviana y cadena pesada, por las secuencias de las cadenas pesada y ligera o por las secuencias de las seis regiones CDR's (3 de la cadena liviana y 3 de la cadena pesada) identificadas por un número de identificación SEQ ID N°;

2) El proceso para producir un anticuerpo descrito en las reivindicación 22 corresponde a un resultado a alcanzar, ya que se busca producir un anticuerpo sin indicar las condiciones específicas en que debiera realizarse el cultivo, las características del medio que le permitieran a la célula producir la proteína de interés, ni los métodos para recuperar el anticuerpo a partir de las células cultivadas. Además, la etapa a) que se refiere a *“proporcionar una célula hospedera...”* no constituye una etapa del método sino las características de la célula cultivada de la etapa b).

Se sugiere hacer las correcciones necesarias y caracterizar dicho proceso a partir de la secuencia lógica de etapas y por características y condiciones físicas, químicas y biológicas asociadas;

3) Las composiciones farmacéuticas mencionadas en los kits de las reivindicaciones 23, 24 y 33 deben definirse teniendo en cuenta sus características técnicas. Se recuerda a la solicitante que una composición debe estar definida por sus ingredientes (compuesto activo y excipientes) y las proporciones de los mismos.

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: enero 20 de 2010, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	Schlaepi <i>et al.</i> 1990	“Preparation of monoclonal antibodies to hirudin and hirudin peptides: A method for studying the hirudin -thrombin interaction”	10 marzo 1990

El documento D1¹ da a conocer anticuerpos monoclonales específicos contra la hirudina, un inhibidor potente y específico de la trombina, que demostraron alta afinidad y propiedades neutralizantes in vitro para este anticoagulante (resumen).

8. Examen de Patentabilidad (Art 14 D486)

Novedad (Art 16 D486)

La reivindicación 1 consiste en *“Una molécula de anticuerpo capaz de neutralizar la actividad de un anticoagulante”*.

1 Schlaepi. J-M., et al. 1990. Preparation of monoclonal antibodies to hirudin and hirudin peptides: A method for studying the hirudin -thrombin interaction. Eur J Biochem 188(2):463-70.

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

Características esenciales	D1
Molécula de anticuerpo que neutraliza un anticoagulante	Anticuerpo que neutraliza un anticoagulante (resumen)

Las características esenciales de la molécula de la reivindicación 1 habían sido divulgadas previamente en el documento D1, el cual enseña anticuerpos que neutralizan o inhiben los efectos del anticoagulante hirudina (resumen).

En consecuencia, la reivindicación 1 no es nueva, según lo divulgado en el documento D1.

Las reivindicaciones dependientes 2 – 6 no mencionan alguna característica que haga nueva a la molécula de la reivindicación 1, por lo cual se considera que no son nuevas.

9. Conclusión

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

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	Schlaeppi <i>et al.</i> 1990	1 - 6	Novedad

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 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 2013-08-27

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Preparation of monoclonal antibodies to hirudin and hirudin peptides

A method for studying the hirudin – thrombin interaction

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A panel of four monoclonal antibodies was obtained against hirudin, a potent and specific inhibitor of thrombin, by immunizing three groups of mice with protein conjugates made of recombinant desulfatohirudin (group I) or two synthetic peptides representing the C-terminal sequences 40–65 (group II) and 52–65 (group III) of hirudin. Only the monoclonal antibody 4049-83-12, obtained from the group I of mice, showed high affinity for hirudin (K_d of 0.6 nM) and *in vitro* neutralizing properties. The anti-peptide monoclonal antibodies bound hirudin with lower affinity (K_d of 1.5–7 nM) and showed lower neutralizing capacities. An epitope analysis performed by competitive ELISA using various hirudin analogues and by limited proteolysis of the hirudin-antibody complex revealed that the binding domains of all the anti-peptide antibodies were located close to the C-terminus of hirudin, since the bond between Glu-61 and Glu-62 was not cleaved by the V8 staphylococcal protease in the presence of these antibodies. The epitope of the antibody 4049-83-12 was strictly conformation-dependent, it recognized neither *S*-carboxymethylated hirudin nor any peptides of hirudin. The cleavage of the bond between Glu-43 and Gly-44 by V8 protease, as well as the cleavage of the bond between Lys-47 and Pro-48 by lysyl endopeptidase, was prevented by the binding of the antibody 4049-83-12 to hirudin. The possibility that this epitope overlapped with a region of hirudin involved in the binding to thrombin is discussed.

Hirudin isolated from the salivary gland of the leech *Hirudo medicinalis* is a potent and specific inhibitor of thrombin, preventing the cleavage of fibrinogen and subsequent fibrin clot formation [1]. Several closely related hirudin variants have been described, each containing 65 or 66 amino acids and designated HV1 [2], HV2 [3] and HV3 [4]. Hirudin reacts very rapidly with α -thrombin to form a very tight noncovalent complex ($K_i \approx 1–0.01$ pM [5, 6]). Its selective inhibition of thrombin in conjunction with its low toxicity and the absence of immunological side effects in humans [7] confer on this compound a great potential for future therapeutic uses [8].

Hirudin has failed to crystallize, but the three-dimensional structure of natural and desulfatohirudin HV1 was determined in solution by NMR [9–12]. These studies show that hirudin is composed of three domains, a central core maintained by the three disulfide linkages, a protruding 'finger' (residues 31–36), and an acidic C-terminal loop (residues 47–65).

Several lines of evidence indicate that hirudin is different from most other naturally occurring inhibitors of serine proteases. Its specificity seems not to be determined primarily by the interaction with the active site of thrombin, but by anionic and hydrophobic interactions, indicating an extended binding region involving residues of the central core and of the C-

terminal domain [5, 10, 13]. Indeed, kinetic data obtained with derivatives of thrombin and the use of sequence-specific anti-thrombin antibodies indicate that hirudin interacts ionically with thrombin in the region of the β -cleavage site [14, 15]. Since the C-terminal region of hirudin is rich in negatively charged residues, this may be the putative region involved in these ionic interactions with thrombin. This conclusion is further confirmed by the observations indicating that a synthetic C-terminal peptide of hirudin still prevents clotting despite its binding outside the catalytic site of thrombin [16–18]. Furthermore, either enzymatic cleavage of the C-terminus or substitution of the acidic residues within this domain drastically abolish hirudin activity [19–21].

