Selection and Characterization of a New Class of Peptide Exosite Inhibitors of Coagulation Factor VIIa

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ABSTRACT: A new series of peptide inhibitors of human Factor VIIa (FVIIa) has been identified and affinity matured from naive and partially randomized peptide phage libraries selected against the immobilized tissue factor·Factor VIIa (TF·FVIIa) complex. These "A-series" peptides contain a single disulfide bond and a 13-residue minimal core required for maximal affinity. They are exemplified by peptide A-183 (EEWEVLCWTWETCER), which binds at a newly identified exosite on the FVIIa protease domain, described in the accompanying report [Roberge, M., Santell, L., Dennis , M. S., Eigenbrot, C., Dwyer, M. A., and Lazarus, R. A. (2001) *Biochemistry 40*, 9522−9531]. A-183 was obtained from a trypsin digest of A-100-Z, a recombinant protein comprising A-183 and the Z domain of protein A. Surprisingly, A-183 was a very potent inhibitor of TF·FVIIa, inhibiting activation of Factor X (FX) and Factor IX and amidolytic activity of Chromozym t-PA with IC_{50} values of 1.6 ± 1.2, 3.5 ± 0.3, and 8.5 ± 3.5 nM, respectively. Kinetic analysis revealed that A-183 was a partial (hyperbolic) mixed-type inhibitor of FX activation having a K_i of 200 pM as well as a partial competitive inhibitor of amidolytic activity. The A-series peptides were also specific and potent inhibitors of TF-dependent clotting as measured in a prothrombin time (PT) clotting assay and had no effect on the TF-independent activated partial thromboplastin time. At saturating concentrations of peptide, the maximal extent by which A-183 and A-100-Z inhibited the rate of FX activation was 78 ± 3 and 89 ± 6%, respectively. The degree of inhibition of the rate of FX activation correlated with a maximum fold prolongation in the PT assay of 1.8-fold for A-183 and 3.3-fold for A-100-Z. The A-series peptides represent a new class of peptide exosite inhibitors that are capable of attenuating, rather than completely inhibiting, the activity of TF·FVIIa, potentially leading to anticoagulants with an increased therapeutic window.

The coagulation cascade is an amplification process of proteolytic events leading to the localized generation of thrombin and ultimately the formation of a fibrin clot (*1*, *2*). Initiation of the cascade occurs when tissue factor (TF),[1] a nonvascular cell membrane protein, is exposed to circulating Factor VII (FVII), an inactive serine protease zymogen (*3*, *4*). Although blood and its constituents are not normally exposed to TF, upon vessel or tissue injury, FVII can bind TF and become activated to FVIIa forming a TF·FVIIa complex (*5*, *6*). This complex binds and activates Factor IX (FIX) to FIXa, Factor X (FX) to FXa, and FVII to FVIIa, ultimately leading to the generation of thrombin. Since the TF·FVIIa complex is paramount in producing enough thrombin to initiate coagulation, it represents an appealing target for therapeutic intervention (*7*, *8*).

Current anticoagulant therapy involves the use of heparins and coumarins (*9*−*11*). Coumarins inhibit an important vitamin K dependent posttranslational modification that enables both procoagulant (FVIIa, FXa, FIXa, thrombin) and anticoagulant (activated protein C and protein S) proteins to interact with the phospholipid membrane, thereby disrupting the coagulation process. Heparins accelerate the inhibition of thrombin and FXa by antithrombin III. In addition to its procoagulant role, thrombin also plays an important role as an anticoagulant in the presence of thrombomodulin by activation of the protein C pathway (*12*). Thus, both of the current therapeutic agents nonselectively perturb the balance between thrombosis and hemostasis, which can lead to undesirable side effects such as bleeding that require close monitoring.

In addition to blocking FVIIa interactions with the membrane as described above, other more selective approaches to inhibiting the function of the TF·FVIIa complex have been described (*8*, *13*). These include blocking the interactions between TF and FVIIa using monoclonal antibodies as well as interfering with FX activation using either TF mutants or FVIIa that has been covalently modified at its active site. Finally, several inhibitors have been described that bind at the active site and inhibit the catalytic

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[1] Abbreviations: TF, tissue factor; FVIIa, Factor VIIa; FIXa, Factor IXa; FXa, Factor Xa; E-76, Ac-ALCDDPRVDRWYCQFVEG-NH_2; HEPES, N-2-hydroxylethylpiperazine-*N*′-2-ethanesulfonic acid; A-183, EEWEVLCWTWETCER; BSA, bovine serum albumin; TF_{1-219}, *E. coli*-derived recombinant human tissue factor encompassing residues 1-219; TF_{1-243}, *E. coli*-derived recombinant human tissue factor encompassing residues 1-243; mTF, 293 cell derived membrane human tissue factor encompassing residues 1-263; HPLC, high-performance liquid chromatography; PT, prothrombin time; APTT, activated partial thromboplastin time; Z, consensus domain of protein A.

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activity of FVIIa. In addition to the naturally-occurring endogenous inhibitors of TF·FVIIa—tissue factor pathway inhibitor and the serpin antithrombin III—selective Kunitz domain inhibitors as well as NAPc2, a naturally-occurring inhibitor from the nematode *Ancylostoma caninum*, have also been characterized (*8*, *13*). To date, limited studies have been reported for small molecule active site inhibitors of TF·FVIIa.

The design of small molecule active site inhibitors is a common approach to inhibiting serine proteases (*14*). Obtaining selective inhibitors, however, is challenging due to the high degree of active site conservation among the many serine proteases present in plasma (*15*, *16*). Substrate specificity is complex and is frequently attained through subsites in the substrate binding cleft (*17*); however, other macromolecular interactions and compartmentalization can be important. The activation of FX by TF·FVIIa occurs through interactions of FX with exosites on the TF·FVIIa complex followed by active site interactions and ultimately bond cleavage (*18*). Many serine proteases utilize additional cofactors that can provide an extended substrate binding site. In the case of FVIIa, TF provides important interactions for binding FX and also serves to anchor FVIIa to the cell membrane (*19*). These extended substrate binding sites provide new opportunities to design highly specific inhibitors.

