A Peptide Derived from a Tissue Factor Loop Region Functions as a Tissue Factor−Factor VIIa Antagonist

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ABSTRACT: Tissue factor (TF) is a transmembrane protein that functions in the initiation of blood coagulation *in vivo*. At sites of vascular injury, TF serves as a cell-surface receptor for the serine protease factor VIIa (FVIIa), forming an enzyme−cofactor complex and enhancing the catalytic activity of FVIIa. Tissue factor, along with the receptors for α- and γ-interferons, is a member of the class 2 cytokine receptor superfamily. Crystallographic analysis demonstrated that the extracellular domain of TF consists of two immunoglobulin-like domains joined by a linker region. Each domain is comprised of two antiparallel β-sheets containing seven conserved β-strands separated by more variable loop regions. Extensive mutagenesis has been performed in order to map the FVIIa binding site on TF. Results indicated that the discontinuous binding site for FVIIa lies at the domain−domain interface and includes residues from extended loops and β-strands within both the N- and C-terminal domains. Our previous study provided evidence that three consecutive residues (D44, W45, K46) within the TF loop region between β-strands C and C′ of the N-terminal domain were important for interactions with FVIIa. We have presently extended our alanine-scanning mutagenesis to include the residues within the flanking β-strands. Thirteen sTF mutants were screened for their ability to enhance FVIIa activity. Three residues within strand C (Y34, Q37, I38) and two residues within C′ (K48, Y51) were shown to be important for TF cofactor function. A series of peptides based on this TF β-strand C−loop region−β-strand C′ motif (residues 33−53) were synthesized and analyzed for their ability to antagonize the TF−FVIIa interaction. Incorporation of the C−C′ loop region into a cyclic peptide template, followed by systematic optimization of the cyclic ring size, has resulted in the generation of a cyclic undecapeptide (11-mer) that functions as a specific inhibitor of TF-dependent clotting. These data clearly indicate that the C−C′ loop and adjacent β-strands in TF play a role in FVIIa binding.

Tissue factor (TF) is an integral membrane glycoprotein that functions as a cofactor for the serine protease factor VIIa (FVIIa). TF is not normally expressed on cells in the vasculature, but is exposed following vascular damage and forms a high-affinity, calcium-dependent complex with FVIIa, thereby increasing the catalytic efficiency of FVIIa several thousand-fold toward peptide substrates as well as its physiological substrates factor IX (Østerud & Rapaport, 1977) and factor X (Nemerson, 1966). The activation of factor IX and factor X leads to the generation of thrombin and the eventual formation of a fibrin clot [reviewed in Bach (1988), Nemerson (1988), and Edgington et al. (1991)]. In a revised model of coagulation (Gailani & Broze, 1991), it was suggested that the thrombin produced through the initial action of the TF−FVIIa complex activates the intrinsic pathway of coagulation by feedback activation of factor XI. This has led to the proposal that TF initiates blood coagulation *in vivo*. Because TF plays a central role in the initiation of the clotting cascade, it has also been implicated in the pathological process of a number of disease states. *In situ* hybridization and immunohistochemistry have localized both TF mRNA and protein to atherosclerotic plaques (Wilcox et al., 1989). Animal experiments using neutralizing antibodies indicate that TF may contribute to the pathogenesis of thrombosis and septic shock (Warr et al., 1990; Taylor et al., 1991; Pawashe et al., 1994).

Tissue factor is comprised of 263 amino acids with a 219 amino acid extracellular domain, a 23 amino acid transmembrane domain, and a 21 amino acid cytoplasmic tail. The extracellular domain contains four cysteine residues, which form two disulfide bonds (Bach et al., 1988), as well as three sites for N-linked glycosylation. Expression of TF in bacteria demonstrated that glycosylation is not required for activity (Paborsky et al., 1989). A soluble form of TF (sTF) containing only the extracellular domain has been generated in a number of expression systems, and the purified truncated recombinant protein appears to be fully functional although the affinity for FVIIa is generally reduced by an order of magnitude (Ruf et al., 1991a,b; Shigematsu et al., 1992; Waxman et al., 1992).

Based on sequence alignments, Bazan (1990a,b) predicted that TF was a member of the cytokine receptor superfamily. Included in this superfamily are receptors for a broad group of growth factors, hematopoietic factors, interleukins, and interferons. TF belongs to the class 2 family, which is distinguished from the class 1 family by the pattern of disulfide bonding. The crystal structure of the extracellular domain of TF was recently solved (Harlos et al., 1994; Muller et al., 1994) and confirmed Bazan's predictions. These analyses demonstrated that the extracellular domain of TF consists of two immunoglobulin-like domains joined by a

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linker region. Each domain folds to form two antiparallel β-sheets containing seven conserved β-strands (A, B, C, C′, D, E, F) separated by more variable loop regions. The two domains both adopt the same topology as the growth hormone receptor, the immunoglobulin superfamily (IgSF) type C2 or fibronectin type III topology.