Binding of hirudin within the active-site region of thrombin is required for optimal interaction [14]. It has been proposed that Lys-47 may be the reactive-site residue P_1 of hirudin, since it is preceded and followed by proline residues. Indeed Pro-46 would enhance binding at the corresponding apolar binding site of thrombin [22] and Pro-48 would make the bond with lysine resistant to proteases such as trypsin [23, 24]. However, data with natural or genetically engineered hirudin variants show that substitution of Lys-47 has only a weak influence on the inhibitory activity of hirudin [3, 6, 21, 25].

In this study, we used hirudin-specific monoclonal antibodies (mAbs) with defined epitopes to identify regions of hirudin likely to be involved in the interactions with thrombin.

EXPERIMENTAL PROCEDURES

Chemicals

Recombinant desulfatohirudin HV1 (rHV1; CGP 39393) [26] was provided by Dr W. Märki (Ciba-Geigy); recombinant

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Abbreviations. BSA, bovine serum albumin; mAb, monoclonal antibody; rHV1, recombinant desulfatohirudin HV1; rHV3, recombinant desulfatohirudin HV3; I_{50} , concentration of hirudin or analogues reducing the ELISA signal to 50% of the control.

Enzymes. α -Thrombin (EC 3.4.21.5); staphylococcal serine protease (EC 3.4.21.19); *Achromobacter* proteinase I (EC 3.4.21.50).

desulfatohirudin HV3 (rHV3) [4] was kindly given by Dr R. Maschler (Plantorgan). The synthetic peptides representing the sequences 1–15, 29–38, 40–65 and 52–65 of rHV1 were synthesized by the solid-phase method.

Bovine serum albumin (BSA) was purchased from Fluka, keyhole limpet hemocyanin (referred to as hemocyanin) from Calbiochem, and poly(ethylene glycol) 4000 from Merck. Biotin-X *N*-hydroxysuccinimide ester and alkaline-phosphatase-conjugated streptavidin were from Calbiochem. Phosphatase-labeled affinity purified goat antibody to mouse IgG was purchased from Kirkegaard & Perry (Gaithersburg, MD). The ELISA kit for isotype and subclass determination was from Bio-Rad.

Staphylococcus aureus strain V8 protease was from Sigma, and lysyl endopeptidase (*Achromobacter lyticus* protease I) was from Wako Chemicals. Human α -thrombin (3000 NIH units/mg) was purchased from the Center for Diagnostic Products (Boston), fibrinogen grade L was from Kabi Vitrum and the mAb EST 6 against human α -thrombin [27] was made available by Bioscot (Edinburgh, UK). All other reagents were of the highest purity grade.

RPMI 1640 medium (Seromed) was supplemented with 15% fetal calf serum (Gibco), 0.01% gentamycin, 4 mM L-glutamine, 1 mM sodium pyruvate, 50 μ M 2-mercaptoethanol, and 5 μ g/ml of insulin, transferrin and selenium (Collaborative Research). The hypoxanthine aminopterin/thymidine (HAT) medium (Boehringer) was prepared as described previously [28].

Preparation of the immunogens

rHV1 was coupled to BSA by the carbodiimide method after protecting the amino groups of hirudin by di-*tert*-butyl dicarbonate. To 1 mg rHV1 in 20 μ l water was added 5 μ l 0.4 M triethylamine, 50 μ l of *N,N*-dimethylformamide and 2 μ l di-*tert*-butyl dicarbonate. After 2 h at 37°C, 50 μ l water and 200 μ l ethyl acetate were added to extract the unreacted di-*tert*-butyl dicarbonate. The extraction was repeated twice. The lower phase was dried out and 250 μ l 0.1 M Mes pH 4.75 was added together with 50 μ l BSA (10 mg/ml) and 100 μ l *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride (20 mg/ml). After 2 h at 22°C, the mixture was dried out. To deprotect the amino groups after coupling, 200 μ l 90% trifluoroacetic acid was added. After 10 min at 22°C, the trifluoroacetic acid was evaporated, and 1 ml of NaCl/P_i (10 mM sodium phosphate pH 7.0, 145 mM NaCl) was added. The solution was extensively dialyzed in NaCl/P_i before using it for immunization. The coupling of rHV1 to BSA was confirmed by a rather diffuse band migrating slower than BSA on SDS gel electrophoresis.

The coupling of the rHV1 peptides 52–65 and 40–65 to keyhole limpet hemocyanin was done by mixing 1 mg of the respective peptide dissolved in 50 μ l water with 5 mg of the hemocyanin dissolved in 500 μ l of NaCl/P_i; 20 μ l 25% glutaraldehyde was then added and the mixture was incubated for 2 h at 22°C. Finally 50 μ l L-lysine (100 mg/ml) was added to stop the reaction.

Immunization and fusion protocol

Three groups of five BALB/c female mice (4–6 weeks old) were given three series of injections as follows: group I, BSA-rHV1 conjugate (15 μ g/injection); group II, hemocyanin-conjugated rHV1 peptide 40–65 (10 μ g/injection); group III, hemocyanin-conjugated rHV1 peptide 52–65 (10 μ g/injec-

tion). The first injection consisted of 0.1 ml of the respective immunogens in NaCl/P_i mixed in a 1:1 ratio with 0.1 ml complete Freund adjuvant; 50 μ l was injected intraperitoneally and 150 μ l subcutaneously. In the second (day 14) and third (day 30) series of injections, incomplete Freund adjuvant replaced the complete adjuvant. One week after the last injection, serum was collected and titers were determined by ELISA using either rHV1 or biotinylated rHV1 complexed to avidin to coat the microtiter plates (see below).