Recently, E-76, an 18 residue peptide selected for binding to TF·FVIIa by phage display, was characterized as a potent and selective inhibitor of FX activation and TF-dependent coagulation (*20*). Consistent with its pure noncompetitive and mixed-type modes of inhibition of FX activation and amidolytic activity, respectively, the X-ray crystal structure of the FVIIa·E-76 complex revealed that E-76 binds to an exosite on the protease domain of FVIIa rather than to the active site. Binding of E-76 to FVIIa caused a large structural change in the 140's loop, which comprises part of the activation domain. The mechanism by which E-76 inhibits TF·FVIIa activity has been described as an "allosteric switch" that may give rise to both steric and allosteric modes of inhibition of FX activation. E-76 may alter the way FX binds to FVIIa as well as disrupt a hydrogen bonding network linked to the oxyanion hole in the active site.

In this report, we describe a new series of peptides also selected for binding to the TF·FVIIa complex by phage display. Remarkably, these "A-series" peptides also potently inhibit FX activation and specifically prolong TF-dependent coagulation. They differ completely from E-76 in their amino acid sequence and structure and do not compete with E-76 or active site inhibitors for binding to FVIIa. In the following paper in this issue, we present the peptide binding exosite for the A-series peptides as well as a discussion of their molecular interactions with FVIIa (*21*).

EXPERIMENTAL PROCEDURES

Phage Libraries and Selection Conditions. The selection of phage-displayed peptides for binding to TF·FVIIa has been described previously (*20*). Briefly, phage libraries expressing random peptide sequences fused to P8 (*22*, *23*) were pooled into three groups: pool A contained $X_iCX_jCX_k$ where $j = 5-7$; pool B contained $X_4CX_2GPX_4CX_4$, X_{20} and $X_iCX_jCX_k$ where $j = 4$; pool E contained $X_iCX_jCX_k$ where $j = 8-10$. In each case $i + j + k = 18$ and $|i - k| < 2$. Each of the 10 libraries has in excess of 10^8 clones.

The library pools were sorted against TF·FVIIa, immobilized directly on maxisorp plates (*20*). Unbound phage were removed by repetitive washing (50 mM HEPES, pH 7.2, 150 mM NaCl, 0.05% Tween 20) and bound phage were eluted with 500 mM KCl, 10 mM HCl, pH 2. Eluted phage were propagated in XL1-Blue cells with VCSM13 helper phage (Stratagene, La Jolla, CA). Enrichment was monitored by titering the number of phage that bound to a TF·FVIIa coated well compared to a well coated with bovine serum albumin (BSA).

Partial and Complete Randomization on Monovalent Phage. A partially randomized library fused to P3 was designed using an oligonucleotide coding for clone A-53, but synthesized with a 70-10-10-10 mixture of bases (*24*). A fully randomized library was designed using NNS codons for residues at position X and holding highly selected residues (in bold) constant: X_3**WEV**X**CW**X**WE**X**C**X_6. This library was then sorted against TF·FVIIa.

Production of A-100-Z and A-183. An expression plasmid for A-100-Z was constructed using Kunkel mutagenesis (*25*) by inserting a DNA sequence encoding A-100 (EEWEVLCWTWETCERGEG) and a linker sequence (GGGSGG) into the vector pZCT between the stII signal sequence and the Z consensus domain from protein A (*26*). The resulting plasmid, pA-100-Z, was confirmed by DNA sequencing, transformed into *Escherichia coli* strain 27C7 and grown in low phosphate minimal media (*27*). A-100-Z was secreted into and harvested from the media and rapidly purified using IgG Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ) essentially as described (*28*). Purified A-100-Z was cleaved using trypsin (1% w/w) and the reaction monitored by HPLC until ~80 % of the fusion was cleaved. The reaction was then stopped by addition of HCl to pH 2. A-183 was purified by reversed-phase HPLC using a water/acetonitrile gradient containing 0.1% TFA.

Peptide Synthesis. Peptides were synthesized by either manual or automated (Perseptive Pioneer) Fmoc-based solid phase synthesis on a 0.25 mmol scale using a PEG-polystyrene resin (*29*). Coupling of each amino acid was accomplished with 2-(H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate and N-hydroxybenzotriazole with diisopropylethylamine in dimethylacetamide. Peptides ending in a carboxy-terminal amide were prepared on Rink amide resin. Side chain protecting groups were removed, and the peptide was cleaved from the resin with 95% trifluoroacetic acid and 5% triisopropylsilane. A saturated iodine solution in acetic acid was added to oxidize the disulfide bonds. Peptides were purified by reversed-phase HPLC using a water/acetonitrile gradient containing 0.1% TFA and lyophilized. Peptides were >95% pure by analytical HPLC and their identity was verified by mass spectrometry.

Inhibition of FX Activation, FIX Activation, and Amidolytic Activity. Inhibition of FX activation by TF·FVIIa was determined with 300 pM relipidated TF_{1-243}, 20 pM FVIIa, and 165 nM FX at 25 °C as a function of peptide concentration essentially as described (*20*, *30*). TF was incorporated into phospholipid vesicles and quantified as previously described (*30*). The linear rates of FXa generation are expressed as fractional activities (v_i/v_o). Controls showed that the peptides tested did not inhibit FXa chromogenic activity.

Inhibition of FIX activation by TF·FVIIa was monitored as a function of peptide concentration essentially as described previously (*31*). Briefly, peptides were incubated with FVIIa and membrane TF_{1-263} (mTF) in 20 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 0.5 mg/mL BSA for 20 min at room temperature. FIX (Haematologic Technologies Inc., Essex Junction, VT) was added to start the reaction. The reaction mixture contained 20 μg/mL mTF (total protein concentration), 1 nM FVIIa, and 400 nM FIX. At timed intervals, 100 μL aliquots were removed and quenched in 125 μL of 30 mM EDTA-60% ethyleneglycol. In the second stage of the assay, 25 μL of 5 mM FIXa chromogenic substrate #299 (American Diagnostica, Greenwich, CT) was added and the increase in absorption at 405 nm measured on a microplate reader (Molecular Devices, Menlo Park, CA). The linear rates of FIXa generation are expressed as fractional activities (v_i/v_o). Peptide A-183 did not inhibit FIXa chromogenic activity.

