Selective Attenuation of the Extrinsic Limb of the Tissue Factor-Driven Coagulation Protease Cascade by Occupancy of a Novel Peptidyl Docking Site on Tissue Factor†

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ABSTRACT: Tissue factor (TF), the receptor and cofactor for factor VIIa (VIIa) for cellular initiation of the coagulation protease cascade, drives thrombogenesis, inflammation, tumor cell metastasis, and the lethality of severe sepsis. To identify TF surface loci that can selectively inhibit substrate zymogen association and activation, TF_{1-218}, the extracellular domain, was used as the target for the phage display search. This resulted in selection of 59 clones from a phage gpVIII surface protein-expressed library of constrained combinatorial peptides. Of these, one encoding the peptide Glu-Cys-Leu-Arg-Ser-Val-Val-Thr-Cys on gpVIII most avidly bound TF_{1-218}, as did the synthetic peptide. Inhibition of binding was selective with an IC_{50} of 30 nM for proteolytic activation of factor X by the TF_{1-218}−VIIa complex. In contrast, there was no inhibition of factor IX activation. The selective inhibition of only factor X association with TF_{1-218} will spare the intrinsic hemostatic pathway while attenuating the extrinsic thrombogenic pathway. This and related peptidyl structures provide the potential for the more precise identification of TF surface loci that mediate selective functional properties of the protein as well as a structural basis for the design of novel molecules for selectively attenuating initiation of the extrinsic limb of the coagulation protease cascade and other functions of TF.

Tissue factor (TF) is the cell surface receptor and cofactor for the plasma serine protease factor VIIa (VIIa). Formation of the TF−VIIa complex initiates the protease cascades that mediate hemostasis and the thrombogenic cascade and induce cell signaling that leads to complex cellular inflammatory responses. It also plays significant roles in the pathophysiology and lethality of severe sepsis, as well as successful implantation of metastatic neoplastic cells (*1*−*5*). Assembly of the TF−VIIa complex (TF−VIIa) results in allosteric transition of the VIIa protease domain to the catalytically functional state and organization of substrate recognition exosites. TF−VIIa mediates presentation of substrate factors X (X) and IX by remote exosite loci on both TF and VIIa (*6*−*10*). TF−VIIa selectively associates with and proteolytically activates zymogens X and IX (*11*). TF, a structural member of the cytokine superfamily (*12*), has a cell surface domain TF_{1-218} with a large contact surface with VIIa (*13*), and it presents the scissile bonds and associated transition state structures of substrates X and IX to the catalytic site of the TF−VIIa complex (*6*−*8*). The transient ternary TF−VIIa−Xa complex proteolytically activates protease-activated receptors 1 and 2 (*5*) to induce cellular signaling involved in the resultant inflammatory responses (*14*, *15*). Thus, the initiated molecular circuits are responsible for a broad array of major pathobiology functions (*14*, *16*−*19*).

A focus on therapeutic attenuation of the TF-initiated cascades has been stimulated by the high frequency of thromboembolic diseases, and by advances in the structural and functional details of the initiation of these protease cascades as well as development of monoclonal antibodies to substrate recognition epitopes of TF (*16*). This has stimulated (*20*−*22*) studies of TFPI, a natural inhibitor of the TF−VIIa−Xa complex (*23*, *24*), and the nematode inhibitor NAPc2 (*25*). The efficacy of inhibition has been explored as well with catalytic site-mutated and inactivated VIIa (*26*). These have been macromolecular except for a peptidyl inhibitor of a functional exosite of VIIa (*27*−*29*). Notably, the current strategies have addressed virtually complete inhibition of both extrinsic and intrinsic limbs of the coagulation protease cascades.

There are no currently recognized small molecule inhibitors of TF, nor does such a discovery appear likely if one attempts to interrupt the large energetic contacts between TF and VIIa (*12*). Less than 3% of the interaction energy of TF−VIIa with substrate X is attributable to the substrate scissile bond and associated transition state structure. However, numerous spatially separate energetic interactions between TF−VIIa and substrates dominate the lower-affinity association of TF−VIIa with substrates X and IX (*3*, *13*). The many dispersed associations between the proteins render

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it unlikely that a small molecule could accomplish significant attenuation of substrate activation. However, *in vitro* selection from a constrained peptide combinatorial library expressed at low density on the surface of bacteriophage has yielded a limited set of peptides that bind the extracellular domain of TF (TF_{1-218}). The most avid of these has demonstrated selective and quantitatively limited attenuation of activation of X and thereby the extrinsic limb of the coagulation protease cascades. Molecules with such selective activity have the potential to provide effective attenuation of thrombotic risk on a long-term basis with minimal risk of diminished hemostatic functions.