Extensive mutagenesis (Ruf et al., 1992a,b, 1994; Gibbs et al., 1994) has been performed to map the FVIIa binding site on the extracellular domain of TF. Ruf et al. (1994) identified five alanine-replacement mutations that caused a greater than 7-fold increase in the apparent dissociation constant for FVIIa, implying that the residues substituted contribute to FVIIa binding (K20, I22, D58, R135, F140). Further analysis using multiple mutations in a single TF molecule suggested that these five residues comprise three entirely independent contact sites on TF for FVIIa (Schullek et al., 1994).

Using the growth hormone receptor crystal structure as a model for TF (de Vos et al., 1992), we conducted a homologous loop swap mutagenesis study in which TF loop regions were systematically replaced with loops from the γ-interferon receptor, which is the closest relative to TF in the cytokine receptor superfamily. Six loop regions that were required for interactions with FVIIa were detected. Alanine-scanning mutagenesis of the 68 residues within these loop regions independently identified K20, R135, and F140 along with 5 additional residues, D44, W45, K46, Q110, and V207, as being important for FVIIa binding (Gibbs et al., 1994).

Based on the crystal structure of sTF (Harlos et al., 1994), K20 is located at the junction between a loop and strand B in the N-terminal domain, and I22 is located in the center of strand B. The three consecutive residues D44, W45, and K46 are situated in the loop region that connects the N-terminal domain β-strands C and C′. D58 is situated near the end of strand E. Q110 is positioned in the linker region that connects the N- and C-terminal domains, and R135 and F140 are located in the C-terminal domain on an extended finger between β-strands B and C. V207 is located on strand G of the C-terminal domain. Therefore, the binding site for FVIIa appears to be located in the domain−domain interface and to involve residues on both β-strands and extended loops.

In this study, we extend our alanine-scanning mutagenesis beyond the loop that contains the D44W45K46 sequence to include residues within the adjacent C and C′ β-strands and demonstrate that several residues within both of these β-strands appear to be important for FVIIa interactions. In addition, we provide evidence that peptides derived from this β-strand−loop region−β-strand motif inhibit FVIIa binding. Optimization of these peptides by cyclization and truncation resulted in the synthesis of a cyclic undecapeptide that functions as an antagonist in a TF-dependent plasma clotting assay.

EXPERIMENTAL PROCEDURES

Materials. Factor VIIa was from Haematologic Technologies and BiosPacific; the chromogenic substrate H-D-isoleucyl-L-propyl-L-arginine-*p*-nitroaniline dihydrochloride (S-2288) was from Chromogenix. The goat anti-TF antibody, the conformationally-dependent monoclonal antibody, and the full-length recombinant tissue factor were from American Diagnostica, Inc.; anti-FLAG M1 monoclonal antibody and anti-FLAG M1 affinity gel were from Kodak; FVII-deficient plasma was from George King Bio-Medical (Overland Park, KS); normal human pooled plasma was from Sigma. The construction of vectors for sTF expression and the expression and purification of *E. coli*-derived sTF as well as the ELISA and monoclonal antibody binding assay were described in detail in our previous publication (Gibbs et al., 1994).

TF Cofactor Functional Assay. Soluble wild-type TF or sTF mutants (0−600 nM) were incubated for 5 min at 37 °C with 2.5 nM FVIIa and 5 mM $CaCl_2$ in 50 mM Tris-HCl, 150 mM NaCl, and 0.1% BSA, pH 7.5. The chromogenic substrate (S-2288) was added and incubated for an additional 10 min at 37 °C. The reaction was stopped by adding acetic acid to 50%, and the amount of *p*-nitroaniline released was quantitated by measuring the absorbance at 405 nm using a Molecular Devices plate reader. The K_D for the interaction between sTF and FVIIa was determined by curve-fitting to the Michaelis−Menten equation using nonlinear regression analysis constrained by estimates of parameters determined from Lineweaver−Burk plots.

To determine whether the synthetic TF peptides inhibited cofactor function, FVIIa (2.5 nM) was preincubated for 5 min at 37 °C in the presence of increasing concentration of the peptides in 150 mM Tris-HCl, 150 mM NaCl, and 0.1% BSA, pH 7.5, containing 5 mM $CaCl_2$. Wild-type sTF (10 nM) was added and incubated for 5 min prior to the addition of S-2288. The absorbance at 405 nm was measured at several time points for each concentration of peptide to determine the rate of cleavage of the chromogenic substrate. The IC_{50} value for the inhibition of cofactor function is the peptide concentration necessary to inhibit 50% of FVIIa activity.