Inhibition of amidolytic activity of TF·FVIIa was monitored as a function of peptide concentration essentially as described previously, except that Tween 20 was used instead of Tween 80 (*20*). The linear rates of the increase in absorbance at 405 nm are expressed as fractional activities (v_i/v_o).

Kinetic Inhibition Assays. Inhibition of FX activation by TF·FVIIa was monitored as a function of peptide concentration essentially as described previously (*20*). Relipidated TF_{1-243} and FVIIa were incubated with varying concentrations of A-183 for 30 min in 100 mM HEPES, pH 7.8, 5 mM $CaCl_2$, 140 mM NaCl, 0.5% BSA, and 0.02% Tween 80. The reaction was initiated by the addition of FX (Haematologic Technologies Inc., Essex Junction, VT), yielding final concentrations of 200 pM TF_{1-243}, 20 pM FVIIa, and from 0.05 to 1 μM FX. Aliquots were removed at 0.5, 1, 1.5, and 2 min and quenched in 15 mM EDTA. The FXa generated in each aliquot was measured by monitoring the hydrolysis of 0.3 mM S-2765 (DiaPharma, West Chester, OH). The concentration of FXa formed as a function of time was calculated from a plot of mOD_{405} min^{-1} versus known concentrations of FXa.

Amidolytic activity assays were carried out as described previously (*20*) using 60 nM soluble TF_{1-219}, 10 nM FVIIa, and varying concentrations of A-183 for 15 min in 100 mM HEPES, pH 7.8, 5 mM $CaCl_2$, 140 mM NaCl, 0.1% PEG-8000, and 0.02% Tween 80. Reactions were initiated by addition of varying concentrations (0.4−3 mM) of Chromozym t-PA (Roche Molecular Biochemicals, Indianapolis, IN). The concentration of *p*-nitroanilide (pNA) formed as a function of time was calculated from a plot of mOD_{405} versus known concentrations of pNA.

Lines for Lineweaver-Burk analysis represent data fit to a linear equation (KaleidaGraph, Synergy Software, Reading, PA). Lines for secondary plots of the slopes or intercepts (from the reciprocal plots) versus A-183 concentration represent data fit to a hyperbolic equation (KaleidaGraph). Lines for secondary plots of $1/\Delta$ slope or $1/\Delta$ intercept (from the reciprocal plots) versus 1/[A-183] concentration were fit to a linear equation (KaleidaGraph).

Clotting Assays. Both the prothrombin time (PT) and activated partial thromboplastin time (APTT) assays were performed in citrated pooled normal human plasma as described previously (*20*). Clotting times were determined using an ACL 300 Automated Coagulation Analyzer (Coulter Corp., Miami, FL) using Innovin (human relipidated TF and Ca^{2+}) to initiate the PT assay and Actin FS to initiate the APTT assay. Innovin and Actin FS were purchased from Dade International Inc. (Miami, FL).

RESULTS

Identification and Maturation of Peptides that Bind to TF·FVIIa. Polyvalent phage libraries of naive 20-residue peptides, displayed on the major coat protein P8 of M13 and constrained by a single disulfide bond, were combined into three pools and sorted against the TF·FVIIa complex. In addition to the enrichment of library pool E, which led to the discovery of peptide E-76 as reported previously (*20*), we observed greater than 1500-fold enrichment of library pool A. The A library pool contained the $X_iCX_jCX_k$ motif where $j = 5$-7, and $i + j + k = 18$ and $|i - k| < 2$. Six representative clones were selected and subjected to DNA sequence analysis revealing a single DNA sequence, encoding for peptide A-53, from the $X_7CX_5CX_6$ library (Table 1).

The A-53 sequence was affinity matured using a "soft randomization" strategy (*23*). An oligonucleotide coding for the A-53 peptide sequence was designed to introduce a mutation rate of 50 percent at each position (*24*). This oligonucleotide was used to generate a monovalent peptide library fused to P3, the minor coat protein of M13. This partially randomized library contained 2 x 10^7 clones and was sorted against immobilized TF·FVIIa. Following four rounds of selection, an enrichment of 10^5 was observed. Sequence analysis revealed that eight amino acid positions were invariant, suggesting that these were important for binding either through direct contact with FVIIa or important for the structural integrity of the peptide (Table 1). In a final monovalent peptide library, these eight positions were fixed and the remaining positions were fully randomized with all 20 amino acids. Following four rounds of selection (enrichment of 10^5), sequences from 23 individual clones were used to calculate amino acid preferences at each position (Figure 1). These preferences led to the peptide consensus sequence, designated C (MEEWEVLCWTWETCERGEGQ).

Expression and Purification of A-100-Z and A-183. We developed a recombinant method of peptide production which incorporated the Z domain from protein A as an affinity handle for rapid purification on IgG sepharose. The carboxy-terminus of the A-series peptides was fused to the Z domain using a GGGSGG linker; these peptide-Z fusions were expressed in *E. coli* and secreted into the culture media in high yield. Generally, 6 mg of the fusion peptide was obtained from a 1 L shake flask culture. Three peptide-Z domain fusions were expressed: A-53-Z, the initial lead identified from the naive libraries; C-Z, the consensus sequence obtained from hard randomization; and A-100-Z, which contained an 18-residue peptide lacking one residue each from the amino- and carboxy-terminus of the consensus sequence (Table 2).

