Fusion of two distinct peptide exosite inhibitors of Factor VIIa

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Highly potent bifunctional inhibitors of Factor VIIa (FVIIa) were generated by linking two distinct peptides, recently shown to bind to two discrete exosites on the FVIIa protease domain [Dennis, Eigenbrot, Skelton, Ultsch, Santell, Dwyer, O'Connell and Lazarus (2000) Nature (London) **404**, 465–470; Dennis, Roberge, Quan and Lazarus (2001) Biochemistry **40**, 9513–9521; Roberge, Santell, Dennis, Eigenbrot, Dwyer and Lazarus (2001) Biochemistry **40**, 9522–9531]. Fusion peptides consisting of an N-terminal A-series peptide followed by flexible linkers, an E-series peptide, and the Z-domain of protein A were expressed in *Escherichia coli* and purified using IgG–Sepharose affinity chromatography. The fusion peptides were potent anticoagulants and had steep concentration dependence curves in tissue factor-dependent prothrombin time (PT) assays in comparison to the individual peptides or their noncovalent combination. This phenomenon was dependent on the length of the linker joining the A- and E-peptides. The fusion of the peptides increased the extent of inhibition of Factor X (FX) activation to 100% at saturating peptide concentrations, but did not improve the binding affinity for Factor VIIa (FVIIa) at the A- and E- binding sites or the IC_{50} for the inhibition of FX activation. Differences between the peptides in the PT fold prolongation in normal and FVII-deficient plasma, in conjunction with the inhibition of ^{125}I-FVII activation, suggest that the enhanced effects of the fusion peptides involve the inhibition of FVII autoactivation.

Key words: anticoagulant, bifunctional inhibitor, prothrombin time, serine protease, tissue factor.

INTRODUCTION

Factor VIIa (FVIIa) is a coagulation serine protease that becomes fully active when bound to its obligate cofactor tissue factor (TF), an integral membrane protein. The TF·FVIIa complex initiates the coagulation pathway by cleaving and activating Factor X (FX) to Factor Xa (FXa) [1], Factor IX (FIX) to Factor IXa (FIXa) [2] and Factor VII (FVII) to Factor VIIa (FVIIa) [3,4], eventually leading to the formation of a blood clot [5–7]. In addition to its role in maintaining normal haemostasis, TF-dependent coagulation is thought to be involved in a variety of thrombotic disorders. Current anticoagulant therapies use heparins and coumarins that block the coagulation pathway by acting directly or indirectly on multiple coagulation enzymes [8–10]. These non-specific inhibitors are known to cause undesirable side effects and consequently patients require close monitoring. Potent and specific inhibitors of the TF·FVIIa complex, which include various protease inhibitors, TF mutants, inactivated FVIIa, and antibodies [11–13], may reduce the side effects associated with the use of currently available anticoagulants.

Recently we described the discovery and characterization of two distinct classes of peptide exosite inhibitors of the TF·FVIIa complex [14–16]. These peptide inhibitors, designated A- and E-series peptides, were selected from phage-display libraries for their ability to bind to the TF·FVIIa complex. Both series of peptides are potent and specific inhibitors of TF·FVIIa enzymic activity, inhibiting FX activation and Chromozym t-PA turnover with low nanomolar IC_{50} values [14,15]. They are also selective anticoagulants inhibiting only TF-dependent coagulation. The A- and E-peptides bind to two distinct exosites on the serine protease domain of FVIIa, both of which are distinct from the active site [14,16]. This was initially based on mutagenesis studies of surface residues on the protease domain of FVIIa, which identified key residues involved in binding these peptides. X-ray crystal structures of the E-peptide E-76 (Ac-ALCDDPRVDR-WYCQFVEG-NH_2) and the A-peptide A-183 (EEWEVLCWT-WETCER), bound to the protease domain of FVIIa and FVII respectively, further refined the location of these exosites [14,16]. These structural and functional studies have suggested both steric and allosteric inhibition mechanisms [14–16].

The structures of these two peptides bound to FVII/FVIIa were used to generate a model of the protease domain of FVIIa with peptides E-76 and A-183 bound to their respective exosites [16]. This model shows that these two peptides were separated by a distance of approx. 25 Å (1 Å = 0.1 nm) (Figure 1). Therefore, it was appealing to consider the idea of making a bifunctional peptide inhibitor of FVIIa that could bind with high affinity, and perhaps increase the prolongation of TF-dependent coagulation, as compared to the A- and E-peptides either individually or in combination. The strategy used to create highly potent bifunctional inhibitors has been explored previously. Notably, a fusion between a Kunitz-type domain and a TF mutant improved the K_i values for the inhibition of FX activation by 200–400-fold in comparison to the individual proteins [17]. Another bifunctional inhibitor ($X_{LC}LACI_{K1}$) of FVIIa was generated by a fusion between the light chain of FX and the first Kunitz domain of tissue factor pathway inhibitor [18]. This fusion resulted in an approx. 100 fold increase in potency in a TF-induced coagulation assay in comparison to TF pathway inhibitor alone. Examples of naturally occurring bifunctional protein inhibitor anticoagulants include hirudin [19],