Solid-Phase Factor VIIa Binding Assay. A 96-well ELISA plate (Corning) was coated overnight at 4 °C with 20 μg/mL anti-FLAG M1 antibody in 50 mM carbonate buffer, pH 9.3. Blocking of nonspecific sites was achieved by incubating the wells with 1% BSA in 50 mM Tris-HCl, 150 mM NaCl, 1 mM $CaCl_2$, and 0.02% Tween-20, pH 7.5, for at least 1 h at 4 °C. The plate was washed with wash buffer (50 mM Tris HCl, 150 mM NaCl, 1 mM $CaCl_2$, 0.02% Tween-20, and 0.1% BSA, pH 7.5). The sTF (3.3 nM) was added to the plate and incubated for 1 h at 4 °C, and then the plate was washed. Biotinylated FVIIa (50 nM; biotinylated using a Molecular Probes kit according to manufacturer's instructions) was preincubated for 1 h at 4 °C with increasing concentrations of peptide in 150 mM Tris-HCl, 150 mM NaCl, and 0.1% BSA, pH 7.5, containing 5 mM $CaCl_2$. The preincubation mixture of peptide and FVIIa was then added to the plate and incubated for an additional 1 h at 4 °C. The plate was washed again and incubated with avidin−horseradish peroxidase (2.5 μg/mL) for 1 h at 4 °C. The plate was washed prior to the addition of the peroxidase substrate, ABTS. The absorbance at 405 nm was measured using a Molecular Devices plate reader. Nonspecific binding was measured by determining the binding of biotinylated FVIIa to M1-coated and BSA-blocked wells and was subtracted from each data point. The IC_{50} value for inhibition of FVIIa binding is the peptide concentration necessary to inhibit 50% of the maximal FVIIa binding.

TF-Dependent Clotting Assay. The clotting assay was performed with full-length recombinant tissue factor (American Diagnostica, Inc.) that was reconstituted into mixed phosphatidylcholine and phosphatidylserine (70:30 w/w) vesicles by incubating the TF at 37 °C for 30 min in 50 mM Tris-HCl, 150 mM NaCl, and 0.1% BSA, pH 7.5, containing

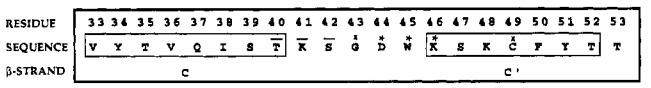

FIGURE 1: Numbering of amino acid residues of TF β-strands C and C′ in the N-terminal domain and loop region connecting the strands. The β-strands are enclosed in boxes. The loop region and β-strands are defined from the crystal structure of soluble TF (Harlos et al., 1994). The single-letter code for amino acids is used; a dash indicates residues that are not important for interaction with FVIIa, an asterisk indicates residues that play a role in FVIIa binding (Gibbs et al., 1994), and X denotes residues that were not substituted with alanine.

2.5 mM $CdCl_2$. Increasing concentrations of the cyclic TF undecapeptide were preincubated for 1 min at 37 °C in normal human pooled plasma. Relipidated TF (4.1 nM) was added to the plasma along with 15 mM $CaCl_2$, and the time to clot was recorded using a manual fibrometer. Clotting times were converted to FVIIa concentrations using a standard curve that was generated in FVII-deficient human plasma. Factor VIIa (0−40 nM) was added to FVII-deficient plasma, and the time to clot was recorded using a manual fibrometer. The log−log plot of FVIIa concentration versus clotting time generated a straight line that was used to convert clotting times to FVIIa concentrations. The IC_{50} value for the inhibition of clotting activity is the peptide concentration necessary to inhibit 50% of the FVIIa activity in normal human plasma.

Peptide Synthesis. Peptides were synthesized by Chiron Mimotopes Peptide Systems using either *tert*-butyloxycarbonyl (*t*BOC) or fluorenylmethyloxycarbonyl (FMOC) chemistry. When synthesis was complete, the peptides were deprotected and cleaved from the resin with hydrogen fluoride (for *tBOC* peptides) or trifluoroacetic acid (for FMOC). The peptides were cyclized by nonspecific air oxidation at pH 8−8.5 in dilute aqueous solutions to form intramolecular disulfide bridges. The peptides were purified by reversed-phase HPLC. The purity of each peptide was assessed by analytical HPLC, and the molecular weight was confirmed by mass spectrometry using a Biolon 20 Mass Analyzer. The purified cyclic peptides were each checked for the presence of free thiols using Ellman's reagent. In addition, the mass spectrometry results confirmed the existence of an internal disulfide bond, the absence of hydrolysis, and the absence of oligomeric forms of the peptide.

RESULTS

Alanine-Scanning Mutagenesis of the TF β-Strands Adjacent to the C−C′ Loop. In our previous study (Gibbs et al., 1994), we determined that the seven amino acid loop region connecting β-strand C to C′ in the N-terminal domain played a role in FVIIa binding. Alanine-scanning mutagenesis was then used to individually identify three amino acids (D44, W45, K46) within the loop region that were important for FVIIa binding. Three other residues within that loop region (T40, K41, S42) did not appear to play a role in FVIIa interactions, and the glycine at position 43 was not substituted. To determine whether amino acid residues in the adjacent β-strands, C and C′, are involved in FVIIa binding, each of the 13 amino acid residues within these 2 TF β-strands was individually substituted with alanine (Figure 1).