We devised a simple method for generating a shorter active peptide from A-100-Z. Since there were no critical amino acid preferences for residues in the carboxy-terminus of A-100 (Figure 1), we reasoned that the loss of these residues would have little effect on binding, which was confirmed with truncated peptides (*vide infra*). Trypsin cleavage of

Table 1. Sequences of Phage Clones Selected from Naive and Partially Randomized Libraries for Binding to TF·FVIIa[a]

Clone	frequency																			
A-53	6/6	S	A	E	W	E	V	L	C	W	T	W	E	G	C	G	S	V	G	L	V
AA/AF	2/12	S	A	E	W	E	V	L	C	W	T	W	E	**E**	C	G	S	V	**W**	**P**	**P**
AB	1/12	**N**	A	**G**	W	E	V	L	C	W	T	W	E	**D**	C	G	**P**	**M**	**D**	**P**	**A**
AC	1/12	S	**G**	E	W	E	V	L	C	W	T	W	E	**A**	C	G	**W**	**E**	**S**	**G**	**E**
AD	1/12	S	**E**	E	W	E	V	L	C	W	T	W	E	**D**	C	**R**	**L**	**E**	G	L	**E**
AE	1/12	S	**D**	E	W	E	V	**V**	C	W	T	W	E	**A**	C	**E**	**T**	V	G	L	**G**
AG	1/12	S	**T**	E	W	E	V	L	C	W	T	W	E	G	C	G	**W**	**G**	G	**I**	**E**
AH	1/12	**G**	A	E	W	E	V	L	C	W	T	W	E	**Q**	C	**E**	**F**	**G**	**S**	L	V
AI	1/12	S	A	E	W	E	V	**I**	C	W	T	W	E	**S**	C	**E**	**W**	**G**	G	L	**G**
AJ	1/12	**R**	**D**	**G**	W	E	V	**V**	C	W	**E**	W	E	G	C	**E**	**R**	**A**	**V**	**D**	V
AK	1/12	**T**	A	**G**	W	E	V	L	C	W	T	W	E	**D**	C	G	**P**	**L**	G	**P**	V
AL	1/12	**G**	A	E	W	E	V	L	C	W	**E**	W	E	G	C	**E**	S	V	**W**	**P**	**G**

[a] Sequence A-53 was identified from the naive peptide library, $X_7CX_5CX_6$. Residues completely conserved during soft randomization are boxed. New amino acid residues that appeared during soft randomization are in bold. Sequences AA through AL were identified during soft randomization of A-53. Clones AA and AF share the same DNA sequence. Peptide A-57 was derived from the sequence in clone AD (*21*).

Table 2. Inhibition of Factor X Activation and A-183b Binding by A-Series Peptides

	A-series peptides		FX Activation IC_{50} (nM)	A-183b Binding[a] IC_{50} (nM)
A-53-Z	S A E W E V L C W T W E G C G S V G L V	-Z	4,400 ± 800	2,500 ± 800
A-57	S E E W E V L C W T W E D C R L E G L E		93 ± 20	n.d.[b]
C-Z	M E E W E V L C W T W E T C E R G E G Q	-Z	5.9 ± 0.9	5.4 ± 3.4
A-100-Z	E E W E V L C W T W E T C E R G E G	-Z	3.8 ± 2.6	9.1 ± 0.1
A-100	E E W E V L C W T W E T C E R G E G	$-NH_2$	1.5 ± 0.7	6.8
A-99	E W E V L C W T W E T C E R G E	$-NH_2$	4.8	n.d.
A-183	E E W E V L C W T W E T C E R		1.6 ± 1.2	6.4 ± 1.1
A-65	W E V L C W T W E T C E R	$-NH_2$	2.5 ± 0.2	n.d.
A-378	E V L C W T W E T C E R	$-NH_2$	470 ± 110	680 ± 130
A-380	V L C W T W E T C E R	$-NH_2$	22,000 ± 700	180,000
A-382	L C W T W E T C E R	$-NH_2$	>50,000	>250,000
A-153	C W T W E T C E R G E G Q	$-NH_2$	>50,000	>100,000
A-383	W E V L C W T W E T C E	$-NH_2$	7,000	25,000
A-386	W E V L C W T W E T C	$-NH_2$	13,000	2,600

[a] The A-183b binding assay used to determine the IC_{50} values for inhibition of a biotinylated version of A-183 binding to FVIIa is described in the following paper (*21*). [b] n.d., not determined.

A-100-Z following the single Arg at position 15 resulted in removal of the linker and the Z domain and generation of a 15-residue peptide, A-183 (EEWEVLCWTWETCER). Cleavage of the fusion was monitored by analytical reversed-phase HPLC. When the reaction was complete, peptide A-183 was then purified from the mixture using reversed-phase HPLC (Figure 2). From 10 mg of A-100-Z, 0.9 mg of A-183 was obtained, resulting in a ca. 50% overall molar yield.

Inhibition of TF·FVIIa Activity. Throughout the maturation process all peptides and peptide-Z fusions tested were found to inhibit the rate of FX activation by TF·FVIIa in a dose dependent manner. In Figure 3A, we compare the inhibition of FX activation by A-53-Z (IC_{50} = 4400 ± 800 nM), the initially selected lead, with C-Z (IC_{50} = 5.9 ± 0.9 nM), A-100-Z (IC_{50} = 3.8 ± 2.6 nM), A-100 (IC_{50} = 1.5 ± 0.7 nM), and A-183 (IC_{50} = 1.6 ± 1.2 nM). This comparison shows that the optimized peptides selected using the full randomization process led to an improvement of greater than 1000-fold. In addition, the Z domain, the amino-terminal Met in C-Z, and the three residues at the carboxy-terminus of A-100 have relatively little influence on the binding affinity (Figure 3A; Table 2).

Although there was little difference in the affinity of peptides and peptide-Z fusions, there was a significant

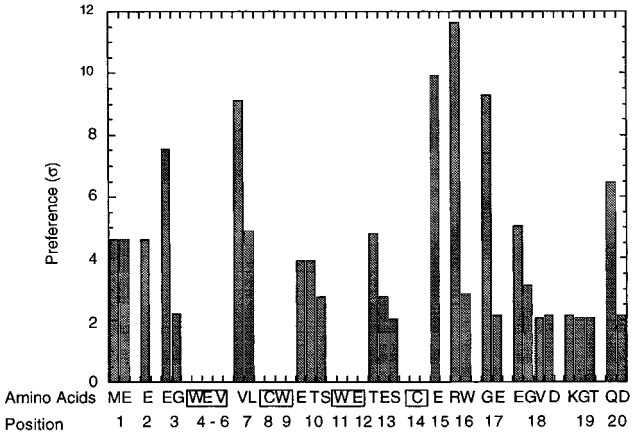


FIGURE 1: Sequence preferences using full randomization. The library X_3**WEV**X**CW**X**WE**X**C**X_6, where amino acids in bold were held constant and all 20 amino acids were substituted at X, was sorted against TF·FVIIa. Amino acids identified at the indicated randomized positions are plotted as a function of their preference. The preference for any amino acid is reported as the number of standard deviation units (σ) above a random chance occurrence of a given residue assuming a binomial distribution and accounts for codon bias and sampling statistics (*41*). Fixed amino acids in the library are boxed.