After a rest period of around two months, the mice were boosted intraperitoneally with either 75 μ g of the BSA-rHV1 conjugate (group I) or 80 μ g of the hemocyanin-peptide conjugates (groups II and III). Three or four days later, the mice were sacrificed and the spleen cells were fused with the murine myeloma cell lines Sp 2/0.Ag14 [29] or PAI [30], using poly(ethylene glycol) 4000, by a modification of the original Köhler and Milstein method [31]. After 2–4 weeks, wells containing growing hybridomas in the hypoxanthine/aminopterin/thymidine medium were tested for specific mAbs by ELISA. Positive hybridomas were cloned by limiting dilution [32] and clones were further selected by ELISA. Positive clones were expanded in mice pretreated with 200 μ l 2,6,10,14-tetramethylpentadecane. Following standard procedures [28], the ascites fluid was purified by centrifugation, ammonium sulfate precipitation and DEAE-cellulose anion-exchange chromatography in order to obtain pure mAbs. The purity was assessed by isoelectric focusing. Specific mAbs were also tested for their ability to neutralize the anticoagulant activity of rHV1 using the clotting assay (see below).

Biotinylation of r-HV1

r-HV1 (0.5 mg in 200 μ l 20 mM acetate pH 6.0) was mixed with 100 μ g biotin-X *N*-hydroxysuccinimide ester dissolved in 40 μ l ethanol/water (1:1, by vol.) and incubated 20 min at 22°C (the molar ratio biotin/hirudin was 3.2:1). Then, 260 μ l NaCl/P_i was added before the dialysis of the solution overnight against NaCl/P_i at 4°C. The structural analysis of biotinylated r-HV1 was performed by quantitative N-terminal analysis [33] after reductive S-carboxymethylation [34] and digestion by lysyl endopeptidase. The biotinylation of each amino group of rHV1 was between 35–50%.

Digestion of rHV1 by V8 staphylococcal protease

The specific cleavage after glutamyl bonds by V8 protease was performed as described previously [35]. Briefly, 70 μ g rHV1 dissolved in 90 μ l 50 mM ammonium bicarbonate pH 8.0, supplemented with 2 mM EDTA, was mixed with 20 μ g (in 20 μ l) of V8 protease, and incubated for different periods of time at 37°C. The reaction was stopped by the addition of 5 μ l 0.01 M diisopropylfluorophosphate. The extent of digestion was measured by quantitative N-terminal analysis [33]. The proteolytic fragments were separated by reverse-phase HPLC eluted with a linear gradient of 20–80% solvent B in solvent A over 30 min; solvent A is 0.1% (by vol.) anhydrous trifluoroacetic acid in water and solvent B 0.1% (by vol.) anhydrous trifluoroacetic acid in acetonitrile/water (6:4, by vol.). The flow rate was 1 ml/min. Peptides were detected by absorbance at 220 nm and identified by amino acid analysis [36].

Digestion of rHV1 by lysyl endopeptidase

To 70 μ g rHV1 dissolved in 97.5 μ l 25 mM Tris/HCl pH 8.3, 2.5 μ g (in 2.5 μ l) lysyl endopeptidase was added and

incubated for different periods of time at 37°C. The proteolytic fragments were separated by HPLC (see above) and the extent of digestion determined by quantitative N-terminal analysis [33].

*Digestion of the complex rHV1-mAbs
by V8 staphylococcal protease or by lysyl endopeptidase*

To 23 µg (3.2 nmol) rHV1, 315 µg (2.1 nmol) mAb was added; after a 15-min incubation at 22°C, 7 µg V8 protease or 2.5 µg lysyl endopeptidase was added. The mixture (total volume 80 µl) was incubated at 37°C for defined periods of time. The reaction was stopped by freezing the mixture at -20°C. Controls were: (a) digestion of rHV1 without anti-hirudin mAbs; (b) digestion of rHV1 in the presence of an unspecific mAb i.e. mAb 4063-21-1, an IgG₁ which binds to the small hapten atrazine, unrelated to hirudin (J.-M. Schlaeppli, unpublished results); (c) digestion of the anti-hirudin mAbs in the absence of rHV1. The proteolytic fragments (2.7 µg) were separated by HPLC (see above).

*Competitive ELISA assay for the determination
of rHV1 and analogues*

For the two-step competitive ELISA, 96-well microtiter plates were coated with either rHV1 (0.3 µg/100 µl 50 mM sodium carbonate pH 9.6) or biotinylated rHV1 (0.5 µg/100 µl) complexed with avidin (1 µg/100 µl) and incubated overnight at 4°C. Unbound antigen was removed by washing five times with NaCl/P_i containing 0.1% (by vol.) Tween 20. The remaining free sites on the solid support were blocked by incubation with NaCl/P_i containing 1% (mass/vol.) BSA. In separate test tubes, 50 µl purified mAb (40–400 ng/ml) or clone supernatant (diluted 1:70 to 1:150) was incubated with 650 µl standard solutions containing increasing amounts of either rHV1 or analogues. After overnight incubation at 4°C (or 1.5 h at 22°C in the case of the rHV1-thrombin complex), 200 µl of the antibody-antigen mixture was transferred to each well and incubated for an additional hour at 22°C. The wells were then washed and 100 µl/well of goat anti-mouse antibody conjugated to alkaline phosphatase (diluted 1:1500) was added and incubated for 1.5 h at 22°C. After washing, 150 µl/well of the substrate *p*-nitrophenyl phosphate (1 mg/ml) in 1 mM diethanolamine pH 9.8, supplemented with 0.5 mM MgCl₂ · 6 H₂O, was added. The change of color was monitored by absorbance at 405 nm. All samples were run in triplicates.

