Factor VII mutant V154G models a zymogen-like form of Factor VIIa

Raffaella TOSO*^{†1}, Francesco BERNARDI*, Theresa TIDD[‡], Mirko PINOTTI*, Rodney M. CAMIRE[‡], Giovanna MARCHETTI*, Katherine A. HIGH[‡] and Eleanor S. POLLAK^{†‡}

*Department of Biochemistry and Molecular Biology, University of Ferrara, Via Luigi Borsari, 46 Ferrara 44100, Italy, [†]Joseph Stokes Research Institute, Children's Hospital of Philadelphia, 3516 Civic Center Boulevard, Philadelphia, PA 19104, U.S.A., and [‡]Department of Pathology and Laboratory Medicine, Hospital of the University of Pennsylvania, 3400 Spruce Street, Philadelphia, PA 19104, U.S.A.

Proteolytic cleavage of the peptide bond between Arg¹⁵² and Ile¹⁵³ converts the procoagulant protein Factor VII (FVII) to an activated two-chain form (FVIIa). The formation of a salt bridge between Ile¹⁵³ and Asp³⁴³ drives the conversion of FVIIa from being zymogen-like to the active form. In the present paper, we describe the novel FVII mutant V154G (Val¹⁵⁴ → Gly mutation; residue 17 in the chymotrypsin numbering system), found in three FVII-deficient patients, which models a zymogen-like form of FVIIa. Recombinant V154G FVIIa, although normally cleaved, shows markedly reduced activity towards peptidyl substrate and undetectable activity towards macromolecular substrates. Susceptibility of Ile¹⁵³ to chemical modification, in either the presence or the absence of tissue factor (TF), suggests that the reduced V154G FVIIa activity is caused by impaired salt-bridge formation, thus resulting in a zymogen-like FVIIa form. The TF-mediated protection from chemical modification of V154A indicated that Gly¹⁵⁴ is responsible for this peculiar feature, and suggests that this region, proximal to the heavy chain

N-terminus, is directly involved in the conversion of FVII into FVIIa. V154G FVII was exploited to study the FVII–TF interaction, together with three additional FVII variants that were expressed to serve as models for different FVII forms. The comparison of binding affinities of full-length TF after relipidation in L- α -phosphatidylcholine for the zymogen FVII (Arg¹⁵² → Gln, $K_d = 1.04 \pm 0.27$ nM), inactive FVIIa (Ser³⁴⁴ → Ala, $K_d = 0.27 \pm 0.06$ nM) and a zymogen-like FVIIa (V154G, $K_d = 1.15 \pm 0.16$ nM) supports the hypothesis that preferential binding of TF to active FVIIa is insufficient to drive the 10⁵-fold enhancement of FVIIa activity. In addition, the inability of V154G FVIIa to accommodate an inhibitor in the active site, indicating an improperly shaped specificity pocket, would explain the low activity of the zymogen-like form of FVIIa, which is predominant in the absence of TF.

Key words: mutation, salt bridge, tissue factor, zymogenicity.

INTRODUCTION

The enzymic complex formed between the procoagulant serine-protease-activated Factor VII (FVIIa) and the integral membrane protein tissue factor (TF) triggers the initiation of blood clotting [1]. The action of the FVIIa–TF complex generates a burst of activated Factors IX (FIXa) and X (FXa) ultimately leading to formation of a stable fibrin clot [2,3]. *In vivo*, only approx. 1 % of Factor VII (FVII) exists as FVIIa [4]. At sites of vascular injury, FVIIa interacts with TF resulting in the rapid formation of the extrinsic complex [5].

Steps involved in the conversion of FVII to a catalytically active FVIIa–TF complex are only partially understood. The initial event, cleavage of the peptide bond between Arg¹⁵² and Ile¹⁵³, converts the zymogen FVII into a two-chain serine protease that exists in equilibrium between a predominant zymogen-like form and an active FVIIa [6–9]. In the zymogen-like conformation, the new N-terminus of Ile¹⁵³ is solvent-exposed and accessible to modification, whereas in the active FVIIa, a salt bridge forms between the α -amino group of Ile¹⁵³ and the β -carboxyl group of Asp³⁴³, stabilizing the insertion of Ile¹⁵³ into the protein [10]. As inferred from studies on trypsinogen, the formation of this salt bridge orders the activation domain and shapes the S1 specificity pocket [11,12] (nomenclature of Schechter and Berger [13]). However, it is not well understood whether the exposure of extended macromolecular interaction sites (exosites) that are necessary for substrate recognition

depends upon this rearrangement. Two-chain FVIIa shows poor activity towards both macromolecular and peptidyl substrates [14,15], possibly due to a predominant zymogen-like conformation in the absence of TF. TF dramatically enhances FVIIa activity (10⁵-fold) either by preferentially binding to the active form of FVIIa or by directly driving the conversion of zymogen-like FVIIa into active FVIIa when bound to the inactive form [7,16]. TF may enhance FVIIa activity by exploiting a combination of these mechanisms.

Understanding the mechanism by which TF can boost FVIIa catalytic efficiency may provide novel insights into designing alternative molecules for therapeutic aims in haemophilia and other bleeding disorders [17,18]. Previous experimental assays used to study the binding of FVII to TF have been limited by the susceptibility of the zymogen FVII to autoactivation in the presence of TF and by inhibitors in the FVIIa active site that are known to constrain FVIIa in the active conformation [7,19].

In the present paper, we characterize a naturally occurring mutation, the substitution of glycine for Val¹⁵⁴ (V154G) at the P2' residue in the serine-protease domain of FVIIa (residue 17 in the chymotrypsin numbering system [20]). This substitution creates a peculiar model of zymogen-like FVIIa, since V154G FVIIa cannot reach the active conformation even in the presence of TF. V154G FVIIa therefore represents a 'stable' form of the FVIIa intermediate, which was exploited to study its interaction with TF compared with other FVII/FVIIa forms. The V154G FVIIa has also allowed us to analyse the competence of the

Abbreviations used: FVII, Factor VII; FVIIa, activated FVII; FVII:Ag, FVII antigen; FVII:C, FVII coagulant activity; FIX, Factor IX; FIXa, activated FIX; FX, Factor X; FXa, activated FX; ITS, insulin/transferrin/sodium selenite supplement; PC, L- α -phosphatidylcholine; PS, L- α -phosphatidylserine; PCPS, 75 % (w/w) PC and 25 % (w/w) PS; PEG 8000, poly(ethylene glycol) with a mean molecular mass of 8000 Da; TAP, tick anticoagulant peptide; TF, tissue factor; TF/PC, TF after relipidation in PC.