Abbreviations used: FVIIa, Factor VIIa; TF, tissue factor; FX, Factor X; FXa, Factor Xa; FIX, Factor IX; Fmoc, fluoren-9-ylmethoxycarbonyl; TF_{1-219} and TF_{1-243}, *Escherichia coli*-derived recombinant human TF encompassing residues 1–219 and 1–243, respectively; HRP, horse radish peroxidase; Z, consensus domain of protein A; PT, prothrombin time; A^{mut}EZ-9, W10A mutant of A-183 in the context of AEZ-9; AE^{mut}Z-9, Y12A mutant of E-151 in the context of AEZ-9.

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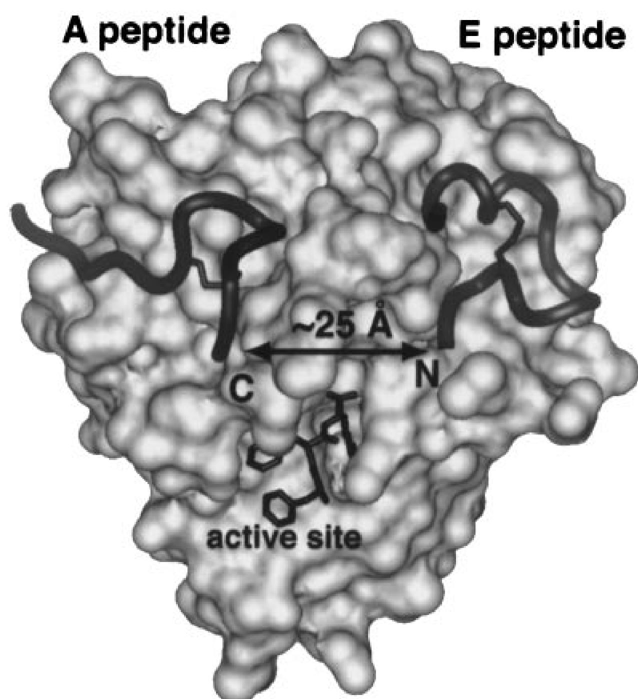

Figure 1 Model of the A- and E-peptides bound to the protease domain of FVIIa

Shown are peptide A-183 (A peptide) at the A-exosite, E-76 (E peptide) at the E-exosite, and D-Phe-L-Phe-L-Arg-chloromethylketone covalently bound to the active site [16]. The distance between the C-terminus (C) of A-183 and the N-terminus (N) of E-76 of approx. 25 Å can be spanned by a 5-residue linear linker.

ecotin [20], and rhodniin [21]. In this work, the A- and E-series peptides have been covalently linked to generate bifunctional peptides. These fusion peptides are potent TF-dependent anti-coagulants, having very steep concentration dependence curves, and also increase the extent of inhibition of the TF·FVIIa complex.

MATERIALS AND METHODS

Peptides

For the A-series peptides, A-183 (EEWEVLCWTWETCER), also referred to as the A-peptide in this report, was isolated from a trypsin digest of the recombinant peptide A-100-Z (EEWEVL-CWTWETCERGEGGGGSGG-Z, where Z represents the 68-residue consensus domain of protein A) as previously described [15]. The A-peptide was biotinylated at its N-terminus to generate A-183b as described previously [16].

E-151 (Ac-ALCDNPRIDRWYCQFVEG-NH_2), also referred to as the E-peptide in this paper, was obtained from further optimization of the E-series peptides (M. S. Dennis, unpublished work) and differs from peptide E-76 (Ac-ALCDDPRVDR-WYCQFVEG-NH_2) by two residues [14]. Peptides were synthesized using methods described previously [15]. E-151 was synthesized with a biotin group at the C-terminus to form peptide E-184. Biotinylation was carried out using reductive amination of BAL-PEG-polystyrene resin (Rapp Polymere GmbH, Tubingen, Germany) with the HCl salt of *N*-allocethylenediamine, which yields a resin in which biotin can be introduced upon release of the alloc group. Standard Fmoc (fluoren-9-ylmethoxycarbonyl) synthesis was carried out beginning with the first coupling of an amino acid to the secondary amine. The recombinant peptide E-274-Z (VGALCDNPRIDRWYCQFVEGGGGGSGG-Z) was used to measure the effect of alanine substitution on the activity of the E-peptide. Importantly, E-151, E-184 and E-274-Z have similar affinities for FVIIa in the FVIIa/peptide binding assay and similar IC_{50} values in the FX activation assay.