A total of 13 new sTF alanine-scanning mutants and wild-type sTF were expressed in *E. coli* as fusion proteins with the FLAG affinity motif (Hopp et al., 1988) at the amino terminal of the coding region of TF (Gibbs et al., 1994). *E. coli* cell extracts were prepared, and the amount of sTF in each extract was quantitated by sandwich ELISA utilizing the M1 anti-FLAG antibody to capture the sTF and a goat anti-TF polyclonal antibody for detection. Extracts containing the alanine-scanning mutants were screened for cofactor function at a concentration of 15 nM. The ability of the mutants to enhance FVIIa activity toward the chromogenic peptide substrate S-2288 was compared to wild-type sTF. Five of these mutants (Y34A, Q37A, I38A, K48A, Y51A) had less than 40% of the activity of wild-type sTF (Figure 2). Three of these residues are located in β-strand C (Y34, Q37, I38), and the other two residues are situated in β-strand C′ (K48, Y51).

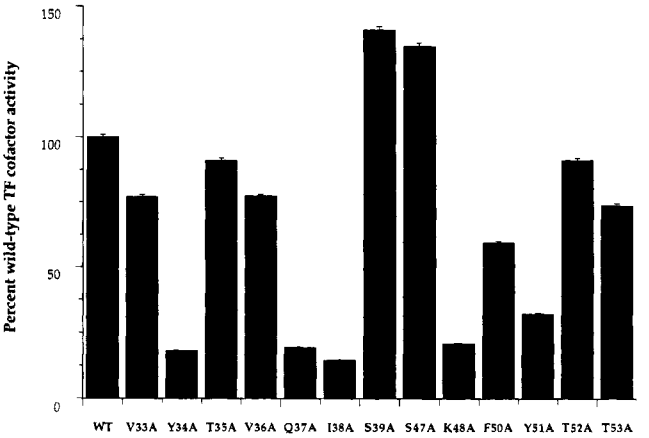

FIGURE 2: Cofactor activity of sTF alanine-scanning mutants. Thirteen residues in β-strands C and C′ of the N-terminal domain were replaced with alanine. Soluble TF mutants, at a concentration of 15 nM, were assayed for cofactor function as described under Experimental Procedures. Activity is the mean of two separate determinations performed in triplicate and expressed as a percentage of wild-type sTF cofactor activity.

Table 1: Affinity of β-Strand C and C′ sTF Mutants for Conformationally-Dependent Monoclonal Antibody and FVIIa[a]

sTF mutant	K_D for conformationally-dependent monoclonal antibody (pM)	maximal cofactor activity at saturating [sTF] (% wild-type)	K_D for FVIIa (nM)
wild-type	121.7	100	9.9
Y34A	30.8	89	59.3
Q37A	100.1	39	220.7
I38A	80.7	98	72.4
K48A	118.0	32	190.8
Y51A	43.0	90	43.4

[a] The K_D for conformationally-dependent TF monoclonal antibody was determined from the concentration dependence of biotinylated antibody binding. The affinity for FVIIa was assessed in the cofactor activity assay, and the maximal cofactor activity was determined at saturating TF concentrations and is expressed as a percentage of wild-type sTF.

These five alanine-scanning sTF mutants were purified from *E. coli* cell extracts utilizing M1 immunoaffinity chromatography as previously described (Gibbs et al., 1994). The purity of these mutants, assessed by SDS−PAGE and silver staining, was at least 90% (data not shown). The TF concentration was determined by ELISA. To evaluate the conformational integrity of these mutants, a modified ELISA was performed using a conformationally-sensitive TF monoclonal antibody (Table 1). In this assay, the K_D for native sTF was 121.7 pM, whereas the K_D for denatured TF was 1664.2 pM. All five sTF mutants had a K_D close to that of

Table 2: Inhibition of FVIIa Binding to sTF by TF Peptides[a]

	Peptide Sequence	Number of Residues	IC_{50} (mM)
Series 1	VYTVQISTKSGDWKSKAFYTT	21	2.6
	YTVQISTKSGDWKSKAFYT	19	ND
	TVQISTKSGDWKSKAFY	17	4.0
	VQISTKSGDWKSKAF	15	10.5
	QISTKSGDWKSKA	13	29.5
	TKSGDWK	7	>50
Series 2	CYTVQISTKSGDWKSKCFYTT	21	>50
	VCTVQISTKSGDWKSKCFYTT	21	>50
	VYCVQISTKSGDWKSKCFYTT	21	7.2
	VYTCQISTKSGDWKSKCFYTT	21	3.5
	VYTVCISTKSGDWKSKCFYTT	21	>50
	VYTVQCSTKSGDWKSKCFYTT	21	2.6
	VYTVQICTKSGDWKSKCFYTT	21	**0.65**
	VYTVQISCKSGDWKSKCFYTT	21	13.8

[a] The peptides were evaluated in the solid-phase binding assay utilizing biotinylated FVIIa. Peptides in series 2 were cyclized; underlined cysteine residues denote participation in disulfide bond formation. The IC_{50} for the 19-mer was not determined because the peptide was insoluble at concentrations tested. Values for IC_{50} were determined by curve fitting using SigmaPlot (Jandel Scientific).