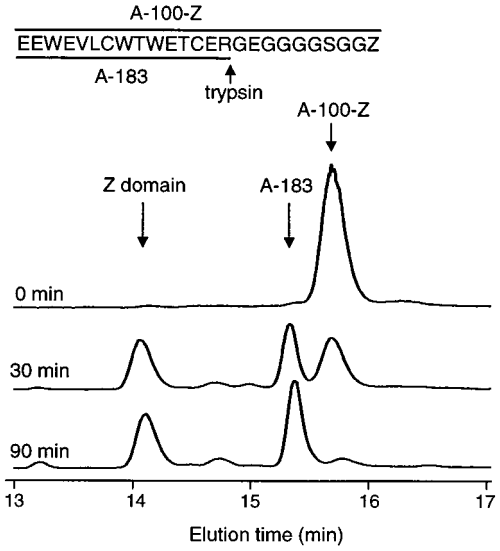


FIGURE 2: Time course of A-100-Z cleavage by trypsin followed by analytical HPLC. Aliquots from a trypsin digest of A-100-Z were monitored by reversed-phase HPLC on a C18 column. Peptides were eluted with a linear gradient from 0 to 60% acetonitrile in 0.1% TFA over 20 min. The elution peaks for A-100-Z, A-183, and the cleaved Z domain are indicated.

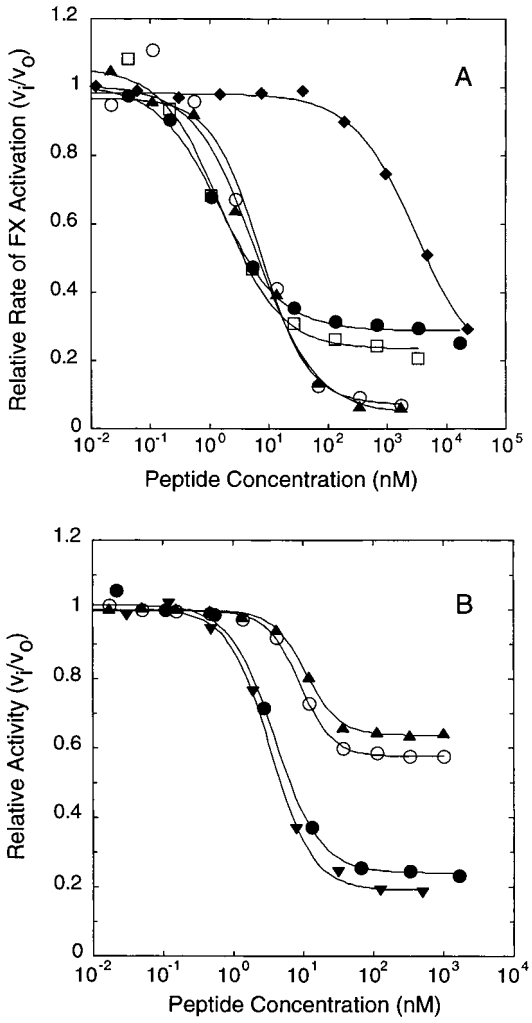


FIGURE 3: Inhibition of TF·FVIIa activity by A-series peptides. (A) FX activation by TF·FVIIa in the presence of varied concentrations of A-53-Z (◆), C-Z (▲), A-100-Z (○), A-100 (□), and A-183 (●). (B) Effect of A-183 on TF·FVIIa during FX (●) and FIX (▼) activation and Chromozym t-PA hydrolysis (▲). The inhibition of Chromozym t-PA hydrolysis is also shown for A-100-Z (○). Data presented are representative of multiple independent experiments; the lines drawn represent the data fit to a four-parameter equation, from which we calculate the IC_{50} and maximal percent inhibition.

difference in the maximal extent of inhibition at saturating concentrations by these peptides. Removal of the Z domain resulted in a lesser extent of inhibition of the rate of FX activation (Figure 3A). At saturating concentrations of A-100-Z, there was 89 ± 6 % inhibition of the FX activation rate. In contrast, peptides A-100 and A-183 maximally inhibited this rate by only 80 ± 3 and 78 ± 3%, respectively.

Peptide A-183 was also a potent inhibitor of FIX activation, exhibiting a similar IC_{50} value and extent of inhibition to those of FX activation (Figure 3B). Furthermore, A-183 was a potent inhibitor of amidolytic activity with the small peptidyl substrate Chromozym t-PA (IC_{50} = 8.5 ± 3.5 nM). At saturating concentrations of A-183, the maximal inhibition of the amidolytic activity rate was only 32 ± 6% compared to the maximal inhibition of the rates of FX activation of 78 ± 3% and of FIX activation of 81 ± 1%. Interestingly, A-100-Z inhibited amidolytic activity with IC_{50} and maximal inhibition values similar those of A-183 (Figure 3B).

Identification of the Minimal Active Sequence. The minimum length for optimum binding to TF·FVIIa and subsequent inhibition of FX activation was determined using a series of synthetic peptide analogs (Table 2). In addition to monitoring the inhibition of FX activation by TF·FVIIa, the effect of changes on the ability of these peptide analogs to bind FVIIa was measured using a FVIIa binding assay (*21*). The results in Table 2 are consistent with the findings described above, where removal of one residue from the N- and the C-terminus (C-Z to A-100-Z) or removal of an additional three C-terminal residues (A-100 to A-183) had minimal effects on inhibiting TF·FVIIa activity. Further deletion of the two N-terminal Glu residues led to the 13-residue peptide A-65, which also had similar inhibitory activity. However, sequential removal of the N-terminal Trp (A-378) and Glu (A-380) residues from A-65 resulted in ca.