¹ To whom correspondence should be addressed (e-mail r_toso@hotmail.com).

specificity pocket in a zymogen-like form of FVIIa, and suggests the importance of the region proximal to the heavy chain N-terminus in the conversion from zymogen-like FVIIa into the active form. This aspect was further investigated by expressing and characterizing an alternate substitution of alanine for Val¹⁵⁴ (V154A) in FVII.

EXPERIMENTAL

Materials

Hepes, poly(ethylene glycol) of a mean molecular mass of 8000 Da (PEG 8000) and Nunc cell factories were from Fisher Scientific (Pittsburgh, PA). L- α -Phosphatidylcholine (PC), L- α -phosphatidylserine (PS) and benzamidine were from Sigma (St. Louis, MO). *N*-Octyl β -D-glucopyranoside was from Calbiochem (La Jolla, CA) and Q Sepharose Fast Flow was from Pharmacia (Piscataway, NJ).

The chromogenic substrates methoxycarbonyl-D-cyclohexylglycyl-glycyl-arginine *p*-nitroanilide acetate (Spectrozyme fXa), methanesulphonyl-D-cyclohexylalanyl-butyl-arginine *p*-nitroanilide acetate (Spectrozyme fVIIa), methylsulphonyl-D-cyclohexylglycyl-glycyl-arginine *p*-nitroanilide acetate (Spectrozyme fIXa) and the IMUBIND® Factor VIIa ELISA kit were from American Diagnostica (Greenwich, CT). Stock solutions (approx. 5 mM) of these substrates were prepared in water and concentrations were determined using a molar absorption coefficient, ϵ_{342} , of $8270 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [21]. Insulin/transferrin/sodium selenite supplement (ITS) was purchased from Roche Diagnostics (Indianapolis, IN). The reaction buffer consisted of 20 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM CaCl₂ and 0.1 % (w/v) PEG 8000.

Phospholipid vesicles, composed of 75 % (w/w) PC and 25 % (w/w) PS (PCPS) were prepared as described in [22] and their concentration was determined by colorimetric phosphate analysis [22].

Proteins

Plasma-derived human FVII, Factor IX (FIX) and Factor X (FX), and human FIXa were from Haematologic Technologies Inc. (Essex Junction, VT). Plasma-derived FXa, soluble human TF (amino acid residues 1–219) and tick anticoagulant peptide (TAP), expressed in *Pichia pastoris*, were generously given by Dr Sai Kumar Buddai and Dr Sriram Krishnaswamy from the Children's Hospital of Philadelphia, PA. Recombinant Novoseven™ FVIIa was from Novo-Nordisk (Gentofte, Denmark). The ELISA kit to detect FVII was from Enzyme Research Laboratories (South Bend, IN). Full-length human TF made in insect cells was purchased from American Diagnostica (Greenwich, CT). Full-length and soluble human TF (amino acid residues 1–219), both produced in *Escherichia coli*, were kindly provided by Dr James H. Morrissey of the University of Illinois College of Medicine, Urbana-Champaign, IL. Full length human TF (25 μg) was reconstituted in 100 % (w/w) PC (1.4 mg) vesicles using 80 mM *N*-octyl β -D-glucopyranoside, as described in [23], for reconstitution of TF in PCPS. The effective concentration of TF after relipidation in PC (TF/PC) was assumed to correspond to 50 % of the total concentration present due to the random orientation of TF during reconstitution [24]. This TF concentration was validated by titration experiments. Reconstituted TF was stored in the dark at 22 °C and used in experiments within 4 days of preparation. Ca²⁺-dependent anti-FVII monoclonal antibody was generously given by Dr Ulla Hedner of Novo-Nordisk, Gentofte, Denmark.

The γ -carboxyglutamic acid content of purified protein, which was determined by alkaline hydrolysis performed at Commonwealth Biotechnologies (Richmond, VA), verified full carboxylation of recombinant proteins.

Genomic studies and site-directed mutagenesis

FVII-exon and splicing-junction scanning was performed by direct sequencing as described in [25]. Population screening for the T⁸⁹⁶⁷ → G mutation, which results in the FVII mutant V154G, was performed by restriction analysis of an amplified genome fragment. Amplification of patient genomic DNA using the oligonucleotide primer 5'-AAACCCCAAGGCCGAAGT-3' (corresponding to residues 8948–8966) created a new *Bsr*I restriction site exclusively in the mutated sequence that results in a different pattern of enzymic digestion in the presence of the mutation from that in its absence (nucleotide numbering is according to FVII DNA sequence published by O'Hara et al. [26]).

The substitutions Arg¹⁵² → Gln (R152Q), V154G, V154A, Asp³⁴³ → His (D343H) and Ser³⁴⁴ → Ala (S344A) were introduced in FVII cDNA cloned in pCDNA3.1 vector (Invitrogen, San Diego, CA) using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The presence of the point mutation was confirmed by DNA-sequence analysis of the entire FVII cDNA.

In vitro expression and purification of FVII recombinant proteins

HEK-293 cells were transfected using FuGene™ 6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN) or Lipofectamine 2000 (Gibco, Gaithersburg, MD) following the manufacturers' instructions. Cells were grown in Dulbecco's modified Eagle's medium/F12 in the presence of 10 % (v/v) foetal bovine serum, 5 $\mu\text{g}/\text{ml}$ vitamin K, 2 mM L-glutamine, 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. Selection was performed as described in [27]. The highest producers (1.5–2 $\mu\text{g}/\text{ml}$ per 10⁶ cells over 24 h) were selected by ELISA, following the manufacturer's instructions, and further expanded to a cell-factory system using medium containing 10 $\mu\text{g}/\text{ml}$ ITS. After 72 h, conditioned medium was collected at 24 h intervals. Benzamidine (10 mM) was added before filtering the medium through a 0.45 μm cellulose nitrate filter and the filtrate was frozen at –20 °C.