Table 3: Inhibition of FVIIa Binding and TF Cofactor Function by TF Peptides[a]

Peptide sequence	FVIIa binding IC_{50} (mM)	Cofactor activity IC_{50} (mM)
VYTVQISTKSGDWKSKAFYTT	2.6	2.9
VYTVQICTKSGDWKSKCFYTT	0.65	0.61
CTKSGDWKSKC	1.1	ND

[a] The IC_{50} values for FVIIa binding were determined as described in Table 2. The peptides were also evaluated in the cofactor activity assay. Underlined cysteine residues denote participation in disulfide bond formation. Values for IC_{50} were determined by curve fitting using SigmaPlot (Jandel Scientific).

native sTF, 30.8−118.0 pM, which suggested that the conformational integrity of these mutants was intact.

The affinity of these mutants for FVIIa was determined using the cofactor functional assay (Table 1). The K_D for these mutants increased between 4.4- and 22.3-fold compared to wild-type sTF, which has a K_D for FVIIa of 9.9 nM. These mutants fell into two groups. At saturating TF concentrations, Y34A, I38A, and Y51A were able to enhance FVIIa activity to nearly the same extent as wild-type TF, whereas Q37A and K48A were not capable of fully stimulating FVIIa activity.

Analysis of Linear and Cyclic Peptides Derived from the TF β-Strand−Loop Region−β-Strand. To determine whether peptides based on the TF β-strand C−loop region−β-strand C′ (residues 33−53, Figure 1) could inhibit the TF−FVIIa interaction, a series of TF peptides were synthesized (Table 2). The first candidate synthesized was a 21 amino acid linear peptide corresponding to TF residues 33−53. The cysteine residue (equivalent to TF residue 49) was substituted with an alanine in the peptide to prevent cross-linking by disulfide bond formation. This peptide was assayed for its ability to inhibit the binding of biotinylated FVIIa to immobilized TF in a solid-phase microtiter assay. This 21-mer inhibited FVIIa binding in a dose-dependent manner with half-maximal inhibition occurring at 2.6 mM. The inhibitory effect of this peptide was also investigated using the cofactor functional assay (Table 3). Similar results were obtained with the activity assay which generated an estimated IC_{50} value of 2.9 mM.

To ascertain the minimal functional peptide, four additional peptides were synthesized (series 1) that had amino acids progressively deleted from both the N- and C-termini to yield linear peptides of 19, 17, 15, 13, and 7 amino acids each. Deletion of a valine (equivalent to TF residue 33) and a threonine (equivalent to TF residue 53) produced a peptide that was not sufficiently soluble to test in the assays; therefore, the IC_{50} for the 19-mer was not determined. The 17-, 15-, and 13-mers showed a 1.5−11-fold increase in IC_{50} as compared to the 21-mer. The heptapeptide, TKSGDWK, with amino acids from the loop region only (residues 40−46) had no inhibitory effect. The successive loss of inhibitory activity observed with the progressive deletion of Y34 from the 17-mer, Y51 from the 15-mer, and Q37, I38, and K48 from the 7-mer is consistent with our mutagenesis data demonstrating that these residues are important for interactions with FVIIa in the context of the intact TF molecule.

In a second series of peptides, the 21-mer was systematically cyclized in an attempt to optimize the conformational display of the C−C′ loop region as well as to determine the contributions of the exocyclic residues. To cyclize these peptides, the alanine residue that had been substituted in series 1 was replaced with the native cysteine residue. A second cysteine residue was positioned at the N-terminus and then was progressively moved one position closer to the C-terminus, generating a series of eight disulfide-bridged 21-mers. The IC_{50} for each of these peptides was determined in the biotinylated FVIIa binding assay (Table 2). Most of the cyclized peptides were less inhibitory than the linear 21-mer. However, one peptide displayed a 4-fold enhancement of potency compared to the linear peptide. This peptide, in which the serine corresponding to the TF residue at position 39 was replaced with a cysteine, had an estimated IC_{50} of 650 μM. This 21-mer with an 11-membered disulfide-bridged ring was also effective at inhibiting TF function as assessed in the cofactor activity assay and yielded an equivalent IC_{50} value of 610 μM (Table 3).

An additional peptide was synthesized based on the sequence of the most potent cyclic 21-mer. The 10 exocyclic amino acids flanking both cysteine residues in the cyclic 21-mer were removed, and a cyclic undecapeptide was synthesized (CTKSGDWKSKC) with a disulfide bridge connecting the carboxyl- and amino-terminal cysteine residues. To increase its solubility, this peptide was diluted in 10% DMSO. When evaluated for its ability to antagonize the TF−FVIIa interaction in the biotinylated FVIIa binding assay, the cyclic undecapeptide had an IC_{50} value of 1.08 mM (Table 3), which represents a slight decrease in potency compared to the disulfide-bridged cyclic 21-mer. The IC_{50} of this peptide was difficult to determine using the cofactor functional assay due to its decreased solubility, which interfered with the absorbance at 405 nm. However, the ability of this peptide to function as an antagonist was evaluated in a TF-dependent plasma clotting assay (Figure 3). This assay utilizes human plasma and full-length TF that has been reconstituted into phosphatidylcholine and phosphatidylserine vesicles and more closely resembles events thought to occur *in vivo*. Clotting times were converted to FVIIa concentrations based on a standard curve generated by supplementing FVII-deficient plasma with FVIIa. Tissue factor-dependent clotting was prolonged in a dose-dependent manner by the conformationally-restricted 11-mer with an IC_{50} value of 1.6 mM.