The concentration of rHV1 or analogues reducing the ELISA signal to 50% of the control (*I*₅₀) was calculated using an adaptation of the curve-fitting program ENZFITTER (R. J. Leatherbarrow, Elsevier) based on a four-parameter logistic curve [37]. The determination by ELISA of the dissociation constant of the antigen – antibody equilibrium was calculated as described previously [38].

*Binding of mAb to rHV1-thrombin complex
tested by sandwich ELISA*

Microtiter plates were coated with the anti-thrombin mAb EST 6 (0.6 µg/100 µl). After blocking and washing, the following substances were added and incubated at 22°C for the indicated time: α-thrombin (0.05 µg/100 µl NaCl/P_i containing 0.1% BSA, 2 h); rHV1 (concentrations ranging over 0–1 µg/100 µl, 1 h), biotinylated anti-hirudin mAbs (0.2 µg/100 µl, 2 h), alkaline-phosphatase-conjugated streptavidin

(diluted 1:2500, 1.5 h), and substrate. Between each step, the plates were washed with NaCl/P_i containing 0.1% Tween. To confirm the binding of rHV1 to thrombin, a positive control was made by adding biotinylated rHV1 (prepared as described above) instead of rHV1. The biotinylated rHV1 was then revealed directly by the streptavidin conjugate (i.e. no anti-hirudin mAbs were added).

Clotting assay

Anti-hirudin mAbs were tested for their ability to neutralize the anticoagulation activity of rHV1 towards α-thrombin by the clotting test [39]. Briefly, 50 µl purified mAb at increasing concentrations was incubated for 10 min at 22°C with 50 µl rHV1 at various concentrations, ranging over 0–85 nM. Then, 50 µl of a freshly prepared α-thrombin solution was added containing 7.2 unit/ml in 0.01 M imidazole pH 7.4 supplemented with 0.15 M NaCl, 0.01 M CaCl₂ and 0.6% (mass/vol.) poly(ethylene glycol) 6000; after 1 min at 37°C, 350 µl prewarmed fibrinogen (3.2 mg/ml) was added. The clotting time was measured in a Amelung coagulometer at 37°C. All determinations were done in triplicate. In some experiments rHV1 was incubated with thrombin for 1 min prior to the addition of increasing concentrations of mAb.

RESULTS

Preparation and characterization of anti-hirudin mAbs

Three groups of mice were immunized with either rHV1 coupled to BSA (group I), or with the rHV1 peptides 40–65 and 52–65 coupled to keyhole limpet hemocyanin (groups II and III respectively). In the first group, rHV1 was coupled to BSA by the carbodiimide method after protecting the amino groups with di-*tert*-butyl dicarbonate. The hirudin C-terminal domain is very rich in acidic residues which are exposed on the surface of the molecule [19]; we therefore assumed that rHV1 would be mainly coupled by its C-terminal domain to BSA and that this would preferentially trigger an immune response against the rest of the molecule. On the other hand, by immunizing mice with the synthetic C-terminal peptides, the cross-reactivity of the antibodies with the native molecule should be restricted to the C-terminal domain.

The analysis by competitive ELISA of the sera of the three groups of mice fully confirmed this hypothesis. All sera bound rHV1 in solution down to nanogram/milliliter concentrations (the *I*₅₀ were 3.5, 39 and 23 ng/ml for groups I, II and III respectively). However, the sera of mice immunized with the BSA-rHV1 conjugate could not bind the C-terminal peptides 40–65 or 52–65 (*I*₅₀ > 1000 ng/ml).

Three fusions experiments were performed on each group of mice. Hybridomas secreting specific anti-hirudin mAbs were screened by ELISA using microtiter plates coated with either rHV1 or biotinylated rHV1 linked to avidin. Since some conformational changes of the rHV1 molecule could occur upon adsorption to the plastic wells, indirect coating was developed to increase the chances for screening positive hybridomas. From the first group of mice (BSA-rHV1 conjugate), we obtained only one hybridoma which secreted an antibody (mAb 4049-83-12) reacting strongly by ELISA. From the two other groups of mice we obtained a total of nine and six mAbs for groups II and III respectively. Two mAbs from group II and one from group III (all IgGs) with high affinity for rHV1 were further selected for closer analysis (Table 1). The overall cross-reactivity patterns of these mAbs,

Table 1. Characterization of mAbs by competitive ELISA

In the entry for rHV3, the last column lists the residues which are different from rHV1 [2, 4]. Results for S-carboxymethylated rHV1 (S-CM-rHV1) are taken from [34]; n.d. = not determined

Protein	I ₅₀ for mAbs of				Characterization of the hirudin analogues
	group I 4049-83-12	group II		group III 4102-21-14	
		4114-96-1	4120-37-7		
	ng/ml				
rHV1	4	23	11	52	Ile-1, Thr-2, Lys-24, Gln-33, Lys-35, Asp-36, Gln-53, Pro-58, Asp-62, Ala-63, Asp-65, Glu-66
rHV3	> 10000	> 1000	> 1000	> 1000	
HV1 peptide 52–65	> 10000	4	1	9	
rHV1 peptide 40–65	> 10000	11	3	20	
rHV1 peptide 29–38	> 10000	n.d.	n.d.	> 1000	97% of Val-1 and 90% of Lys-27 modified 97% cleavage at Glu-43, 50% cleavage at Glu-61
rHV1 peptide 1–15	> 10000	n.d.	n.d.	> 1000	
S-CM-rHV1	> 1000	6	13	22	
Val-1, Lys-27 modified rHV1	56	3	1	5	
rHV1 + V8 protease (2 h)	100	> 1000	95	200	
rHV1 1–43	> 1000	n.d.	n.d.	n.d.	
rHV1 1–61	4.2	1000	105	> 1000	
rHV1 + lysyl endopeptidase (1.5 h)	29	28	29	35	
	nM				
K _d (rHV1)	0.6	3.2	1.5	7.1	
Sub-isotype	IgG ₁	IgG ₁	IgG _{2b}	IgG ₁	