Purification of FVII from conditioned medium was carried out as described in [28]. The homogeneity of all protein preparations was evaluated by SDS/4–12 % PAGE [29]. FVII concentration was determined using a molecular mass of 50000 kDa and a molar absorption coefficient, ϵ , of $69500 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

FVII cleavage and functional assays

Recombinant FVII derivatives (2 μM) were incubated for 30 min in reaction buffer at 37 °C with 400 μM PCPS and 20 nM human FXa. TAP, at a concentration of 2 μM , was used to specifically inhibit FXa. The formation of two-chain FVIIa was evaluated by SDS/PAGE analysis after Coomassie Blue staining.

Peptidyl substrate cleavage

FVIIa (10 nM) was incubated in reaction buffer at 22 °C in the presence or absence of 50 nM soluble TF and 200 μM PCPS. Spectrozyme fVIIa was added at concentrations ranging from 0 to 1 mM immediately before monitoring the A_{405} as a function of time using a VMax plate reader (Molecular Devices, Menlo Park, CA). Initial rates were expressed in concentration terms using a molar absorption coefficient, ϵ_{405} , of $9650 \text{ M}^{-1} \cdot \text{cm}^{-1}$ and an

effective path length of 0.59 cm calculated for a 200 μ l reaction volume. Due to their markedly reduced activity, K_m values of V154G and V154A FVIIa for the chromogenic substrate were evaluated using 200 nM or 40 nM FVIIa in the presence or absence of 1 μ M or 200 nM soluble TF respectively. Amidolytic activity of R152Q FVII, D343H FVIIa and S344A FVIIa was tested using 10 nM, 200 nM and 500 nM FVII/FVIIa in the presence of 200 μ M PCPS and 50 nM, 400 nM and 1 μ M soluble TF respectively.

Macromolecular substrate cleavage

Activity towards FX

FVIIa (1 nM) was incubated in reaction buffer at 37 °C with 10 nM TF and 60 μ M PC, or with 10 nM soluble TF and 100 μ M PCPS; the reaction was initiated by the addition of 100 nM human FX. Aliquots of the reaction were quenched at several time points up to 3.5 min in 20 mM Hepes, pH 7.4, 150 mM NaCl, 50 mM EDTA and 0.1% (w/v) PEG 8000. The FXa chromogenic substrate (Spectrozyme fXa) was added at a final concentration of 100 μ M. FXa activity was monitored by measuring the A_{405} at 22 °C. The concentration of FXa formed as a function of time was determined by interpolation from the linear dependence of the initial rate of Spectrozyme fXa hydrolysis on known concentrations of FXa that were determined separately. The initial steady-state rate of FXa formation was determined from the slope of plots that documented the linear appearance of FXa with time. The same experiment was performed using different concentrations of FVIIa (1 or 10 nM) in the presence of soluble TF (10 or 100 nM respectively).

Activity towards FIX

FVIIa (1 nM) was incubated at 37 °C in reaction buffer with 200 nM soluble TF and 500 μ M PCPS. The reaction was initiated by the addition of 400 nM human FIX. Aliquots of the reaction were quenched at several time points within 2 min in 30 mM EDTA and 60% (v/v) ethylene glycol. FIXa activity was monitored at 405 nm after the addition of the chromogenic substrate Spectrozyme fIXa (500 μ M). The concentration of FIXa that formed as a function of time was determined by interpolation from the linear dependence of the initial rate of Spectrozyme fIXa hydrolysis on known concentrations of FIXa that were determined separately. The initial steady-state rate of FIXa formation was determined from the slope of plots that documented the linear appearance of FIXa with time.

Carbamylation of FVIIa

FVIIa (4 μ M), in the presence or absence of 20 μ M soluble TF, was incubated with 0.2 M NaNCO at 22°C in reaction buffer, pH 7.2. Aliquots from the reaction were taken at 0, 15, 30, 60, 90, 120, 150 and 180 min. Residual activity of FVIIa towards Spectrozyme fVIIa (800 μ M) was measured using 20 nM FVIIa, 100 nM soluble TF for wild-type; 200 nM FVIIa, 1 μ M soluble TF for V154G FVIIa and 60 nM FVIIa, 300 nM soluble TF for V154A FVIIa.

In order to rule out the possibility that carbamylation affects TF cofactor activity, 2 μ M soluble TF was incubated at 22°C in the absence or presence of 0.2 M NaNCO in reaction buffer, pH 7.2. After 0, 1 and 2 h time intervals, TF cofactor activity was tested using 100 nM soluble TF in the presence of 20 nM FVIIa, 100 μ M PCPS in Reaction Buffer. The amidolytic activity of the soluble TF–FVIIa complex was assayed adding Spectrozyme fVIIa (375 μ M).

FVII interaction with an active-site inhibitor

The ability of wild-type and mutated FVIIa to accommodate an inhibitor in the active site was tested using IMUBIND® Factor VIIa ELISA kit that employs a biotinylated enzyme inhibitor of FVIIa and an anti-FVII/FVIIa monoclonal antibody as the capture antibody. The assay was performed following instructions of the manufacturer. Wild-type and Novoseven™ FVIIa concentrations ranged from 0.031 nM to 2 nM, whereas V154G FVIIa concentrations ranged from 0.5 nM to 8 nM. The active-site inhibitor provided was a Phe-Pro-Arg chloromethylketone.

TF-binding competition assay for recombinant proteins

Novoseven™ FVIIa (10 nM) was incubated in Reaction Buffer and 100 μ M PCPS, with different fixed concentrations of soluble TF (5, 10 and 20 nM) in order to form a functional FVIIa–TF complex. Novoseven™ FVIIa was displaced from the complex by adding increasing concentrations of each of the inactive FVII/FVIIa mutants (R152Q uncleavable FVII, V154G FVIIa, D343H zymogen-like FVIIa or S344A inactive FVIIa) ranging from 0 to 1 μ M, during a 30 min incubation at 37 °C. Residual amidolytic activity of soluble TF–FVIIa complex was assayed adding Spectrozyme fVIIa. Figure 3(C) refers to the experiment using the different FVII mutants in the presence of 10 nM soluble TF. In order to rule out a possible difference in dissociation constant values among FVII forms due to the presence of phospholipid vesicles, the same competition experiment was carried out in the absence of membrane using R152Q FVII as competitor. Binding of FVII/FVIIa to full length TF reconstituted in PC was performed under the same conditions but in the presence of 5 nM Novoseven™ FVIIa, and different fixed concentrations of full length TF (3, 5 and 15 nM TF relipidated in 12, 20 and 60 μ M PC, respectively). FVII/FVIIa mutants ranged from 0 to 200 nM.