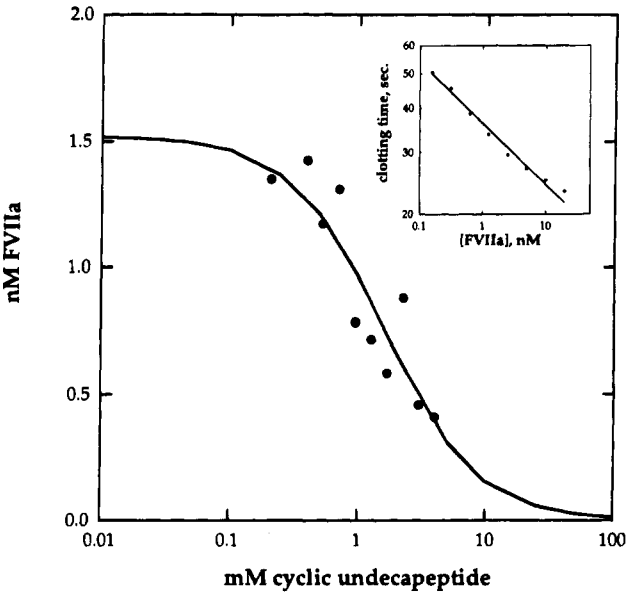

FIGURE 3: Inhibition of TF-dependent clotting activity by cyclic 11-mer TF peptide. The inhibitory effect of TF peptide CTKSGDWKSKC was assessed in a clotting assay as described under Experimental Procedures. The peptide was solubilized in 10% DMSO, and the final concentration of DMSO in the assay was 5%. This concentration of DMSO had no effect on clotting activity. Insert: a standard curve was generated with FVII-deficient human plasma and was used to convert clotting times to FVIIa concentrations. The IC_{50} value was determined by curve fitting to the data using SigmaPlot (Jandel Scientific) and represents the peptide concentration necessary to inhibit 50% of the FVIIa activity in a TF-dependent clotting assay.

DISCUSSION

Our previous mutagenesis study demonstrated that the C−C′ loop region in the N-terminal domain of TF was important for interactions with the ligand, FVIIa. Individually substituting the residues within this loop with alanine identified three consecutive residues (D44, W45, K46) that play a role in the formation of the cofactor−enzyme complex. In the current study, we report that five additional residues within the β-strands adjacent to the C−C′ loop region may also be important for FVIIa binding. Three residues (Y34, Q37, I38) are located in the C β-strand, and two residues (K48, Y51) are located in the C′ β-strand. These residues appear to fall into the same two groups that were observed for the previous set of alanine-scanning mutants. Three mutants, Y34A, I38A, and Y51A, display a modest decrease in their affinity for FVIIa while maintaining their ability to fully enhance FVIIa activity at saturation. The residues substituted in these mutants appear to be involved in simple FVIIa binding interactions. The other two mutants, Q37A and K48A, have a greater than 19-fold increase in their estimated K_D value for FVIIa and are not capable of maximally enhancing the activity of FVIIa. The residues replaced in these mutants may participate in inducing the allosteric change in the enzyme that has been postulated to be responsible for the increased catalytic function of the enzyme−cofactor complex (Nemerson & Gentry, 1986).

Our initial mutagenesis study involved only residues within TF loop regions because they tend to be exposed on the surface of proteins and would therefore be likely to make important contributions to ligand binding (Kuntz, 1972; Rose et al., 1985). The identification of residues within TF β-strands is consistent with the mutagenesis results of Ruf et al. (1994) which demonstrated that residues within both loop regions and β-strands can contribute to FVIIa binding. However, the residues within the C−C′ loop region and residues within the adjacent β-strands have not previously been shown to play a role in FVIIa binding (Ruf et al., 1994; Schullek et al., 1994). Four (Y34, Q37, I38, K48) of the five residues identified in this study are conserved in human, bovine, rabbit, rat, and mouse TF sequences. The tyrosine at position 51 is conserved in human and bovine TF but is replaced with serine in mouse TF, leucine in rabbit TF, and glycine in rat TF. Although the global conformation of the sTF mutants in which these residues were substituted with alanine appeared to be preserved as assessed by the binding of a conformationally-dependent TF monoclonal antibody, the observed effects of substituting these residues may result from a localized structural perturbation rather than the disruption of a direct interaction with FVIIa. This is especially possible for the hydrophobic β-strand residues Y34, I38, and Y51 which may be buried in the molecule and contribute to β-sheet packing.