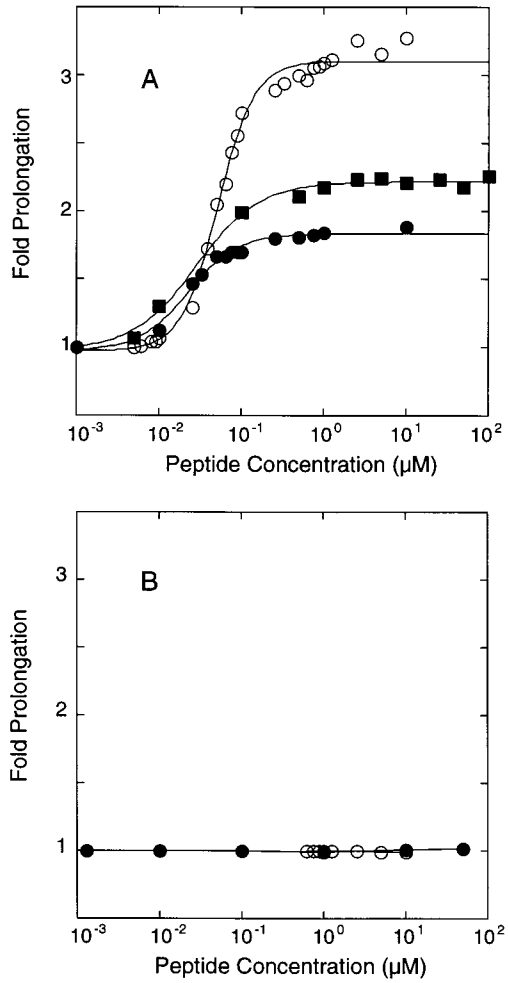


FIGURE 4: Effect of A-series peptides on the prothrombin time (PT) and activated partial thromboplastin time (APTT) in human plasma. The fold prolongation of the clotting times upon (A) initiation by Innovin (human relipidated TF and Ca^{2+}) in the PT assay or (B) initiation by Actin FS in the APTT assay are plotted versus the concentration of A-183 (●), A-100 (■), and A-100-Z (○). Uninhibited clotting times were 9.6 and 30.3 s for the PT and APTT, respectively.

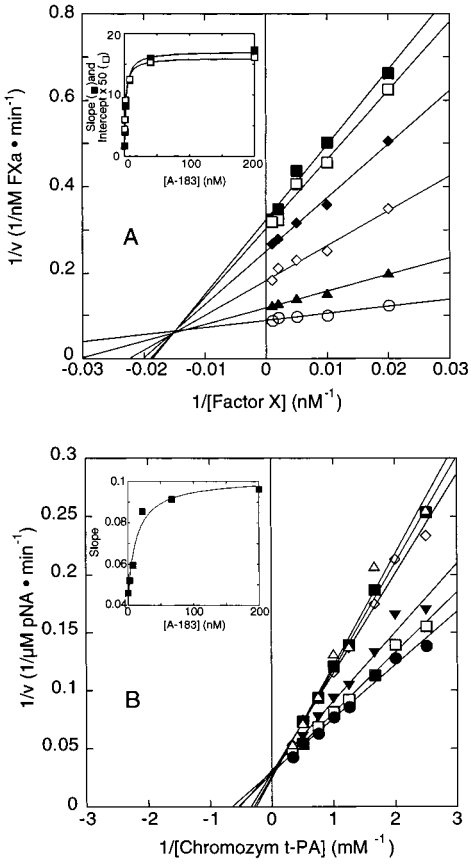


FIGURE 5: Lineweaver-Burk plots for A-183 inhibition of FX activation and amidolytic activity. (A) Inhibition of TF·FVIIa catalyzed activation of FX was monitored with various concentrations of A-183; 200 nM (■), 40 nM (□), 8 nM (◆), 1.6 nM (◇), 320 pM (▲), and uninhibited (○). (Inset) Plot of the slopes (■) and intercepts times 50 (□) of the reciprocal plot (of $1/v$ versus 1/[FX]) versus [A-183]. (B) Inhibition of TF·FVIIa amidolytic activity was monitored with various concentrations of A-183; 200 nM (△) 66.7 nM (■), 22.2 nM (◇), 7.4 nM (▼), 2.5 nM (□), and uninhibited (●). (Inset) Plot of the slopes (■) of the reciprocal plot (of $1/v$ versus 1/[Chromozym t-PA]) versus [A-183]. Lines for Lineweaver-Burk analysis for both plots represent data fit to a linear equation. Lines for both inset plots represent data fit to a hyperbolic equation.

200- and 9000-fold decreases in activity in the FX activation assay, respectively. Further deletion at the N-terminus resulted in peptides without any detectable activity (Table 2). Addition of the four C-terminal residues (A-153) present in the original consensus sequence did not restore activity. Finally, deletion of the C-terminal Arg residue (A-383) from A-65 caused a ca. 3000-fold decrease in activity for inhibition of FX activation while further removal of the Glu residue led to A-386, which was ca. 5000-fold less active than A-65 (Table 2).

Effects of A-183, A-100, and A-100-Z on Clotting Times in Normal Human Plasma. The effect of inhibiting TF·FVIIa by A-183, A-100, and A-100-Z in normal human plasma was examined using assays to determine the TF-dependent prothrombin time (PT) and the TF-independent activated partial thromboplastin time (APTT). All three peptides showed a dose dependent prolongation of the PT, consistent with their ability to inhibit TF·FVIIa activity (Figure 4A). Importantly, neither A-183 nor A-100-Z prolonged the clotting times in the APTT assay (Figure 4B) indicating their high degree of specificity for TF·FVIIa over serine proteases in the intrinsic arm of the coagulation pathway. At high concentrations of peptide, A-100-Z, A-100, and A-183 did not prolong clotting beyond 3.3-, 2.2-, and 1.8-fold, respectively. Interestingly, this plateau at saturating concentrations of peptide correlates with the maximal extent of inhibition of the rates of FX activation (Figure 3A).

Kinetic Analysis of A-183 Inhibition of TF·FVIIa Activity. While A-183 potently inhibited the activation of FX by TF·FVIIa, the observed incomplete inhibition, most notable in the hydrolysis of Chromozym t-PA, led us to investigate the mechanism of inhibition by kinetic analysis. A Lineweaver-Burk plot shows that A-183 acts as a mixed-type inhibitor of FX activation (Figure 5A), affecting both the maximal velocity of FX turnover (V_{max}) and the affinity (K_m) for FX. In the absence of any inhibitor, K_m and V_{max} values of 18.4 ± 2.4 nM and 11.0 ± 0.9 nM FXa min^{-1}, respectively, were determined by fitting the data to the Michaelis−Menten equation using nonlinear least squares analysis. The K_m value above and calculated k_{cat} value of 9.2 ± 0.8 s^{-1} are in reasonable agreement with those of others (*18*, *32*). In order to further characterize the inhibition, we analyzed the data using secondary plots from the Lineweaver-Burk analysis.