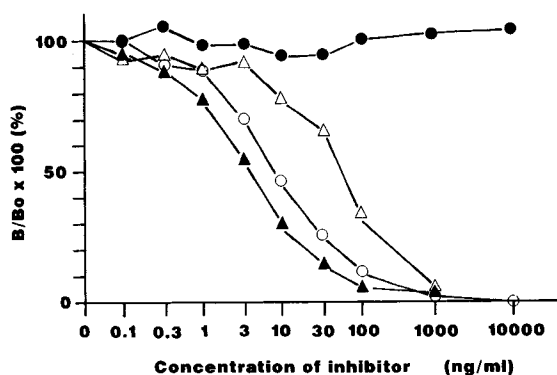


Fig. 1. Competitive ELISA. mAb 4049-83-12 binding to rHV1 (▲), and rHV1 peptide 52–65 (●); mAb 4102-21-14 binding to rHV1 (△), and rHV1 peptide 29–38 (○). B/Bo × 100 (%) represents the percentage of mAb bound to the plate

as determined by competitive ELISA, reflected those observed with the corresponding animal sera. The mAb 4049-83-12 showed the highest affinity for rHV1 (K_d of 0.6 nM) and showed no cross-reactivity with the C-terminal peptides (Fig. 1). The three anti-peptide mAbs recognized rHV1, however with a lower affinity (K_d of 1.5–7 nM) (Table 1, Fig. 1). By double mAb sandwich ELISA [40], mAb 4049-83-12 combined with any anti-peptide mAbs was able to bind rHV1 in a dose-dependent manner, whereas a combination of anti-peptide mAbs only failed to bind rHV1 (data not shown), indicating that the epitopes of the anti-peptide mAbs overlapped but were distinct from that of mAb 4049-83-12 (see below). In the clotting assay, only the mAb 4049-83-12 could completely neutralize rHV1 (Fig. 2). Indeed, even at an rHV1 concentration of 85 nM (corresponding to a slight excess of rHV1 over thrombin) where clotting time is infinite, the

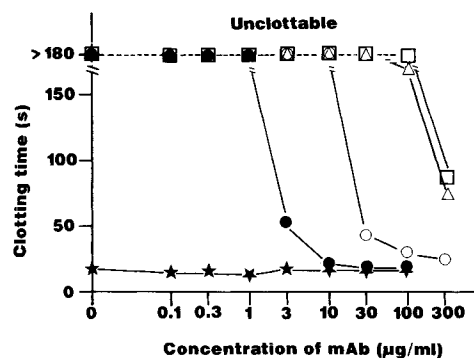


Fig. 2. Neutralizing capacity of the anti-hirudin mAbs. The clotting assay was performed by mixing increasing concentrations of mAbs to rHV1 and then by adding the mixture to thrombin and fibrinogen. mAb 4049-83-12 (●); mAb 4114-96-1 (△); mAb 4120-37-7 (○); mAb 4102-21-14 (□); mAb 4049-83-12 without rHV1 (★)

anticoagulant activity of rHV1 was totally neutralized by an equimolar concentration of mAb binding sites (around 5–10 μg/ml). The same neutralizing activity of mAb 4049-83-12 was observed using chromozym-TH as a thrombin substrate (data not shown). Furthermore, the addition of an excess of this mAb (100 μg/ml) to rHV1 already complexed to thrombin could partially restore the clotting activity of thrombin (data not shown). The anti-peptide mAb 4120-37-7 could also neutralize rHV1 activity, although the mAb concentration needed to neutralize the rHV1 completely was ten times higher than that of mAb 4049-83-12 (Fig. 2).

Epitope mapping

The epitopes on rHV1 recognized by these mAbs were determined either by competitive ELISA using rHV1 ana-

Table 2. Identification of rHV1 fragments separated by HPLC after a 30-min digestion with V8 protease

The peak numbers refer to those of Fig. 3. The proteolytic fragments in the peaks were assigned to the peptides listed on the basis of the best fit of the amino acid composition to the known primary structure of rHV1 [2]

Amino acid	Amount in											
	peak 1	peptide 62–65	peak 2	peptide 1–43	peak 3	peptide 44–61	peak 4	peptide 1–61	peak 5	peptide 1–65	peak 6	peptide 44–65
	residues/molecule											
Asp/Asn			5.0	6	2.0	3	5.6	9	6.1	9	2.6	3
Glu/Gln	1.8	2	6.8	7	3.2	4	9.2	11	9.8	12	4.9	6
Cys			7.0	6			4.2	6	3.9	6		
Gly			7.0	7	2.6	2	10.1	9	10.0	9	2.4	2
His					0.7	1	1.2	1	1.3	1	0.7	1
Ile			1.4	1	0.8	1	2.8	2	2.6	2	1.0	1
Leu	1.1	1	3.3	3			4.2	3	3.9	4	1.2	1
Lys			1.8	2	0.7	1	2.9	3	2.5	3	0.9	1
Phe					0.7	1	1.7	1	1.7	1	0.8	1
Pro					1.5	3	2.5	3	2.4	3	2.3	3
Ser			3.7	3	1.7	1	6.2	4	6.9	4	1.4	1
Thr			2.8	3	0.8	1	3.3	4	2.8	4	1.1	1
Tyr	0.5	1	0.7	1			0.9	1	1.7	2	0.7	1
Val			3.3	4			3.4	4	3.4	4		