Rehemtulla (1992) identified a tripeptide motif, WKS, that occurs 3 times within the extracellular domain of TF. Substitution of the second repeat, W45, K46, and S47, with the residues RKG resulted in a TF mutant that had a specific activity comparable to wild-type TF, suggesting that the motif itself was not required for function. The substitution of tryptophan for arginine which has significant hydrophobic character rather than for alanine, as was the substitution in our study, may account for this difference. In addition, the sequence W45K46S47 was identified as being part of the epitope of an antibody that blocks FVIIa binding to TF, implying that the FVIIa binding site is in close proximity to this sequence.

Additional evidence that the C−C′ loop region and the adjacent β-strands in the N-terminal immunoglobulin-like domain of TF are important for FVIIa binding comes from the demonstration that synthetic peptides derived from this sequence (residues 33−53) can function as antagonists of this interaction and inhibit TF-dependent clotting in plasma. The linear 21-mer that was used as the peptide scaffold on which our functional analysis was based inhibited the binding of FVIIa to immobilized TF with an estimated IC_{50} value of 2.6 mM. Removal of the tyrosine (equivalent to TF residue 34) resulted in a slight increase in the IC_{50} value which is consistent with the modest decrease in affinity for FVIIa observed for the Y34A mutant. Deletion of the tyrosine at the C-terminal end of the peptide (equivalent to TF residue 51) resulted in a 2.5-fold increase in the IC_{50} value which is also in agreement with our mutagenesis results demonstrating that Y51 may play a role in FVIIa interactions.

Because the linear 21-mer may occupy many conformational states that vary in their ability to associate with FVIIa, we attempted to enhance the potency of the 21-mer by cyclization to constrain its conformation. The 21 amino acid scaffold (residues 33−53) contained a cysteine residue in the native protein (TF residue 49), and therefore all the cyclic peptides were synthesized with a cysteine at this position. Optimizing the cyclization of this peptide by systematically contracting the size of the disulfide-bridged ring while retaining the exocyclic residues resulted in the generation of a peptide with enhanced inhibitory activity. This peptide contained an 11-membered ring flanked by 6 amino acids at the N-terminus and 4 amino acids at the C-terminus and displayed a 4-fold increase in potency. In addition to optimizing the position of the disulfide bond, the evaluation

of this series of cyclic peptides also provided some structure–function information. The results from the cysteine-scanning analysis of this peptide series were concordant with our mutagenesis data. A substantial increase in the IC_{50} value was observed for the peptide in which the glutamine (equivalent to TF residue 37) was replaced with a cysteine. The alanine substitution of Q37 in TF caused a 22-fold decrease in its affinity for FVIIa.

Although the concentration of peptide required to inhibit the TF–FVIIa interaction was relatively high, the evaluation of both the deletion series of peptides (Table 2, series 1) and the cysteine-scanning analysis (Table 2, series 2) established a structure–activity relationship. While several peptides in series 2 had no inhibitory effect (IC_{50} value was >50 mM), a single amino acid change resulted in more than 2 orders of magnitude increase in potency (IC_{50} = 0.65 mM) for one of the peptides. These results demonstrated that the inhibitory activity of these synthetic peptides was sequence-specific and not due to a nonspecific binding effect.

Unlike the RGD-containing cyclic peptides which are at least an order of magnitude more potent inhibitors of the platelet receptor, glycoprotein IIb–IIIa, than the linear RGD-containing peptides (Pierschbacher & Ruoslahti, 1987; Samanen et al., 1991; Barker et al., 1992), the DWK-containing peptides derived from the C–C′ loop region of TF exhibited only a 4-fold increase in potency following cyclization. However, this may be due to not having achieved the optimal conformation of this peptide. Further optimization of the position of the disulfide-bridged ring would require the synthesis of a much larger set of cyclic peptides in which the positions of both cysteines within the 21 amino acid scaffold are varied and all possible cyclic analogs are evaluated.

The removal of the residues flanking the cysteine residues in the 21-mer resulted in an undecapeptide with a slight decrease in potency. This may be due to the decreased solubility observed for this peptide. However, removal of those 10 amino acid residues does represent a significant decrease in the molecular weight of the peptide without substantial loss of activity which is a desirable outcome in the development small molecule therapeutics. Thus, although this peptide lacks the potency for a therapeutic candidate, molecular modeling of this peptide based on the TF crystal structure should aid in the design of either more potent peptides or a nonpeptide mimetic that could effectively inhibit TF-mediated coagulation and have potential applications in a number of vascular disease states.