FVII autoactivation

The time course of wild-type FVII autoactivation was performed in reaction buffer using concentrations of the soluble TF–FVII complex above (100 nM FVII and 100 nM soluble TF in the presence of 2.5 nM FVIIa, either with or without 430 μ M PCPS) and below (32 nM FVII and 30 nM soluble TF in the presence or absence of 2.5 nM FVIIa, either with or without 430 μ M PCPS) the K_d value we calculated in the present study. Aliquots of the reactions were tested for the amidolytic activity of the soluble TF–FVIIa complex at 0, 10, 20, 40, 60, 90 and 120 min by adding Spectrozyme fVIIa (375 μ M).

Data analysis

Steady-state kinetic constants for synthetic substrate hydrolysis were determined from duplicate measurements of the initial velocity that were obtained using different substrate concentrations. Initial velocity data were fit to the Michaelis–Menten equation [30] by nonlinear least-squares regression analysis using the Marquardt algorithm [31]. When saturation of enzyme by substrate was not achieved ($K_m > [S]$), catalytic efficiency (k_{cat}/K_m) was extracted with reasonable accuracy from the slope of the linear dependence of initial velocity on substrate concentration.

For competition experiments with the inactive FVII/FVIIa mutants, titration curves with different concentrations of TF were simultaneously analysed by fitting the initial velocity of synthetic peptide hydrolysis to the cubic Equation 17 of Olson et al. [32] as described in [33]. Nonlinear least-squares regression

yielded values of ΔV , V_t , K_d , and n , which described the binding of Novoseven™ FVIIa to TF, and $K_{d,comp}$ and n_{comp} , which described the equivalent interaction of inactive mutant FVIIa with TF. In these analyses ΔV is the decrease in velocity of substrate cleavage when competitor FVIIa concentration saturates the fixed concentration of TF; V_t is the velocity at infinite concentration of competitor, equal to the activity of the fixed concentration of Novoseven™ FVIIa in the absence of TF; K_d is the equilibrium dissociation constant for the FVIIa–TF interaction, and n represents the number of moles of FVIIa bound per mole of TF at saturation. According to previous results, in this analysis n was set at 1 [15].

Reduction in activity after carbamylation was fitted to first-order exponential decay through nonlinear least-squares regression analysis.

RESULTS

Identification and characterization of mutant FVII

Two asymptomatic patients from Northern Italy were referred for a prolonged prothrombin time (> 2 S.D. above the prothrombin time of the reference plasma) detected in pre-surgery screening. FVII antigen (FVII:Ag) and coagulant activity (FVII:C) levels were 125%, and 60% for patient 1, and 128% and 51% for patient 2, compared with pooled normal plasma respectively. A third patient, also of Italian descent, was referred for a mild bleeding tendency from childhood including recurrent epistaxis. His FVII:Ag and FVII:C values were 42% and $< 1\%$ respectively. Genomic DNA was isolated, after obtaining informed consent, from leucocytes of the patients. Sequence analysis of exon 6 showed heterozygosity for a T⁸⁹⁶⁷ → G substitution in the three patients. This point mutation causes the V154G mutation. No additional mutations were found in the other FVII exons or promoter region. Patient 3 was also heterozygous for the substitution G to A at the fifth nucleotide of intron 7 (IVS7+5, FVII Lazio mutation) [25]. This mutation, which affects the donor splice site in intron 7 of FVII, determines the insertion of 37 bp in the mature mRNA. The frameshift, caused by the abnormal splicing, results in a premature termination of translation at residue 231, thus abolishing most of the C-terminal catalytic domain [34]. Population screening using a mutagenized primer that produced a BsrI site excluded the presence of the T⁸⁹⁶⁷ → G change, which is responsible for the V154G substitution, in 100 normal Italian subjects, thus indicating that this transversion is not a common polymorphism.

FVII mutants

The substitution V154A was expressed and characterized to test whether Gly¹⁵⁴ was responsible for the impaired salt-bridge formation in V154G FVIIa.

In order to compare the interaction of TF with V154G and different FVII forms, we expressed three additional representative FVII mutants, R152Q, D343H and S344A. R152Q FVII results in a molecule that cannot be cleaved, thus providing a stable zymogen form [27]; D343H FVII is a naturally occurring substitution of the aspartic residue that is involved in salt-bridge formation, as reported in [35], but is not yet characterized; S344A FVII, owing to the substitution of the serine residue in the catalytic triad, leads to an inactive molecule that is properly cleaved and has normal TF-binding properties [16,36].

FVII proteolytic activation and FVIIa activity

Proteolytic activation of purified recombinant proteins was tested using human FXa. The cleavage pattern and rate of activation of

Table 1 Catalytic efficiency of FVIIa for the hydrolysis of the peptidyl substrate (Spectrozyme fVIIa)

The experiment was performed in reaction buffer in the presence of 200 μ M PCPS with FVIIa and TF as indicated. Spectrozyme fVIIa ranged from 0 to 1 mM.

	FVIIa (nM)	Soluble TF (nM)	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
Wild-type	10	50	2.5×10^4
V154G	200	1000	5×10^2
V154A	40	200	2.5×10^3

Figure 1 Effect of carbamylation on FVIIa activity

Residual activity of FVIIa after carbamylation of the α -amino group of Ile¹⁵³. NaCO₃ (0.2 M) was used to carbamate 4 μ M wild-type FVIIa (triangles), V154G FVIIa (circles) or V154A FVIIa (squares) in the presence (filled symbols) or absence (open symbols) of 20 μ M soluble TF, at 22 °C in reaction buffer.

Figure 2 Binding of FVIIa to an active-site inhibitor

Interaction of FVIIa with a Phe-Pro-Arg chloromethylketone active-site inhibitor, detected by a horseradish peroxidase conjugated to streptavidin that recognized the biotinylated inhibitor–FVIIa complex (IMUBIND® Factor VIIa ELISA). FVIIa concentrations ranged from 0.031 to 2 nM for Novoseven™ (■) and wild-type (▲) FVIIa, whereas V154G FVIIa (●) concentrations ranged from 0.5 to 2 nM.