Generation of fusion peptide constructs

The fusion peptides were generated using the plasmid pA-100-Z that codes for the peptide A-100-Z, where the Z-domain was used to facilitate expression and purification [15]. Kunkel mutagenesis [22] was used to insert the coding sequence for the E-peptide E-274 (VGALCDNPRIDRWYCQFVEG) and a 5-residue linker (GGGGT) between peptide A-100 [15] and the Z-domain of protein A to form plasmid pAEZ-9, which encodes peptide AEZ-9. Fusion peptides with longer linker lengths were generated by inserting a sequence coding for $(GGGGS)_{1-4}$ (AEZ-14 to AEZ-29) in pAEZ-9. The numerical portion in the name of the fusion peptide refers to the number of residues separating the C-terminal Arg of A-183 and the N-terminal Ala of E-151. Variants of AEZ-9 containing alanine substitutions at Trp^{10} of A-183 and Tyr^{12} of E-151, as well as fusion peptides with shorter linker lengths were also generated by Kunkel mutagenesis of plasmid pAEZ-9.

Expression and purification of the fusion peptides

The Z-domain containing fusion peptides were secreted into the culture fluid from *Escherichia coli* 27C7 and purified using IgG–Sepharose beads as described for A-100-Z [15]. All fusion peptides were quantified using a calculated molar absorption coefficient at 280 nm, ([23], but see [23a]). Further analyses included fast atom bombardment MS and SDS/PAGE.

FVIIa/peptide binding assays

The relative binding affinities of the peptides with FVIIa at the A- and E-sites were determined by competition against A-183b (2.5 nM) and E-184 (2 nM), respectively, in solid-phase binding assays as described previously [16].

TF·FVIIa activity assays

Inhibition of FX activation by TF·FVIIa was determined with 300 pM relipidated TF_{1-243}, 20 pM FVIIa, and 165 nM FX (Haematologic Technologies Inc., Essex Junction, VT) at 25 °C as a function of peptide concentration, as described previously [15]. Controls showed that the peptides tested did not inhibit FXa chromogenic activity. The linear rates of FXa generation are expressed as fractional activities (v_i/v_o) and the lines drawn represent data fit to a 4-parameter equation (KaleidaGraph, Synergy Software, Reading, PA, U.S.A.). Inhibition of FIX activation by TF·FVIIa was monitored as a function of peptide concentration essentially as described previously [24], but using 4.4 nM relipidated TF_{1-243} instead of membrane TF. Inhibition of the hydrolysis of Chromozym t-PA (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.) was monitored as a function of peptide concentration essentially as described previously, except that Tween 20 was used instead of Tween 80 [14].

Autoactivation assay with ^{125}I-FVII

This assay was performed with minor modifications of the assay described previously [25]. FVII was labelled with ^{125}I using the Iodogen method as described [25]; the specific activity of the

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labelled material was 0.6 μCi/μg FVII. ^{125}I-FVII and peptides were added to cell membranes of TF_{1-263}-expressing 293 cells in 20 mM Hepes, pH 7.5 containing 150 mM NaCl, 0.5 mg/ml BSA and 5 mM $CaCl_2$ (HBSA buffer). The concentrations of the reactants in the reaction mixture were as follows: 1 μM peptides, 100 μg/ml membrane TF_{1-263} (total protein concentration) and 10 nM ^{125}I-FVII. The ^{125}I-FVII preparation contained approx. 7% ^{125}I-FVIIa, which served as the catalyst [25]. At different time points aliquots were removed and added to sample buffer (Bio-Rad Laboratories, Hercules, CA, U.S.A.) containing the reducing agent dithiothreitol (Bio-Rad). After a brief heating, samples (approx. 10^5 c.p.m./lane) were loaded onto a 4–20% gradient polyacrylamide gel (Invitrogen Corp., Carlsbad, CA, U.S.A.). After electrophoresis, the dried gels were exposed on X-ray films (X-OMAT AR, Eastman Kodak Company, Rochester, NY, U.S.A.) for 12–24 h. Films were developed (Kodak M35A X-OMAT Processor), scanned (Umax S-12, Umax Data Systems, Inc., Fremont, CA, U.S.A.) and further processed with Adobe V5.5 Photoshop software (Adobe Systems Inc., San Jose, CA, U.S.A.).

FXa-mediated activation of ^{125}I-FVII

This assay was carried out basically as described previously [25]. Briefly, ^{125}I-FVII was incubated in HBSA buffer with TF_{1-219} in the presence of membrane fractions of human wild-type 293 cells that do not express TF for 20 min, after which FXa was added. The concentrations in this reaction mixture were: 100 μg/ml (total protein concentration) 293 cell membranes, 10 nM ^{125}I-FVII, 100 nM TF_{1-219} and 0.2 nM FXa. At different time points aliquots were removed and added to sample buffer and further analysed by SDS/PAGE as described above.