Secondary plots of both the slopes and intercepts versus A-183 concentration were hyperbolic (Figure 5A, inset), indicative of partial inhibition. Thus, we can classify A-183 as a partial (or hyperbolic) mixed-type inhibitor (also referred to as intersecting, slope-hyperbolic, intercept-hyperbolic, noncompetitive inhibitor) of FX activation (*33*).

Lineweaver-Burk analysis of the amidolytic activity with Chromozym t-PA as the substrate shows that the inhibition by A-183 is competitive in nature, reducing the substrate affinity (K_m) for TF·FVIIa without affecting the V_{max} (Figure 5B). A secondary plot of the slopes from the Lineweaver-Burk analysis versus A-183 concentration was also hyperbolic (Figure 5B, inset), again indicative of partial inhibition. Therefore, A-183 is a partial (or hyperbolic) competitive inhibitor (also referred to as simple intersecting hyperbolic competitive inhibitor) of the amidolytic activity of TF·FVIIa (*33*).

DISCUSSION

The A-series peptides described herein represent a novel class of peptides that specifically bind to the TF·FVIIa complex. These peptides share no sequence similarity to the "E-series" peptide E-76, another recently described exosite peptide inhibitor of FVIIa (*20*). As described in the following paper in this issue (*21*), the A-series peptides bind to a new exosite on FVIIa distinct from both the E-76 peptide binding exosite and the active site. Remarkably, although both the E-series and A-series peptides were selected from totally different naive peptide libraries and only for their ability to bind to TF·FVIIa, peptides from both of these classes potently and selectively inhibit the TF·FVIIa catalyzed activation of FX. Furthermore both peptides are potent anticoagulants, specifically prolonging the clotting time only in the TF-dependent PT assay. This is consistent with their lack of binding to other serine proteases in the intrinsic pathway, including thrombin, FXa, FIXa, FXIa, FXIIa, and plasma kallikrein (*21*).

It is striking that we have now selected two different classes of peptides solely on their ability to bind TF·FVIIa, where both classes found distinct exosites on the FVIIa protease domain and both affected the function of the enzyme (*21*). This demonstrates that peptides selected from naive peptide phage libraries are capable of, and perhaps biased towards, identifying functionally relevant sites on proteins. Support for this idea is derived from numerous phage-selected peptides that not only bind to their target, but block natural ligands from binding as well (*34*). This has been further explored by specifically looking for phage-derived peptides as enzyme inhibitors, where 13 of the 17 peptides tested were specific inhibitors of enzyme function (*35*). For these enzymes, peptides displayed kinetic behavior indicative of competitive, noncompetitive, or mixed-type inhibition.

The A-series peptide A-53, identified from a naive peptide library on polyvalent phage, was rapidly matured on monovalent phage using a "soft randomization" strategy. The fully matured peptide (A-183) resulted in an improvement in the inhibition of TF·FVIIa activity of greater than 1000-fold. This phage selection strategy was also employed with the E-series peptides, where the affinity of the initially selected peptide E-56 was improved 15000-fold to yield E-76, which had an IC_{50} of 1 nM for inhibition of FX activation (*20*).

A BLAST search of the Dayhoff protein database for proteins with sequences similar to peptide A-183 found no obvious human proteins containing both cysteines with high sequence similarity. However, we did find a 46% identity and 67% similarity to nematode anticoagulant proteins AcaNAP45 and AcaNAP47 (*36*). These proteins are related to other anticoagulant proteins from the hookworm *A. caninum* that inhibit TF·FVIIa in a FX dependent manner (*37*), suggesting that they may bind to FVIIa in a similar manner to A-183. However, the NMR structure of NAPc2 would suggest that the disulfide found in A-183 may not be present in these related proteins (*38*).

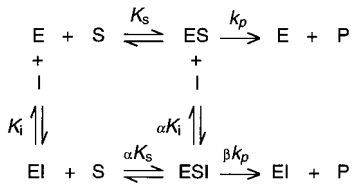
Fusion of the peptides to the Z domain of protein A allowed for their high expression and rapid purification using IgG Sepharose affinity chromatography and reversed-phase HPLC. Comparison of A-183 with A-100-Z shows that the Z domain did not affect binding of the peptides to FVIIa as measured in the A-183b peptide binding assay (Table 2) or potency in the inhibition assays for FX activation or amidolytic activity (Figure 3, Table 2). However, there was a significant difference in the extent by which the rate of FX activation was inhibited at saturating concentrations (Figure 3A). Removal of the C-terminal Z domain from A-100-Z by cleavage with trypsin to generate A-183 resulted in a decrease in the extent of inhibition of FX activation from 89 to 78%. This does not appear to be the case for inhibition of amidolytic activity where the Z domain had little effect (Figure 3B). Since A-183 binds at an exosite on FVIIa close to its active site (*21*), we speculate that while the Z domain might not result in adverse steric interactions with a small peptidyl substrate, it could adversely affect binding of the macromolecular substrate FX. This could alter the position of the FX scissile bond, resulting in a greater extent of inhibition.

The increase in the extent of inhibition of the rate of FX activation was also correlated with the clotting times in the PT assay. In this case, addition of residues to the carboxy-terminus of A-183, either GEG for A-100 or GEG-GGGSGG-Z for A-100-Z, resulted in a higher plateau for the fold prolongation at saturating concentrations of peptide (Figure 4A). Interestingly, all three forms of the matured sequence began to prolong the PT at similar concentrations, perhaps reflecting their similar affinity for TF·FVIIa. The correlation of the greater extent of inhibition of FX activation with a greater PT fold prolongation suggests that these are related. The possibility that saturating concentrations of inhibitor may result in a specific and predictable degree of anticoagulation is attractive from the perspective of being able to regulate the effects of an anticoagulant in vivo.