Figure 3 Binding of FVII variants to soluble TF

Binding of FVII mutants to soluble TF was performed in reaction buffer by incubating 10 nM NovosevenTM FVIIa in the presence of 100 μM PCPS with different fixed concentrations of soluble TF, at 5 (■), 10 (▲) and 20 nM (●). S344A FVIIa (A) and V154G FVIIa (B) were used as competitors at concentrations ranging from 0 to 1000 nM. After 30 min of incubation at 37 °C, the residual activity of the FVIIa–TF complex was assayed using Spectrozyme fVIIa (375 μM). (C) Competition assay using 10 nM soluble TF and 10 nM NovosevenTM FVIIa using increasing concentrations of functional FVIIa competitors, R152Q FVII (●), V154G FVIIa (◆), D343H FVIIa (▲) and S344A FVIIa (■).

wild-type, V154G, V154A, D343H and S344A FVII were indistinguishable, whereas R152Q was not cleaved after Arg¹⁵², even after a prolonged incubation for 4 h.

The resulting activated products were characterized with respect to their activity towards the FVIIa peptidyl substrate, Spectrozyme fVIIa, in the presence of soluble TF. The kinetic constants that were calculated for wild-type FVIIa were $K_m = 0.67 \pm 0.05$ mM and $k_{cat} = 17$. Whereas the catalytic efficiency parameters for wild-type, V154G and V154A FVIIa are shown in

Table 1, R152Q FVII, D343H FVIIa and S344A FVIIa did not show any activity at concentrations of 10 nM, 200 nM and 500 nM.

Recombinant wild-type FVIIa showed similar activity towards FX as compared with NovosevenTM FVIIa (33.8 and 19.6 nM FXa generated/min for wild-type and NovosevenTM, respectively) and FIX (24.9 and 21.4 nM FIXa generated/min for wild-type and NovosevenTM, respectively). R152Q FVII, V154G FVIIa, D343H FVIIa and S344A FVIIa, tested under different sets of conditions,

Table 2 Dissociation constant (K_d) for soluble TF, or TF/PC, and FVII/FVIIa

Values (means $\pm$ S.D.) were calculated from curves of TF binding to different forms of FVII, simultaneously fitting the data to equation 17 in [32]. N.M., not measured.

FVII/FVIIa type	K_d (nM)	
	TF–FVII/FVIIa	TF/PC–FVII/FVIIa
R152Q FVII	65.2 $\pm$ 5.4	1.04 $\pm$ 0.27
V154G FVIIa	75.8 $\pm$ 6.4	1.15 $\pm$ 0.16
D343H FVIIa	68.1 $\pm$ 8.9	N.M.
S344A FVIIa	4.8 $\pm$ 1.6	0.27 $\pm$ 0.06

were characterized by undetectable activity towards FIX and FX. V154A FVIIa showed reduced proteolytic activity towards FX (1.4 nM FXa generated/min) as compared with wild-type FVIIa, and undetectable activity towards FIX, most probably owing to the low sensitivity of the chromogenic substrate for FIXa.

Carbamylation of Ile¹⁵³ in FVIIa

Previous studies have demonstrated that free FVIIa is susceptible to chemical modification of the exposed α -amino group of Ile¹⁵³ by cyanate ions [7,10]. In the presence of TF, structural rearrangements protect the FVIIa Ile¹⁵³ N-terminus from carbamylation. Since carbamylation of Ile¹⁵³ results in a loss of activity, the rate of modification of the N-terminus of FVIIa can be used to estimate the formation and the stability of the Ile¹⁵³–Asp³⁴³ salt bridge [7].

Cyanate ions did not perturb TF-cofactor activity, as indicated by the unaltered capability of soluble TF to enhance FVIIa activity after carbamylation (results not shown).

In the presence of NaNCO, soluble TF protected wild-type and V154A FVIIa from carbamylation, whereas it failed to protect V154G FVIIa from inactivation beyond that seen with free enzyme (Figure 1). This finding suggests strongly that V154G maintains a zymogen-like conformation, even in the presence of TF.

FVII interaction with an active-site inhibitor

Wild-type or mutated FVIIa (2 nM and 8 nM respectively) was incubated with the active-site inhibitor Phe-Pro-Arg chloromethylketone; whereas wild-type FVIIa showed normal binding of the inhibitor as compared with NovosevenTM FVIIa, V154G FVIIa was unable to accommodate the inhibitor in the active site (Figure 2).

TF binding to FVII and FVIIa

In order to evaluate equilibrium parameters for the FVIIa–TF complex assembly in the absence of a macromolecular substrate, we exploited the increased efficiency of FVIIa amidolytic activity in the presence of TF. It has been described previously that dissociation constants that were calculated in the presence of either a peptidyl or macromolecular substrate were comparable [15].

We compared the ability of R152Q FVII, V154G FVIIa or D343H FVIIa to displace functional FVIIa from the soluble TF–FVIIa complex with that of S344A FVIIa (Figure 3). R152Q FVII, V154G FVIIa and D343H FVIIa bound soluble TF with approx. 15-fold lower affinity as compared with S344A FVIIa (Table 2). Similar K_d values were calculated using soluble TF

Figure 4 Binding of FVII variants to full-length TF/PC

Binding of FVII variants to full-length TF/PC was performed in reaction buffer by incubating 5 nM NovosevenTM FVIIa with 3 nM TF/12 μ M PC (■), 5 nM TF/20 μ M PC (▲) or 15 nM TF/60 μ M PC (●). S344A FVIIa (A) and V154G FVIIa (B) were used as competitors at concentrations ranging from 0 to 200 nM. After 30 min of incubation at 37 °C, the activity of the FVIIa–TF complex was assayed using Spectrozyme fVIIa (375 μ M).

produced in either *E. coli* or *P. pastoris*. The assay performed in the absence of membranes gave similar results (not shown), supporting the hypothesis that phospholipids do not significantly influence the affinity of soluble TF for FVII/FVIIa [37].

Both V154G FVIIa and R152Q FVII bound full-length TF, produced in *E. coli* and relipidated in PC (Figure 4), with approx. 4-fold decreased affinity as compared with S344A FVIIa (Table 2). Similar results were obtained using full-length TF made in insect cells. Because of sensitivity limitations, the fixed FVIIa concentration (5 nM) used in competition assays for binding to

reconstituted TF was significantly higher than the inferred dissociation constant between FVIIa and TF. Therefore, calculated K_d values may only provide an upper limit value of these constants, which, however, agree well with previous results [15].