Clotting assays

Prothrombin time (PT) assays were performed in pooled citrated human plasma or in plasma from FVII-deficient patients (George King Bio-Medical, Overland Park, KS, U.S.A.). Clotting times were determined using an ACL 300 Automated Coagulation Analyzer (Coulter, Miami, FL, U.S.A.). FVII-deficient plasma was supplemented with 600 pM FVIIa to mimic the clotting time of normal plasma, typically approx. 9 s. Peptides were incubated in plasma for at least 30 min at 20 °C before the assay was initiated by mixing 50 μl plasma/peptide with 100 μl Innovin (Dade International, Miami, FL, U.S.A.). For the reverse PT assay, peptides were incubated with 100 μl Innovin and coagulation was initiated by the addition of 50 μl plasma.

RESULTS

Construction, expression and purification of the fusion peptides

The A- and the E-peptides were linked by flexible linkers of various lengths (Figure 2). In addition, residues Trp[10] in the A-peptide and Tyr[12] in the E-peptide, both previously shown to be important for binding to FVIIa [14,16], were replaced individually, or in combination, by Ala in the context of the fusion peptide AEZ-9 to form A^{mut}EZ-9, AE^{mut}Z-9 and $A^{mut}E^{mut}$Z-9 respectively. All of the constructs were confirmed by DNA sequencing. The Z-domain containing fusion peptides were expressed in *E. coli* and purified from the culture supernatant using IgG-Sepharose affinity chromatography. The yields obtained for the various constructs varied from 1 to 5 mg of purified peptide from a 1 litre culture. The purified material was then confirmed by SDS/PAGE and MS.

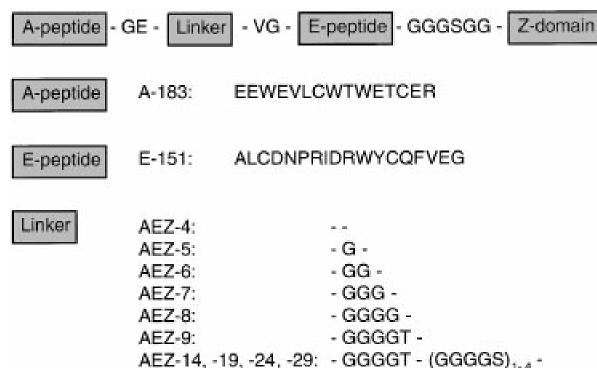

Figure 2 Amino acid sequences of the different peptides used in this work

Fusion peptides were generated by inserting flexible linkers of various lengths. The numerical portion in the name of a fusion peptide refers to the number of residues separating the A- and the E-peptides.

Table 1 Relative affinities of various peptides for FVIIa in binding and activity assays

The relative IC_{50} values are shown for the FVIIa/peptide binding assays at the A- and E-exosites of FVIIa. The values in the A- and E-peptide binding assays have been normalized to peptides A-183 and E-151, respectively. The absolute IC_{50} value for A-183 in the A-site assay is 6.4 nM and the absolute IC_{50} value for E-151 in the E-site assay is 2.5 nM. The IC_{50} values and percentage maximal inhibition (in parentheses) are shown for the inhibition of FX activation. The values in parenthesis represent the extent of inhibition of the rate of FX activation at saturating concentrations of peptide. n.d., not determined.

Peptides	A-site binding relative IC_{50}	E-site binding relative IC_{50}	FX activation IC_{50} (nM) (% maximal inhibition)
A-183	1	n.d.	1.6 (78)
E-151	n.d.	1	0.9 (92)
A-100-Z/W10A	> 1000	n.d.	> 1000 (n.d.)
E-274-Z/Y12A	n.d.	> 1000	> 1000 (n.d.)
A-183 + E-151	0.6	0.5	0.7 (100)
AEZ-4	2.2	6.6	16 (100)
AEZ-5	1.7	3.0	21 (100)
AEZ-6	1.0	5.2	23 (100)
AEZ-7	0.6	6.5	20 (100)
AEZ-8	0.3	2.7	15 (100)
AEZ-9	3.8	4.6	11 (100)
AEZ-14	5.3	4.7	3.5 (100)
AEZ-19	3.7	4.3	2.1 (100)
AEZ-24	4.7	4.1	3.5 (100)
AEZ-29	2.9	3.1	5.0 (100)
A^{mut}EZ-9	3.3	4.9	9.5 (100)
AE^{mut}Z-9	1.7	2.4	14 (100)
$A^{mut}E^{mut}$Z-9	530	> 1000	> 1000 (n.d.)