Assessment of the kinetic mechanism of inhibition of TF·FVIIa activity by A-183 shows that A-183 is a partial mixed-type inhibitor of FX activation, comprising both partial competitive and partial noncompetitive components, whereas it is partial competitive inhibitor of amidolytic activity with the small molecule substrate Chromozym t-PA (Figure 5). These modes of inhibition are best understood in the context of Scheme 1 (*33*).

In this scheme, α is the factor by which the K_s (or K_i) changes upon complexation with inhibitor (or substrate) and β refers to a factor relating to the catalytic efficiency of the inhibited complex. For inhibition of FX activation, α and β can be determined from the slope and intercept limits at high

Scheme 1



inhibitor concentration of the secondary plots (Figure 5A, inset), where the slope replot limit = $\alpha K_s / \beta V_{max}$ and the intercept limit = $1/\beta V_{max}$. From these plots and the kinetic constants we calculate that $\alpha = 2.6$ and $\beta = 0.27$, which are indicative of partial mixed-type inhibition. Further analysis of the data obtained by plotting $1/\Delta$ slope or $1/\Delta$ intercept from the reciprocal plot in Figure 5A versus 1/[A-183] results in straight lines from which we can extract values for the K_i of ca. 200 pM for A-183 inhibition of FX activation (*33*). For this calculation, we have assumed that $K_s = K_m$, since turnover to product is most likely the rate limiting step. In addition, we have assumed that E in Scheme 1 is TF·FVIIa and not FVIIa. Therefore, although unlikely, any potential effects on the association of TF with FVIIa due to A-183 binding have not been taken into account.

For inhibition of amidolytic activity, we observe partial competitive inhibition where $1 < \alpha < \infty$ and $\beta = 1$. Thus, the E·S and E·S·I complexes have the same catalytic efficiency. From the slope limit at high inhibitor concentration of the secondary plot (Figure 5B, inset), we calculate that $\alpha = 2.2$. However, we cannot calculate an accurate K_i value from a plot of $1/\Delta$ slope versus 1/[A-183] since this assay contained 10 nM FVIIa which was necessary to observe a sufficient signal; the value of 10 nM obtained by such a calculation simply represents a titration of FVIIa.

The fact that we see partial mixed-type (affecting both the K_m and V_{max}) or partial competitive inhibition (affecting only the K_m) for FX and Chromozym t-PA, respectively, is consistent with the data in Figure 3B where the inhibition of activity is incomplete at saturating concentrations of inhibitor. The observation that the E·I complex is catalytically competent is reflected by the fact that $\beta \neq 0$ for either substrate. The finding that $\beta = 1$ for amidolytic activity and $\beta = 0.27$ for FX activation shows that A-183 binding to the E·S complex results in a different catalytic efficiency for the two E·S·I complexes.

Even though A-183 is a partial competitive inhibitor with Chromozym t-PA as a substrate, the lack of complete inhibition of amidolytic activity suggests that A-183 does not bind to the active site. The same is true for A-100-Z. In the following paper (*21*), we describe the localization of the A-series peptide on the FVIIa protease domain and probe the relative importance of individual residues on the A-series peptides for their structure and interactions with FVIIa. In agreement with the inhibition data, A-183 binds to an exosite that is distinct from, but close to, the active site. This is also confirmed by a crystal structure of A-183 complexed with a version of zymogen FVII (*39*). Furthermore, the A-series peptide binding exosite does not overlap with the E-76 peptide binding exosite described previously (*20*).

Although we cannot present a detailed mechanism of inhibition, the structural and kinetic data imply that peptide binding to an exosite induces subtle changes near the active site, i.e., an allosteric effect, that affect the binding of both substrates in a manner such that catalysis is impaired. To explain the V_{max} effect with FX, we propose that A-183 adversely affects the macromolecular substrate FX, but not the smaller peptidyl substrate, such that only the scissile bond of FX is suboptimal for cleavage. This is potentially due to allosteric changes in subsite residues in FVIIa upon A-183 binding that disrupt FX interactions on the C-terminal side of the scissile bond, which are absent in Chromozym t-PA (*21*). Consistent with the increased extent of inhibition for A-100-Z, A-183 may also sterically interfere with FX binding. A mechanism involving dissociation of TF in the presence of A-183 is disfavored since peptide phage were selected against FVIIa in complex with immobilized TF, not FVIIa alone, and TF only modestly affects the affinity of A-183 for FVIIa (*21*). In agreement with this, preliminary calorimetry data suggest only a modest ca. 2-fold increase in the affinity of TF for FVIIa in the presence of A-183 (R. Kelley, personal communication). Finally, we cannot rule out the possibility that FX binds to TF·FVIIa in such a manner that, in the presence of A-183, the FVIIa active site is structurally different compared to that with Chromozym t-PA as the substrate.

The inhibition of TF·FVIIa activity that we observe for A-183 differs from that observed for active site inhibitors of this complex (*18*). Both *p*-aminobenzamidine and an alternate peptidyl substrate exhibited kinetics consistent with pure noncompetitive inhibition against FX and pure competitive inhibition with a small peptidyl substrate. The inhibition patterns with macromolecular and the small peptidyl substrate have been explained based on the fact that turnover for both of these would be prevented sterically; however, only binding of the macromolecular substrate FX still occurs owing to its extended interactions on exosites on the TF·FVIIa complex as well as phospholipid membranes. It is interesting to note that the anti-FVIIa Mab 12D10, which was mapped to a region of FVIIa (Arg 62, Asn 63, and Arg 84) that is relatively close to the A-183 binding exosite (*21*), also exhibited mixed-type of inhibition of FX activation (*40*).

In contrast to most other approaches to inhibiting TF·FVIIa activity, the A-series peptides offer the potential to modulate or attenuate rather than completely block its activity. Heparins and coumarins rely on proper dosing to achieve the desired degree of anticoagulation with minimal side effects. A therapeutic that acts in the manner similar to the A-series peptides offers the potential to selectively attenuate the TF·FVIIa activity at saturating concentrations. This strategy offers the possibility to develop a molecule having the desired degree of inhibition and anticoagulation, potentially with minimal side effects, but now without the risk of overdosing, thus dramatically extending the therapeutic window.

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