The dissociation constant that we measured for soluble TF and FVII ($K_d = 65.2 \pm 5.4$ nM) was higher than previously reported [38]. This difference could explain the absence of autoactivation of FVII using concentrations of soluble TF and of FVII below the K_d value that we determined [38]. Autoactivation occurs when the soluble TF–FVII concentration exceeds this K_d value, indicating that, as already shown for relipidated TF [39], FVII needs to be bound to soluble TF to be an appropriate substrate of the soluble TF–FVIIa enzymic complex.

In addition, the results shown in Table 2 suggest that the looser binding of all FVII forms to soluble TF, compared with full-length TF, amplifies the range of K_d values, thus making soluble TF useful to estimate differences in TF binding among FVII forms.

DISCUSSION

Conversion of the zymogen FVII to fully active FVIIa has undergone extensive scrutiny, in part because of potential benefits that may be gained by a better understanding of the steps required to form enzymically active FVIIa [40–42]. After conversion into the two-chain form, FVIIa remains in a zymogen-like state that renders coagulation initiation dependent on TF exposure. Production of TF-independent FVIIa or the synthesis of inhibitors specific to FVIIa may result in improved strategies for treatment of haemorrhagic or thrombotic disorders [17,18,43,44].

Previous work has shown that the events leading to FVIIa catalytic enhancement include (i) interaction with TF, (ii) salt bridge formation between Ile¹⁵³ and Asp³⁴³, and (iii) ordering of the activation domain. However, the mutual and tight linkage among these steps has hampered the precise determination of their chronological sequence. More importantly, the lack of efficient models to represent zymogen-like FVIIa has hindered clarification of the TF mechanism of catalytic enhancement in the FVIIa–TF complex.

In the present paper, we describe the naturally occurring FVII mutant protein V154G, which shows properties consistent with the proposed zymogen-like form of FVIIa, a form that exists between the zymogen FVII and fully active FVIIa and which does not present a salt bridge between Ile¹⁵³ and Asp³⁴³ [7]. Since salt-bridge formation is expected to partially protect FVIIa from chemical modifications, the FVIIa carbamylation experiments suggest that the majority of V154G FVIIa molecules maintain a zymogen-like form even when associated with TF.

We propose that impairment of salt-bridge formation and/or stability in V154G FVIIa is due to the introduction of a glycine residue at position P2' producing a series of three glycine residues in a row (I¹⁵³GGG) at the activation site. This sequence at the N-terminus should be extraordinarily flexible and could easily give rise to a variety of interchangeable structures, thus not favouring the insertion of the newly formed N-terminus into the protein. Consistent with this hypothesis, the V154A mutation did not impair the ability of the heavy chain N-terminus to form a salt bridge. Nevertheless, V154A FVIIa showed reduced catalytic efficiency compared with wild-type FVIIa, suggesting an important role of Val¹⁵⁴ in the conversion of FVIIa into a fully active enzyme.

The 'immature' V154G FVIIa molecule was exploited to investigate the active-site competence of this salt-bridge-less

Scheme 1 Effect of TF/PC binding to (A) S344A FVIIa and (B) V154G FVIIa with respect to salt-bridge formation

See the text for details.

form of FVIIa. Its inability to accommodate an active-site inhibitor, even at high FVIIa concentrations, would suggest that the specificity pocket is not properly formed. This result may help to interpret the low catalytic efficiency of FVIIa in the absence of TF as the predominance of an inactive zymogen-like form in the equilibrium after FVII cleavage at Arg¹⁵².

The V154G FVIIa variant, modelling a salt-bridge-less form of FVIIa, allowed us to analyse the binding of TF to this intermediate form of FVIIa, therefore representing a more physiological interaction between TF and FVIIa compared with previous studies [7,16]. V154G FVIIa also differs from previously described FVIIa mutants that could be defined as zymogen-like FVIIa [9,41]. These mutations either impaired the contact region between FVIIa and TF [41], or had all the residues in Loop 170s replaced with those of trypsin [9], thus not representing models to study the interaction of a salt-bridge-less FVII form with other macromolecules of interest in blood-clotting initiation.

To investigate TF binding to V154G FVIIa, we also used the FVII mutants R152Q, D343H and S344A to represent zymogen FVII, zymogen-like FVIIa, and inactive FVIIa respectively. V154G FVIIa bound soluble TF with an affinity comparable with that of mutants that lacked the salt bridge (R152Q and D343H). Schemes 1(A) and 1(B) depict binding of TF to mutant S344A and V154G FVIIa respectively, and provide models for interpreting the measured dissociation constants reported in Table 2. In the presence of TF, the equilibrium may be shifted from a zymogen-like FVIIa (white) towards active FVIIa (grey) by TF preferential binding to the species that has already formed the Ile¹⁵³–Asp³⁴³ salt bridge ($K_1 + K_2$). Alternatively, TF could bind zymogen-like FVIIa and active FVIIa with comparable affinity, without influencing their equilibrium, leading to an inactive and an active complex (lower left and lower right TF–FVIIa complexes), respectively. Conformational changes due to TF interaction with the zymogen-like form stabilize salt-bridge formation and order the activation domain, giving rise to the active enzymic complex FVIIa–TF [$(K_2) + (K_4 + K_3)$].

In the case of S344A FVIIa (Scheme 1A), the calculated dissociation constant (0.27 nM) between TF and FVIIa would reflect the combination of the equilibrium constants of all the steps involved in the two different pathways [$K_1 + K_2$; $(K_2) + (K_4 + K_3)$]. In contrast, binding of TF to V154G FVIIa (Scheme 1B), which retains characteristics of a zymogen-like form, would be insufficient to drive this FVIIa variant into the active conformation. In this case, the quasi-homogeneity, with respect to salt-bridge formation, of zymogen-like V154G FVIIa molecules and the negligible value of K_3 would simplify the scheme to a single pathway. It is possible to assert, with a reasonable approximation, that the calculated dissociation constant (1.15 nM) represents the mere interaction of TF with the zymogen-like V154G FVIIa (K_4).

Based on the comparison of dissociation constants between full-length TF and the different FVII species, and the above mentioned interpretation, the preferential TF binding to the active FVIIa form, as suggested by Bach et al. [24], cannot account for the entire FVIIa catalytic enhancement by TF. Conformational changes driven by the interaction with TF must contribute to FVIIa conversion into the active form.