Determination of the binding affinity for FVIIa

The purified fusion peptides were tested for their ability to bind FVIIa in competition binding assays using biotinylated versions of the A- or E-peptides. None of the fusion peptides with various linker lengths ranging from 4 to 29 residues had significantly lower IC_{50} values compared with the individual peptides A-183 or E-151 (Table 1). When changed to Ala, residues Trp[10] in A-100-Z and Tyr[12] in E-274-Z resulted in peptides with > 1000-fold reduced affinity for FVIIa (Table 1) [14,16]. However, fusion peptides A^{mut}EZ-9 and AE^{mut}Z-9 bound to FVIIa at both the A- and E-sites with similar IC_{50} values to A-183 and E-151, respectively (Table 1), suggesting an improvement in binding

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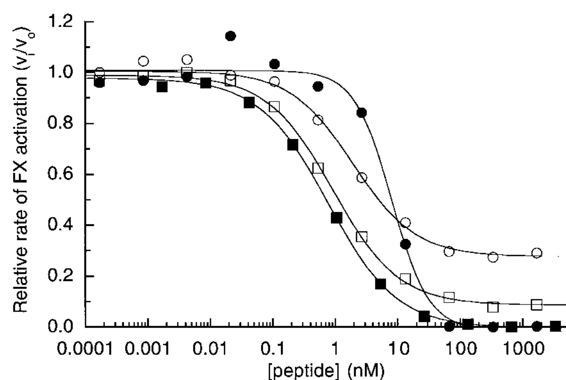

Figure 3 Inhibition of TF·FVIIa-catalysed activation of FX

The inhibition of FX activation is shown using various concentrations of A-183 (○), E-151 (□), a noncovalent combination of A-183 + E-151 (■), and the fusion peptide AEZ-9 (●). Data presented are representative of multiple independent experiments; the lines drawn represent the data fit to a 4-parameter equation, from which the IC_{50} and % maximal inhibition were calculated.

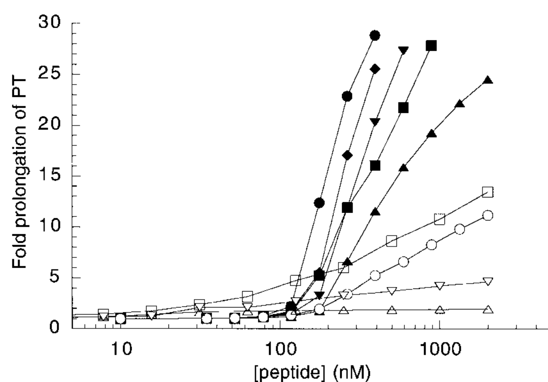

Figure 4 Concentration dependence of the individual, combination, and fusion peptides on the prothrombin time (PT) in normal human plasma

The fold prolongation of the clotting times was determined upon initiation by Innovin (see Materials and methods section); the uninhibited clotting time was 9 s. The fold prolongation of the PT are shown for A-183 (△), E-151 (▽), the noncovalent combination of A-183 + E-151 (□), AEZ-4 (○), AEZ-5 (■), AEZ-6 (▲), AEZ-7 (▼), AEZ-8 (◆), and AEZ-9 (●).

affinities of 200-fold provided by fusion of these peptides. Peptide $A^{mut}E^{mut}Z$-9 bound to the A- and E-sites with 530- and 1000-fold reduction in affinity compared to A-183 and E-151, respectively (Table 1).

Inhibition of TF·FVIIa activity

We previously showed that both the A- and the E-peptides inhibit the enzymic activity of the TF·FVIIa complex [14,15]. When tested for their ability to inhibit the activation of FX, all fusion peptides showed IC_{50} values ranging between 2 and 23 nM, slightly higher than the individual peptides (Table 1). Peptides $A^{mut}EZ$-9 and $AE^{mut}Z$-9 also inhibited FX activation with IC_{50} values similar to AEZ-9, while the IC_{50} value of $A^{mut}E^{mut}Z$-9 was increased by more than 1000-fold, consistent with the results from the FVIIa binding assays (Table 1). All the fusion peptides increased the extent of inhibition of FX activation to 100 % as compared to only 78 % and 92 % for A-183 and E-151, respectively (Table 1, Figure 3). A combination of A-183 and E-151 in a 1:1 molar ratio, also resulted in a complete

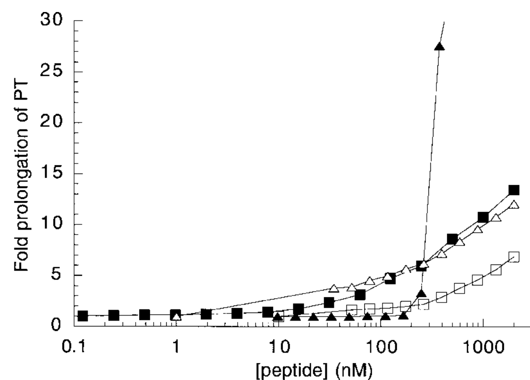

Figure 5 Effect of peptides in TF-dependent coagulation in normal and FVII-deficient human plasma

The concentration dependence on the PT fold prolongation is shown for AEZ-19 in normal (▲) and FVII-deficient (△) human plasma and the noncovalent combination of A-183 + E-151 in normal (■) and FVII-deficient (□) human plasma. The FVII-deficient plasma was supplemented with 600 pM FVIIa to mimic the clotting time of normal plasma. For fusion peptide AEZ-19, plasma did not coagulate at concentrations above the last symbol shown within the time limit of the assay of 300 s.