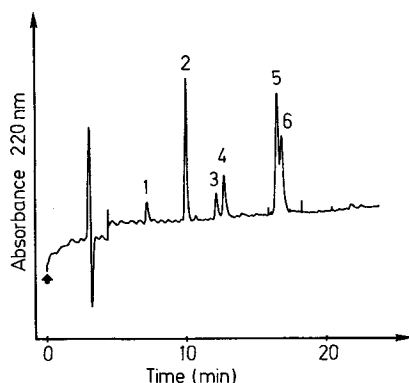


Fig. 3. Separation by reverse-phase HPLC of rHV1 fragments obtained after 30-min digestion by V8 protease. Samples of 7.6 µg were applied on the column. Peaks 1–6 were collected and identified by amino acid analysis (Table 2)

logues, or by the proteolysis of the rHV1-antibody complex, as described previously [41]. The competitive ELISA was performed on microtiter plates coated with biotinylated rHV1 linked to avidin in the case of mAb 4049-83-12, since this mAb bound poorly to rHV1 directly adsorbed on the plates; rHV1-coated plates were used for the other three mAbs. For mAb 4049-83-12, no cross-reactivity was observed between rHV1 and the four peptides 1–15, 29–38, 40–65 and 52–65 which encompassed most of the sequence of rHV1 (Table 1). Furthermore, reduction and *S*-carboxymethylation of rHV1, which destroyed the three-dimensional structure of hirudin by cleavage of the disulfide linkages, completely prevented the binding of the mAb, suggesting a conformationally well ordered epitope. On the other hand, the anti-peptide mAbs recognized both C-terminal peptides 40–65 and 52–65; they showed equal or even higher affinity for the *S*-carboxymethylated rHV1 compared to that of the native molecule. None of the four mAbs recognized rHV3, despite more than 80% similarity between both hirudins [4].

The epitopes were further defined by chemical modification of rHV1 with 4-*N,N*-dimethylaminobenzene-4'-isothiocyano-2'-sulfonic acid. This treatment modified more than 90% of Val-1 and Lys-27 of rHV1 [34] and resulted in a significant decrease of the binding of mAb 4049-83-12 (Table 1). When the modified rHV1 was kept in trifluoroacetic acid for 10 min at 50 °C, des-Val-1, Lys-27-rHV1 was obtained [34]; however, this treatment could not restore the full binding of mAb 4049-83-12 ($I_{50} = 33$ ng/ml) suggesting that the modification of Lys-27 was probably responsible for the reduced binding of mAb 4049-83-12 (see Discussion).

The C-terminal domain of hirudin is sensitive to proteolysis by V8 protease [19]. The influence of this treatment on the binding of the mAbs was tested by two kinds of experiments. In a first approach, rHV1 was incubated for 0.5–4 h with the enzyme in ammonium bicarbonate buffer in order to restrict the cleavage to the glutamyl bonds [35]. The digest was then tested by competitive ELISA and subjected to N-terminal analysis. After a 2-h proteolysis, 97% and 50% of cleavage occurred after Glu-43 and Glu-61, respectively, and the binding of all four mAbs was considerably reduced (Table 1), indicating that the cleavage of the C-terminal domain destroyed the mAb epitopes. In a second approach, rHV1 was digested for 0.5 h and the fragments were separated by reverse-phase HPLC and identified by amino acid analysis (Fig. 3, Table 2). The comparatively higher discrepancy between the amino acid composition and the integers of peak 4 was due to the very low yield of peptide 1–61. Indeed the bond between Glu-43 and Gly-44 is the most sensitive to the protease, implying that the peptide is rapidly cleaved to peptides 1–43 and 44–61 during the course of the digestion (see below). The fragments corresponding to rHV1 1–43 and 1–61 were purified by HPLC and analyzed by competitive ELISA. Only mAb 4049-83-12 could bind to rHV1 1–61 (Table 1), showing that the epitopes of the anti-peptide mAbs, were located close to the C-terminus of rHV1. mAb 4049-83-12 failed to bind rHV1 1–43, suggesting that the epitope of this mAb, although clearly distinct from the anti-peptide mAbs, was located near the C-terminal domain of rHV1 (see below).

Table 3. Quantitative analysis of the V8 proteolysis of the rHV1-mAb complex

The values listed are peak-height ratios of free rHV1/mAb-bound rHV1 of the HPLC-fractionated peptides obtained after digestion with V8 protease for 30 min and 120 min. The peak numbers refer to those of Fig. 3. The peptides were assigned as described in Table 2. mAb 4063-21-1 is the control IgG₁ (anti-hapten mAb). The values are average values from two experiments

HPLC peak	rHV1 peptide	Peak-height ratios after									
		30-min digestion					120-min digestion				
		mAb 4049-83-12	mAb 4114-96-1	mAb 4120-37-7	mAb 4102-21-14	mAb 4063-21-1	mAb 4049-83-12	mAb 4114-96-1	mAb 4120-37-7	mAb 4102-21-14	mAb 4063-21-1
1	62–65	1.2	> 5	> 5	> 5	1.0	1.4	> 10	> 9	> 9	1.0
2	1–43	> 17	1.1	0.7	1.0	0.9	> 31	1.1	0.7	0.9	0.9
3	44–61	> 3	> 4	> 4	> 4	1.0	> 15	> 17	> 14	> 15	1.0
4	1–61	0.8	> 7	> 8	> 7	1.0	0.1	> 3	> 2	> 3	1.2
5	1–65	0.6	0.7	0.6	0.7	1.0	0	0	0	0	(0) ^a
6	44–65	> 10	0.7	0.5	0.7	0.9	> 2	0.1	0.2	0.2	1.0

^a After 120 min of V8 digestion, no peak 5 was detected with either rHV1 or with mAb 4063-21-1 + rHV1 (see Fig. 4A).

Hirudin is resistant to trypsin digestion since Lys-47 is followed by Pro [19]; however, the Lys-Pro bond of rHV1 could be readily cleaved by *Achromobacter lyticus* protease I, a lysyl endopeptidase [42]. After 90 min of digestion, the cleavage after Lys-47 and Lys-36 was complete (since Cys-28 is linked to Cys-16, the cleavage after Lys-27 could not be determined by one-step N-terminal analysis). This treatment significantly reduced the binding of mAb 4049-83-12, whereas almost no effect was observed using the anti-peptide mAbs (Table 1).