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REFERENCES

Bach, R. R. (1988) *CRC Crit. Rev. Biochem. 23*, 339–368.
Bach, R. R., Konigsberg, W. H., & Nemerson, Y. (1988) *Biochemistry 27*, 4227–4231.
Barker, P. L., Bullens, S., Bunting, S., Burdick, D. J., Chan, K. S., Deisher, T., Eigenbrot, C., Gadek, T. R., Gantzos, R., Lipari, M. T., Muir, C. D., Napier, M. A., Pitti, R. M., Padua, A., Quan, C., Stanley, M., Struble, M., Tom, J. Y. K., & Burnier, J. P. (1992) *J. Med. Chem. 35*, 2040–2048.
Bazan, J. F. (1990a) *Proc. Natl. Acad. Sci. U.S.A. 87*, 6934–6938.
Bazan, J. F. (1990b) *Cell 61*, 753–754.
de Vos, A. M., Ultsch, M., & Kossiakoff, A. A. (1992) *Science 255*, 306–312.
Edgington, T. S., Mackman, N., Brand, K., & Ruf, W. (1991) *Thromb. Haemostasis 66*, 67–79.
Gailani, D., & Broze, G. J., Jr. (1991) *Science 253*, 909–912.
Gibbs, C. S., McCurdy, S. N., Leung, L. L. K., & Paborsky, L. R. (1994) *Biochemistry 33*, 14003–14010.
Harlos, K., Martin, D. M. A., O'Brien, D. P., Jones, E. Y., Stuart, D. I., Pollkarpov, I., Miller, A., Tuddenham, E. G. D., & Boys, C. W. G. (1994) *Nature 370*, 662–666.
Hopp, T. P., Prickett, K. S., Price, V. L., Libby, R. T., March, C. J., Cerretti, D. P., Urdal, D. L., & Conlon, P. J. (1988) *Biol Technology 6*, 1204–1210.
Kuntz, I. K. (1972) *J. Am. Chem. Soc. 94*, 4009.
Muller, Y. A., Ultsch, M. H., Kelley, R. F., & de Vos, A. M. (1994) *Biochemistry 33*, 10864–10870.
Nemerson, Y. (1966) *Biochemistry 5*, 601–608.
Nemerson, Y. (1988) *Blood 71*, 1–8.
Nemerson, Y., & Gentry, R. (1986) *Biochemistry 25*, 4020–4033.
Østerud, B., & Rapaport, S. I. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5260–5264.
Paborsky, L. R., Tate, K. M., Harris, R. J., Yansura, D. G., Band, L., McCray, G., Gorman, C. M., O'Brien, D. P., Chang, J. Y., Swartz, J. R., Fung, V. P., Thomas, J. N., & Vehar, G. A. (1989) *Biochemistry 28*, 8072–8077.
Pawashe, A. B., Golino, P., Ambrosio, G., Migliaccio, F., Ragni, M., Pascucci, I., Chiariello, M., Bach, R., Garen, A., Konigsberg, W. K., & Ezekowitz, M. D. (1994) *Circ. Res. 74*, 56–63.
Pierschbacher, M. D., & Ruoslahti, E. (1987) *J. Biol. Chem. 262*, 17294–17298.
Rehemtulla, A., Ruf, W., Miles, D. J., & Edgington, T. S. (1992) *Biochem. J. 282*, 737–740.
Rose, G. D., Geselowitz, A. R., Lesser, G. J., Lee, R. H., & Zehfus, M. H. (1985) *Science 229*, 834–838.
Ruf, W., Kalnik, M. W., Lund-Hansen, T., & Edgington, T. S. (1991a) *J. Biol. Chem. 266*, 15719–15725.
Ruf, W., Rehmulla, A., & Edgington, T. S. (1991b) *J. Biol. Chem. 266*, 2158–2166.
Ruf, W., Miles, D. J., Rehemtulla, A., & Edgington, T. S. (1992a) *J. Biol. Chem. 267*, 22206–22210.
Ruf, W., Miles, D. J., Rehemtulla, T. S. (1992b) *J. Biol. Chem. 267*, 6375–6381.
Ruf, W., Schullek, J. R., Stone, M. J., & Edgington, T. S. (1994) *Biochemistry 33*, 1565–1572.
Samanen, J., Ali, F., Romoff, T., Calvo, R., Sorensen, E., Vasko, J., Storer, B., Berry, D., Bennett, D., & Strohsacker, M. (1991) *J. Med. Chem. 34*, 3114–3125.
Schullek, J. R., Ruf, W., & Edgington, T. S. (1994) *J. Biol. Chem. 269*, 19399–19403.
Shigematsu, Y., Miyata, T., Higashi, S., Miki, T., Sadler, J. E., & Iwanaga, S. (1992) *J. Biol. Chem. 267*, 21329–21337.
Taylor, F. B., Jr., Chang, W. R., Morrissey, J. H., Hinshaw, L., Catlett, R., Blick, K., & Edgington, T. S. (1991) *Circ. Shock 33*, 127–134.
Warr, T. A., Rao, L. V. M., & Rapaport, S. I. (1990) *Blood 75*, 1481–1489.
Waxman, E., Ross, J. B. A., Laue, T. M., Guha, A., Thiru-vikraman, S. V., Lin, T. C., Konigsberg, W. H., & Nemerson, Y. (1992) *Biochemistry 31*, 3998–4003.
Wilcox, J. N., Smith, K. M., Schwartz, S. M., & Gordon, D. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 2839–2843.

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