(100 %) inhibition of FX activation (Table 1). Finally, $A^{mut}EZ$-9 and $AE^{mut}Z$-9 also increased the extent of inhibition of FX activation to 100 % (Table 1).

The same phenomenon was also observed for the activation of FIX where the fusion peptides increased the extent of inhibition to 100 %, while having a minimal effect on the IC_{50} values (results not shown). In addition, the fusion peptides increased the maximal extent of inhibition of TF·FVIIa amidolytic activity using Chromozym t-PA. For A-183, E-151, and the combination of A-183 + E-151, the extent of inhibition was 32 %, 71 %, and 88 %, respectively, and the fusion peptide AEZ-19 had 92 % maximal inhibition.

Inhibition of TF-dependent clotting

The fusion peptides were then tested for their ability to inhibit TF-dependent coagulation *in vitro*. The PT-fold prolongation showed very steep concentration dependence curves for the fusion peptides (Figure 4). In comparison, A-183 and E-151 prolonged the PT to a maximum of 1.8- and approx. 5-fold [15]. The non-covalent combination of A-183 and E-151 resulted in an increase in the fold prolongation of the PT, as compared to the individual peptides, but this concentration dependence curve was not nearly as steep as those of the fusion peptides (Figure 4).

There was essentially no effect of the linker length on the PT-fold prolongation from AEZ-6 to AEZ-9 (Figure 4). Addition of subsequent 5-residue linker cassettes (AEZ-14, AEZ-19, AEZ-24 and AEZ-29), up to a total of 29 amino acids in the linker, resulted in similarly steep PT-fold prolongation curves (results not shown). However when the linker length was shortened to four residues, the PT-fold prolongation curve was no longer steep, similar to that of the combination of the A- and E-peptides (Figure 4). The addition of one Gly residue to form AEZ-5 significantly increased the steepness of the concentration dependence curve for the PT-fold prolongation. Furthermore, neither $A^{mut}EZ$-9 nor $AE^{mut}Z$-9 showed a steep prolongation of the PT (results not shown). As expected, the double Ala mutant $A^{mut}E^{mut}Z$-9 did not cause any significant prolongation of the PT at concentrations up to 2 μM. In summary these results indicate that both peptides need to be present, and linked together with

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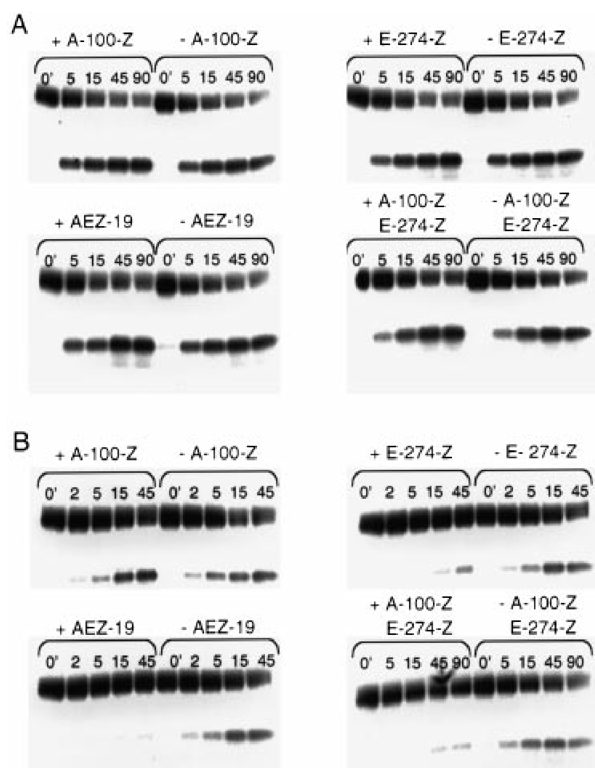

Figure 6 Effect of peptides on the activation of ^{125}I-FVII

The activation of ^{125}I-FVII by FXa (**A**) or TF·FVIIa (**B**) in the presence of 1 μM of the various peptides was measured over time by SDS/PAGE in reducing conditions followed by autoradiography of the dried gel. The effect of each peptide or combination of peptides was compared with uninhibited activation as a control. On each of the autoradiograms, the higher band is ^{125}I-FVII and the lower band is the heavy chain of ^{125}I-FVIIa.

a linker of at least five residues, to cause the steep prolongation of the PT.