These results were further confirmed by analyzing the proteolysis of the rHV1-mAb complex. Indeed, it has been shown previously that an mAb bound to a protein antigen decreases the rate of proteolytic cleavage of the antigen, having the greatest effect on those regions involved in antibody contact [41]. We subjected rHV1-mAb complexes to V8 proteolysis for 0.5 h and 2 h, and separated the resulting fragments by HPLC. Controls included digestion of rHV1 without anti-hirudin mAbs, digestion of rHV1 in the presence of an unspecific mAb (an IgG₁ binding to atrazine, a small hapten unrelated to rHV1), and digestion of the four anti-hirudin mAbs in the absence of rHV1. When the rHV1-mAb 4049-83-12 complex was subjected to V8 proteolysis for 2 h, peaks 2, 3 and 6 (corresponding to the peptides 1–43, 44–61 and 44–65, respectively) completely disappeared from the chromatogram whereas peak 5 (undigested rHV1), which was no longer detectable in the controls, increased drastically (Fig. 4 B, Table 3). This clearly demonstrated that the cleavage after Glu-43 was prevented by the presence of mAb 4049-83-12, whereas cleavage after Glu-61 occurred to the same extent as in the controls. Since the latter cleavage was not complete (i.e. only 50% after 2 h), undigested hirudin appeared on the chromatogram. A different pattern was observed when the anti-peptide mAb-rHV1 complexes were subjected to V8 proteolysis: peaks 1, 3 and 4 disappeared from the chromatogram (corresponding to the peptides 62–65, 44–61, 1–61 respectively), whereas peak 6 (peptide 44–65) increased significantly, indicating that, in this case, the cleavage occurred after Glu-43 but not after Glu-61 (Fig. 4 C and Table 3). Protection from V8 cleavage after Glu-61 was also observed when the anti-peptide mAbs were bound to the peptides 40–65 or 52–65 (data not shown). It followed that, despite the size of the antibodies, it was possible to differentiate between the shielding capacity of the anti-peptide mAbs

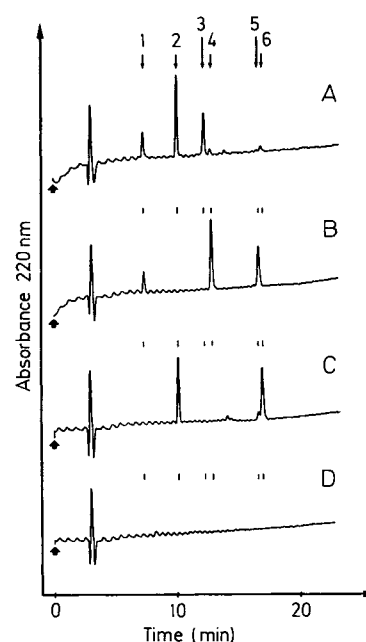


Fig. 4. Proteolysis of the hirudin-mAb complex. HPLC separation of rHV1 fragments obtained after a 2-h digestion by V8 protease. The components of the digest were as follows: rHV1 and an unrelated mAb (A); rHV1 and mAb 4049-83-12 (B); rHV1 and the anti-peptide mAb 4114-96-1 (C); mAb 4114-96-1 (D). Samples of 2.9 µg rHV1 with 39 µg mAb were applied on the column. The peak numbers refer to those of Fig. 3

and that of mAb 4049-83-12. Protection experiments where mAb-rHV1 complexes were exposed to lysyl endopeptidase confirmed these results. As shown in Fig. 5 B, rHV1 bound to mAb 4049-83-12 was fully protected from proteolysis during the 6-h incubation with the enzyme. It eluted as one peak at the same retention time as untreated rHV1 (Fig. 5C). After a 6-h digestion, unprotected rHV1 eluted in two peaks (Fig. 5A). They corresponded to the fragments 48–65 and 1–47, the latter fragment being held together by its disulfide linkages. Therefore, we concluded that the cleavage after Lys-47 was completely prevented by the binding of mAb 4049-83-12. On the other hand, when the anti-peptide mAb 4102-21-

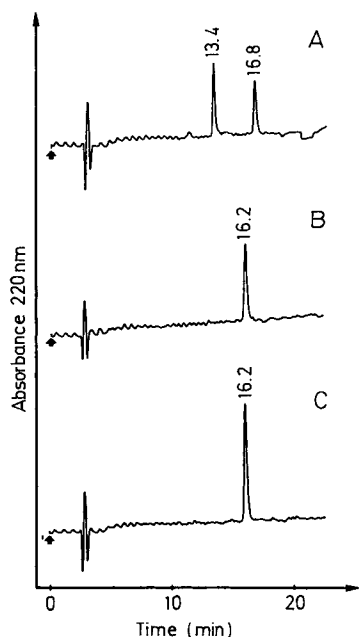


Fig. 5. Proteolysis of the hirudin-mAb complex. HPLC separation of rHV1 fragments obtained after a 6-h digestion by lysyl endopeptidase: 2.9 μ g rHV1 (A); 2.9 μ g rHV1 and 39 μ g mAb 4049-83-12 (B); 2.9 μ g undigested rHV1 (C)

14 was bound to rHV1, proteolysis occurred as in the controls (data not shown).

Binding of the mAbs to the hirudin-thrombin complex

The binding of the mAbs to rHV1-thrombin complex was tested by competitive ELISA and by a double-antibody sandwich assay (see Materials and Methods). In the competition experiment, a 2.1 molar excess of thrombin was added to rHV1 prior to binding to the mAbs. In these conditions, the binding of mAb 4049-83-12 and mAb 4120-37-7 to rHV1 was drastically reduced (I_{50} of 73 ng/ml and >100 ng/ml respectively). In the sandwich ELISA, we used the anti-thrombin mAb EST 6 as a capture antibody, in order to avoid any denaturation of thrombin upon direct binding on the plates. This mAb did not compete with rHV1 for binding to thrombin (S. Vekemans, J.-Y. Chang, and J.-M. Schlaeppli, unpublished results). We observed that none of the four mAbs could bind rHV1 once bound to thrombin. Also, no binding of the mAbs was observed when the thrombin-rHV1 complex was directly applied to the plates (without using mAb EST 6).