It is worth noting that the dissociation constants between active FVIIa and TF reconstituted in phospholipid vesicles calculated in this study agree well with previous investigations using bovine and human proteins [7,15], but differ from results obtained in non-equilibrium conditions [24]. The difference in affinity between FVII/FVIIa forms and TF (truncated or reconstituted forms) is consistent with previous reports [7,24] and correlates well with the described tighter binding of TF to

active site modified FVIIa [7]. However, these findings do not mirror results obtained by surface plasmon resonance, probably owing to the differences between experimental approaches [16].

In the present study, we characterized a FVII mutant that, after cleavage, still shares characteristics with the FVII zymogen form, thus representing a model of the zymogen-like FVIIa not previously described. Since this form of FVIIa is predominant in the absence of TF, the interaction of V154G FVIIa with an active-site inhibitor and with TF mimics, to date, the most realistic interaction that triggers coagulation initiation.

This work was supported by National Institutes of Health (NIH) grant HL-K0803661 and by Doris Duke Charitable Foundation grant T98062B (to E.S.P.) and by Telethon, Italy grant GP02182 (to F.B.). We thank Dr Sriram Krishnaswamy and Dr James H. Morrissey for their gifts of tissue factor, Dr Else Marie Nicolaisen and Dr Ulla Hedner for kindly providing us with anti-FVII monoclonal antibody and Dr Sai Kumar Buddai for the preparation of FXa. We thank Dr Giancarlo Castaman (Department of Haematology and Haemophilia and Thrombosis Center, San Bortolo Hospital, Vicenza, Italy) and Dr Rosaria Redaelli (Haematology Division of Niguarda Hospital, Milan, Italy) for referring FVII-deficient patients to us and for the coagulation laboratory data. We acknowledge Dr Sriram Krishnaswamy and his co-workers for their helpful suggestions and excellent support.

REFERENCES

- Rao, L. V. and Rapaport, S. I. (1988) Activation of Factor VII bound to tissue factor: a key early step in the tissue factor pathway of blood coagulation. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6687–6691.
- Østerud, B. and Rapaport, S. I. (1977) Activation of Factor IX by the reaction product of tissue factor and Factor VII: additional pathway for initiating blood coagulation. *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5260–5264.
- Nemerson, Y. (1966) The reaction between bovine brain tissue factor and Factors VII and X. *Biochemistry* **5**, 601–608.
- Morrissey, J. H., Macik, B. G., Neuenschwander, P. F. and Comp, P. C. (1993) Quantitation of activated Factor VII levels in plasma using a tissue factor mutant selectively deficient in promoting Factor VII activation. *Blood* **81**, 734–744.
- Rao, L. V., Williams, T. and Rapaport, S. I. (1996) Studies of the activation of Factor VII bound to tissue factor. *Blood* **87**, 3738–3748.
- Hagen, F. S., Gray, C. L., O'Hara, P., Grant, F. J., Saari, G. C., Woodbury, R. G., Hart, C. E., Insley, M., Kisiel, W., Kurachi, K. and Davie, E. W. (1986) Characterization of a cDNA coding for human Factor VII. *Proc. Natl. Acad. Sci. U.S.A.* **83**, 2412–2416.
- Higashi, S., Matsumoto, N. and Iwanaga, S. (1996) Molecular mechanism of tissue factor-mediated acceleration of Factor VIIa activity. *J. Biol. Chem.* **271**, 26569–26574.
- Higashi, S., Matsumoto, N. and Iwanaga, S. (1997) Conformation of Factor VIIa stabilized by a labile disulfide bond (Cys-310–Cys-329) in the protease domain is essential for interaction with tissue factor. *J. Biol. Chem.* **272**, 25724–25730.
- Soejima, K., Mizuguchi, J., Yuguchi, M., Nakagaki, T., Higashi, S. and Iwanaga, S. (2001) Factor VIIa modified in the 170 loop shows enhanced catalytic activity but does not change the zymogen-like property. *J. Biol. Chem.* **276**, 17229–17235.
- Higashi, S., Nishimura, H., Aita, K. and Iwanaga, S. (1994) Identification of regions of bovine Factor VII essential for binding to tissue factor. *J. Biol. Chem.* **269**, 18891–18898.
- Bode, W., Schwager, P. and Huber, R. (1978) The transition of bovine trypsinogen to a trypsin-like state upon strong ligand binding. The refined crystal structures of the bovine trypsinogen-pancreatic trypsin inhibitor complex and of its ternary complex with Ile–Val at 1.9 Å resolution. *J. Mol. Biol.* **118**, 99–112.
- Bode, W. (1979) The transition of bovine trypsinogen to a trypsin-like state upon strong ligand binding II. The binding of the pancreatic trypsin inhibitor and of isoleucine-valine and of sequentially related peptides of trypsinogen and to p-guanidinobenzoate-trypsinogen. *J. Mol. Biol.* **127**, 357–374.
- Schechter, I. and Berger, A. (1967) On the size of the active site in proteases. I. Papain. *Biochem. Biophys. Res. Commun.* **27**, 157–162.
- Komiyama, Y., Pedersen, A. H. and Kisiel, W. (1990) Proteolytic activation of human Factors IX and X by recombinant human Factor VIIa: effects of calcium, phospholipids, and tissue factor. *Biochemistry* **29**, 9418–9425.
- Krishnaswamy, S. (1992) The interaction of human Factor VIIa with tissue factor. *J. Biol. Chem.* **267**, 23696–23706.
- Dickinson, C. D. and Ruf, W. (1997) Active site modification of Factor VIIa affects interactions of the protease domain with tissue factor. *J. Biol. Chem.* **272**, 19875–19879.
- Persson, E., Bak, H. and Olsen, O. H. (2001) Substitution of valine for leucine 305 in Factor VIIa increases the intrinsic enzymatic activity. *J. Biol. Chem.* **276**, 29195–29199.