To characterize further the effect of the fusion peptides on the PT-fold prolongation, FVII-deficient plasma supplemented with FVIIa was used. This contrasts with normal plasma which contains approx. 99 % of the total FVII in the zymogen form [26]. As shown in Figure 5, the fusion peptide AEZ-19 did not cause a steep prolongation of the PT with FVII-deficient plasma supplemented with FVIIa. In fact, the concentration dependence of AEZ-19 in FVII-deficient plasma supplemented with FVIIa was comparable to that of the combination of A- and E-peptides (Figure 5). These results show that the improvement in the PT-fold prolongation observed with the fusion peptides in normal human plasma is directly related to the activation of zymogen FVII.

Inhibition of FVII activation

In plasma, the enzymes most likely to activate FVII to FVIIa include FXa [27–29] and TF·FVIIa via autoactivation [3,4]. ^{125}I-FVII was used to measure the inhibition of FVII activation by the fusion peptides, where gel autoradiographs provide a qualitative assessment of the role of the fusion peptides in the activation of FVII. The results show that none of the peptides tested at a concentration of 1 μM (A-100-Z, E-274-Z, A-100-Z + E-274-Z, and AEZ-19) inhibited the activation of FVII to FVIIa by FXa (Figure 6A). On the other hand, the assays using TF·FVIIa as the activator showed that the fusion peptide and the combination of the A- and E-peptides were more effective than the individual peptides used alone (Figure 6B); however, this assay was not able to distinguish between the fusion peptide and the combination of peptides.

DISCUSSION

In this work, two unrelated peptides that were previously shown to bind at two distinct exosites on the protease domain of FVIIa, were linked to create a bifunctional inhibitor of FVIIa. At the time this work was initiated, only the structure of E-76 bound to FVIIa was known [14]. Although the residues of FVIIa which are important for A-peptide binding had been identified [16], the exact location of the peptide on the protease domain of FVIIa was not. Thus, it was impossible to design a bifunctional molecule based on structural information only. For this reason, fusion peptides with different linker lengths were generated and characterized for their ability to inhibit the TF·VIIa complex. This strategy resulted in a dramatic improvement in the prolongation of TF-dependent coagulation as compared to the individual as well as a combination of the A- and E-peptides.

Comparison of different linker lengths from 4 to 29 residues in the PT assay indicated that the minimum linker length for the steep PT-fold prolongation was five residues between the C-terminal Arg of A-183 and the N-terminal Ala of E-151 (Figure 4). To explain these results, a model of the protease domain of FVIIa with bound A- and E-peptides was generated [16] (Figure 1) based upon the recently described crystal structures of E-76 bound to FVIIa and A-183 bound to a truncated version of zymogen FVII [14,30]. In this model, the distance between the C-terminus of A-183 and the N-terminus of E-76 is approx. 25 Å, which corresponds to the distance covered by a 5-residue linear peptide. This agrees with our results where a 5-residue linker (AEZ-5) was shown to be the minimal length that improves the PT prolongation over the combination of peptides (Figure 4). This linker length dependence strongly suggests that the fusion peptides bind both exosites simultaneously. Additional evidence for simultaneous binding of both peptides is provided by data showing an improvement in the extent of inhibition of FX activation (Table 1) and in the PT-fold prolongation (Figure 4) for the non-covalent combination of peptides relative to the individual peptides.

In an effort to understand the mechanism of the increased PT-fold prolongation, the fusion peptides were further tested for their ability to bind FVIIa. To our surprise, fusion of the peptides did not appear to increase the affinity for FVIIa (Table 1). To investigate this phenomenon further, fusion peptides were generated in which either the A-peptide or the E-peptide component had a substantially weaker affinity for FVIIa. Alanine substitution of Trp[10] in peptide A-100-Z and Tyr[12] in peptide E-274-Z resulted in a > 1000-fold increase in the IC_{50} values in the A-site and E-site binding assays, respectively [16] (Table 1). If these same two mutations in the fusion peptides would similarly affect binding to FVIIa at the A- and E-sites, respectively, a similar reduction in binding affinity should occur. However, this was not the case (Table 1), suggesting that linking these peptides did increase their affinity for FVIIa.

We propose two different ways to explain these somewhat paradoxical results. First, it is possible that fusion of the two exosite peptides did improve the binding affinity to FVIIa, but the assays used were not sensitive enough to detect binding in the picomolar range. Second, we postulate that the fusion peptides did not improve the affinity for FVIIa, and the results obtained for the Ala substituted fusion peptides were due to the method

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used to measure binding. It is likely that the wild-type half of the single mutant bifunctional peptide is providing the binding affinity for FVIIa. Because of the proximity effect, this enables the mutant part to now compete for binding at its exosite. For instance, in the peptide A^{mut}EZ-9, the E-peptide half binds to the E-site on FVIIa with low nanomolar affinity, which may enable the A-peptide containing the W10A mutation to efficiently compete for binding at the A-site. The latter explanation is supported by FX activation and PT data where both the IC_{50} for the inhibition of FX activation, and the concentration at which the peptides start to prolong PT, were similar to the individual peptides (Table 1 and Figure 4). Furthermore, preliminary data comparing peptide binding to immobilized FVIIa using surface plasmon resonance showed K_d values between 0.5 and 2.5 nM for peptides A-183, E-151, AEZ-9, A^{mut}EZ-9 and AE^{mut}Z-9 (M. Roberge, unpublished results), also indicating that fusion of the two exosite peptides did not improve binding affinity towards FVIIa. Altogether, these results suggest that the enhancement in the PT-fold prolongation was not caused by an increase in binding affinity to FVIIa and subsequent decrease in the IC_{50} for the inhibition of FX activation.