DISCUSSION

Antibodies were raised against rHV1 and its C-terminal peptides. In the clotting assay, the anti-peptide mAbs showed limited neutralizing capacities, whereas the mAb 4049-83-12 could completely neutralize rHV1 in equimolar concentrations (Fig. 2). The detailed epitope analysis revealed that the binding sites of the three anti-peptide mAbs were located very close to the C-terminus of rHV1 (Fig. 6). The evidence for this assignment was: (a) the cleavage of the bond between Glu-61 and Glu-62 by the V8 protease was inhibited by the binding of the mAbs to rHV1 (Fig. 3, Table 3); (b) we observed no cross-reaction with rHV1 1–61 (Table 1); (c) rHV3 which differs from rHV1 at positions 62, 63, 65 and 66, could not

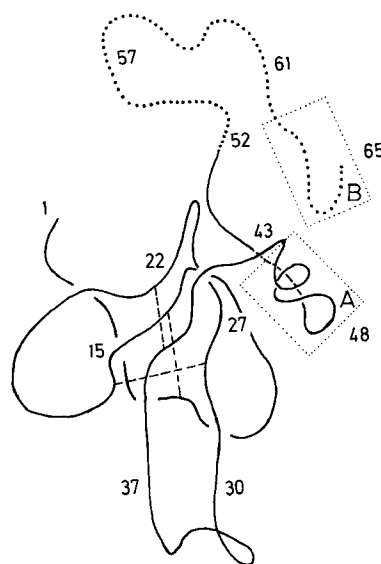


Fig. 6. Primary binding site of mAb 4049-83-12 (A) and of the three anti-peptide mAbs (B) on the rHV1 molecule. The three-dimensional structure determined by NMR is from [12]. The dotted line represents the C-terminal peptide 52–65

bind the mAbs (Table 1). These results indicate that the final residues of the C-terminal domain of rHV1 are readily accessible on the surface of the molecule and can adopt a conformation which can be mimicked by the linear peptides 40–65 or 52–65, in accordance with NMR data showing that the residues 58–65 form an irregular strand [9, 12]. In addition, the partial neutralizing capacity of rHV1 by mAb 4120-37-7 (Fig. 2) and the absence of binding of the anti-peptide mAbs to the thrombin-rHV1 complex fully support previous observations indicating that the C-terminal region of rHV1 interacts with thrombin [16–18].

Competitive ELISA experiments, double-mAb sandwich assays and proteolysis of the mAb-rHV1 complex clearly demonstrated that the epitope of mAb 4049-83-12 did not overlap with those of the anti-peptide mAbs, and that the mAb bound primarily to the connecting domain (residues 41–47) between the central core and the exposed C-terminal loop of rHV1 as shown by NMR studies [9–12] (Fig. 6). This conclusion is supported by the following results. (a) The epitope is conformation-dependent, being present only in the native rHV1 molecule, since *S*-carboxymethylation of rHV1 significantly affected the binding of the mAb (Table 1). (b) The mAb prevented the cleavage after Glu-43 by V8 protease and after Lys-47 by lysyl endopeptidase (Figs 4 and 5, Table 3). (c) Competitive ELISA showed that rHV1 cleaved by either enzyme reduced the affinity to the mAb. (d) The mAb could bind rHV1 1–61 but not rHV1 1–43 (Table 1). The failure of rHV3 to bind the mAb, despite having the same primary structure as rHV1 between residues 37–52 [4], could result from minor folding differences in this region. However we cannot rule out the possibility that other residues located in the N-terminal core domain participate in the binding to the mAb. From X-ray crystallography studies of antibody-antigen complexes, it appears that the average surface complementarity of an antigen is around 7.5 nm² and can accommodate up to 15 amino acids [43]. It follows that the chemical modification of Lys-27 which reduced the binding of rHV1 to the mAb (Table 1) may indi-

cate that this residue, which is close to the connecting domain 41–47 in the folded molecule (Fig. 6), is indeed still enclosed in the epitope.

This report describes for the first time the generation of an mAb which binds to the region 41–47 of rHV1. All the anti-hirudin mAbs reported previously bind close to the C-terminus of rHV1 [44], very probably reflecting the poor immunogenicity of hirudin [45]. Interestingly, within the region 40–48, Lys-47 has been proposed as the putative reactive-site residue P_1 of hirudin: firstly, it is preceded and followed by proline residues (i.e. Pro-46 would enhance binding at the corresponding apolar binding site of thrombin and Pro-48 would make the bond with Lys-47 resistant to proteases); secondly, the amino acid sequence 40–48 is similar to the α -thrombin cleavage site in prothrombin (residues 149–157) [46]. However, point mutations of residue 47 [6, 21, 25] influenced the inhibitory activity of hirudin only weakly, indicating that an extended binding region of the inhibitor is involved in ionic and hydrophobic interactions with thrombin [5, 10, 13].

Our results with mAb 4049-83-12 support the idea that the rHV1 domain 41–47 nevertheless interacts with thrombin. Indeed, the mAb can completely neutralize the rHV1 anticoagulant activity, it cannot bind to rHV1 once bound to thrombin; and preliminary results with anti-idiotypic sera raised in syngeneic mice show that these sera bind specifically to thrombin and that this binding is prevented by rHV1 (J.-M. Schlaeppli, unpublished results). Current models suggest that the C-terminal domain of hirudin binds initially to a site distinct from the thrombin active site and, through a conformational rearrangement of the protease, a tighter complex is formed in which hirudin is also bound to the active site [5, 16, 47]. Therefore, we can infer that the latter interaction is blocked upon binding of the mAb to the rHV1 domain 41–47.

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