- 18 Persson, E., Kjalke, M. and Olsen, O. H. (2001) Rational design of coagulation Factor VIIa variants with substantially increased intrinsic activity. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 13583–13588
- 19 Dickinson, C. D., Shobe, J. and Ruf, W. (1998) Influence of cofactor binding and active site occupancy on the conformation of the macromolecular substrate exosite of Factor VIIa. *J. Mol. Biol.* **277**, 959–971
- 20 Bode, W., Mayr, I., Baumann, U., Huber, R., Stone, S. R. and Hofsteenge, J. (1989) The refined 1.9 Å crystal structure of human alpha-thrombin: interaction with D-Phe-Pro-Arg chloromethylketone and significance of the Tyr-Pro-Pro-Trp insertion segment. *EMBO J.* **8**, 3467–3475
- 21 Lottenberg, R. and Jackson, C. M. (1983) Solution composition dependent variation in extinction coefficients for *p*-nitroaniline. *Biochim. Biophys. Acta* **742**, 558–564
- 22 Higgins, D. L. and Mann, K. G. (1983) The interaction of bovine Factor V and Factor V-derived peptides with phospholipid vesicles. *J. Biol. Chem.* **258**, 6503–6508
- 23 Lawson, J. H., Krishnaswamy, S., Butenas, S. and Mann, K. G. (1993) Extrinsic pathway proteolytic activity. *Methods Enzymol.* **222**, 177–195
- 24 Bach, R., Gentry, R. and Nemerson, Y. (1986) Factor VII binding of tissue factor in reconstituted phospholipids vesicles: induction of cooperativity by phosphatidylserine. *Biochemistry* **25**, 4007–4020
- 25 Bernardi, F., Patraccini, P., Gemmati, D., Ferrati, M., Arcieri, P., Papacchini, M., Redaelli, R., Baudo, F., Mariani, G. and Marchetti, G. (1993) Molecular analysis of Factor VII deficiency in Italy: a frequent mutation (FVII Lazio) in a repeated intronic region. *Hum. Genet.* **92**, 446–450
- 26 O'Hara, P. J., Grant, F. J., Haldeman, B. A., Gray, C. L., Insley, M. Y., Hagen, F. S. and Murray, M. J. (1987) Nucleotide sequence of the gene coding for human Factor VII, a vitamin K-dependent protein participating in blood coagulation. *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5158–5162
- 27 Chaing, S., Clarke, B., Sridhara, S., Chu, K., Friedman, P., VanDausen, W., Roberts, H. R., Blajchman, M., Monroe, D. M. and High, K. A. (1994) Severe Factor VII deficiency caused by mutations abolishing the cleavage site for activation and altering binding to tissue factor. *Blood* **83**, 3524–3535
- 28 Toso, R., Pinotti, M., High, K. A., Pollak, E. S. and Bernardi, F. (2002) A frequent human coagulation Factor VII mutation (A294V, c152) in Loop 140s affects the interaction with activators, tissue factor and substrates. *Biochem. J.* **363**, 411–416
- 29 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. *Nature (London)* **227**, 680–685
- 30 Segel, I. H. (1975) Simple inhibition systems. In *Enzyme Kinetics: Behaviour and Analysis of Rapid Equilibrium and Steady State Enzyme System*, pp. 100–135, John Wiley & Sons, Inc., New York
- 31 Bevington, P. R. and Robinson, K. D. (1992) Least-squares fit to an arbitrary function. In *Data Reduction and Error Analysis for the Physical Sciences*, pp. 141–167, McGraw-Hill Inc., New York, NY
- 32 Olson, S. T., Bock, P. E. and Sheffer, R. (1991) Quantitative evaluation of solution equilibrium binding interactions by affinity partitioning: application to specific and nonspecific protein–heparin interactions. *Arch. Biochem. Biophys.* **286**, 533–545
- 33 Betz, A. and Krishnaswamy, S. (1998) Regions remote from the site of cleavage determine macromolecular substrate recognition by the prothrombinase complex. *J. Biol. Chem.* **273**, 10709–10718
- 34 Pinotti, M., Toso, R., Redaelli, R., Berrettini, M., Marchetti, G. and Bernardi, F. (1998) Molecular mechanisms of FVII deficiency: expression of mutations clustered in the IVS7 donor splice site of Factor VII gene. *Blood* **92**, 1646–1651
- 35 Wulff, K. and Herrmann, F. H. (2000) Twenty two novel mutations of the Factor VII gene in Factor VII deficiency. *Hum. Mutat.* **15**, 489–496
- 36 Nakagaki, T., Foster, D. C., Berkner, K. L. and Kiesel, W. (1991) Initiation of the extrinsic pathway of blood coagulation: evidence for the tissue factor dependent autoactivation of human coagulation Factor VII. *Biochemistry* **30**, 10819–10824
- 37 Ruf, W., Rehmtulla, A. and Edgington, T. S. (1991) Phospholipid-independent and -dependent interactions required for tissue factor receptor and cofactor function. *J. Biol. Chem.* **266**, 2158–2166
- 38 Neuenschwander, P. F. and Morrissey, J. H. (1992) Deletion of the membrane anchoring region of tissue factor abolishes autoactivation of Factor VII but not cofactor function. Analysis of a mutant with a selective deficiency in activity. *J. Biol. Chem.* **267**, 14477–14482
- 39 Neuenschwander, P. F., Fiore, M. M. and Morrissey, J. H. (1993) Factor VII autoactivation proceeds via interaction of distinct protease-cofactor and zymogen-cofactor complexes. *J. Biol. Chem.* **268**, 21489–21492
- 40 Butenas, S. and Mann, K. G. (1996) Kinetics of human Factor VII activation. *Biochemistry* **35**, 1904–1910
- 41 Petrovan, R. J. and Ruf, W. (2000) Role of residue Phe²²⁵ in the cofactor-mediated, allosteric regulation of the serine protease coagulation Factor VIIa. *Biochemistry* **39**, 14457–14463
- 42 Petrovan, R. J. and Ruf, W. (2001) Residue Met¹⁵⁶ contributes to the labile enzyme conformation of coagulation Factor VIIa. *J. Biol. Chem.* **276**, 6616–6620
- 43 Dennis, M. S., Roberge, M., Quan, C. and Lazarus, R. A. (2001) Selection and characterization of a new class of peptide exosite inhibitors of coagulation Factor VIIa. *Biochemistry* **40**, 9513–9521
- 44 Örnning, L., Fischer, P. M., Hu, C., Agner, E., Engebretsen, M., Husby, M., Petersen, L. B., Ørving, U., Llinas, M. and Sakariassen, K. S. (2002) A cyclic pentapeptide derived from the second EGF-like domain of Factor VII is an inhibitor of tissue factor dependent coagulation and thrombus formation. *Thromb. Haemostasis* **87**, 13–21