To explain the difference in the PT-fold prolongation between the fusion and the combination of A- and E-peptides, the kinetics of the PT assay were investigated. In a standard PT assay, the peptides are first incubated in citrated plasma and allowed to bind FVII/FVIIa to reach equilibrium. Clotting is then initiated by adding a solution containing TF and Ca^{2+}. E-peptide binding to FVIIa is Ca^{2+}-dependent whereas binding of the A-peptide is not [14,16]. The A-peptide also binds with high affinity to both FVII and FVIIa [15]. In the case of the fusion peptide, it is possible that A-peptide binding brings the E-peptide to the E-site during incubation in citrated plasma. This would provide a kinetic advantage to the fusion peptide over the combination of peptides, resulting in an increased fold prolongation of the PT. To rule out this hypothesis, the peptides were tested in a reverse PT assay in normal human plasma, where the peptides were mixed with the TF-Ca^{2+} reagent (Innovin) rather than plasma. Under these conditions there was no kinetic advantage provided by A-peptide binding to FVII/FVIIa. The results were very similar to the PT assay where the fusion peptides, but not their non-covalent combination, showed steep PT-fold prolongation curves (data not shown). These results indicate that the proximity effect provided by the fusion peptides is not responsible for the higher fold prolongation of the PT.

We also considered the role of zymogen FVII in trying to address the mechanism by which the fusion peptides caused the steep prolongation of the PT when compared to the combination of peptides. PT assays were carried out using FVII-deficient plasma supplemented with FVIIa. Thus, the initial step of TF-dependent coagulation where FVII is activated to FVIIa was eliminated. Under these conditions, the concentration dependence of the fusion peptide on the PT-fold prolongation was now similar to the combination of A- and E-peptides (Figure 5). We can therefore conclude that it is the conversion of FVII into FVIIa in normal plasma that gives rise to the difference in the PT-fold prolongation between the fusion and the combination of peptides. The apparent difference in potency of the fusion peptide in normal versus FVII-deficient plasma (Figure 5) is difficult to interpret due to differences in assay conditions, more specifically the total FVIIa concentration.

The activation of FVII to FVIIa in plasma is catalysed by FXa [27–29] and/or the TF·FVIIa complex [3,4]. The fusion peptide AEZ-19 was compared to the combination of peptides for their ability to inhibit the activation of ^{125}I-FVII by FXa or TF·FVIIa. None of the peptides inhibited the activation of FVII by FXa (Figure 6A). This was not unexpected since the peptides are specific inhibitors of FVIIa, but the data also indicated that binding of these peptides, individually, in combination, or as a fusion peptide did not prevent FVII from being a substrate for FXa. In contrast, all combinations of these peptides were good inhibitors of FVII autoactivation (fusion or combination $>$ E-274-Z $>$ A-100-Z) (Figure 6B). However, this assay was not sensitive enough to show a difference between the fusion and the combination of peptides.

How do we explain the difference in the PT-fold prolongation between the fusion and the combination of peptides? Binding of the bifunctional peptide may 'lock' FVIIa into an inactive state, while the combination of peptides may allow FVIIa to breathe and still activate FX and FIX. This could affect the extent of inhibition of FVIIa activity in a subtle manner that is not observable in the assays used here. A difference between 99.5 and 100 % inhibition in the rate of FX activation or FVII autoactivation would not be detectable, yet may be sufficient to cause the significant difference observed in the PT-fold prolongation between the fusion and the combination of peptides.

Conclusions

Two peptides that bind two distinct exosites on the protease domain of FVIIa were fused to generate a bifunctional inhibitor. Compared to the individual peptides, the fusion peptides increased the extent of inhibition of the enzymic activity of TF·FVIIa and resulted in a greater inhibition of TF-dependent coagulation *in vitro*. Careful comparison of the characteristics of the fusion peptides suggested that the autoactivation of FVII was involved in the mechanism by which they prolong coagulation. We have previously suggested that the individual A- or E-series peptides may be useful themselves or lead to useful therapeutic agents [14,15]. The fusion peptides represent opportunities to develop potential anticoagulants for therapeutic situations where complete inhibition of the TF·FVIIa complex is desired. The degree of anticoagulation desired for clinical use, by inhibition of TF·FVIIa activity, is unknown. In addition the fusion peptides described herein may also prove to be useful agents to investigate the role of TF·FVIIa in signalling [31].

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