MATERIALS AND METHODS

Protein and Reagents. The extracellular domain of human TF (TF_{1-218}) was produced by recombinant methods in *Escherichia coli* as described previously (*30*). Human X was isolated from plasma and purified as described previously (*31*). Human plasma-derived IX was from Haematologic Technologies (Essex Junction, VT). The monoclonal anti-TF antibody mixture has been described previously (*32*). Purified human plasma VIIa protein was from Haematologic Technologies. Spectrozyme fXa and fIXa amidolytic substrates were from American Diagnostica Inc. (Greenwich, CT), and chromozyme t-PA amidolytic substrate for VIIa was from Boehringer Mannheim GmbH (Mannheim, Germany). *E. coli* strain XL1-Blue was from Stratagene (La Jolla, CA), and restriction enzymes were from Roche (Basel, Switzerland) unless otherwise stated. Isopropyl β-D-thiogalactopyranoside (IPTG) was from Roche. Polyethylene glycol (PEG) and other chemicals were from Sigma (St. Louis, MO).

Heptapeptide Phage Display Library pJCM13-88-loop6. Disulfide-constrained heptapeptide library pJCM13-88-loop6 was constructed by expressing the amino acid sequence XCX_6C via a GGGGS linker fused to the amino terminus of an inserted additional copy of coat protein gpVIII encoded by M13 gene VIII. Library construction was achieved in two stages. First, the oligonucleotides [5′-ACAGCGATAGC-TAGCGTAGCTCAGGCCGGT$(N)_3$TGT$(N)_{18}$TGCGGTG-GCGGTGGCTCCCAAGCTTCGATACGTG-3′ and 5′-AGCTTGGGAGCCACCGCA-3′ (Operon, Alameda, CA)] were annealed, extended by Klenow, and digested with *Nhe*I and *Hin*dIII. The insert was cloned into the intermediate vector pORFES (*33*) and electroporated into XL1-Blue, followed by ampicillin (Ap) selection to eliminate constructs encoding peptides bearing a stop codon or unable to be expressed or secreted properly. The library diversity was 5×10^9 independent clones prior to Ap selection and 3×10^9 expressing clones following selection. Second, the recovered pORFES library was digested with *Xba*I and *Hin*dIII, and the gene VIII peptide fusion inserts were isolated and cloned into pJCM13-88 (*33*). This results in expression of random peptides incorporated at the N-terminus of the introduced synthetic copies of gpVIII by bacteriophage M13 upon transformation into *E. coli* XL1-Blue cells. The yield was 6.4×10^{10} pfu measured by a plaque assay (*34*). Since this number is much larger than that of the prior step, the overall library diversity is estimated to be $\sim 3 \times 10^9$ independent clones. The library was analyzed by sequencing of DNA from randomly selected phage clones. No stop codons were observed, and the frequency of all amino acid codons was the same as the theoretical frequency.

Phage Selection by in Vitro Panning. The phage library was screened in fluid phase against biotinylated TF_{1-218}. The TF_{1-218} (2 mg) was biotinylated by incubation in the presence of 0.1 mg/mL sulfo-NHS-LC-Biotin (Pierce Laboratories, Rockford, IL) in 0.50 mL of 50 mM $NaHCO_3$ (pH 8.5) for 1 h. Unreacted free biotin reagent was removed by dialyzing with a Spectra/Por Molecularporous Membrane, 5000 MW cutoff (The Spectrum Companies, Gardena, CA), against PBS [20 mM sodium phosphate and 150 mM NaCl (pH 7.4)]. The first three rounds of selection used phage that had been propagated at 25 °C with 1 mM IPTG to induce multivalent expression of the added library gene VIII. In the first round of selection, 10^{12} pfu of multivalent phage was incubated with 5 μM biotin-TF_{1-218} in 1 mL of PBSA (PBS with 1% BSA) at 4 °C overnight. Streptavidin-coated magnetic beads (10^7) (Dynal, Oslo, Norway) were added, and the solution was mixed at 4 °C for 15 min to trap biotin-TF_{1-218}, then recovered with a magnetic particle concentrator (Dynal), and extensively washed with PBST (PBS with 0.1% Tween 20). Bound phage were recovered and propagated by adding *E. coli* XL1-Blue ($OD_{600} = 0.6$) and incubating for 15 min. Recovered phage were propagated multivalently and used for the following two rounds of selection with biotin-TF_{1-218} with concentrations reduced to 500 and 50 nM, respectively.

The fourth and subsequent rounds of selection used monovalent phage which had been propagated in *E. coli* XL1-Blue at 37 °C in the absence of IPTG. An internal control phage-expressing peptide (YNSSMFCLFEGP) which does not bind to TF_{1-218} was propagated monovalently (*33*) and added at a 1:1 ratio to the phage pool from the third round of selection. The control phage DNA has an additional *Nae*I susceptible cleavage site, thus permitting differentiation from the pJCM13-88-loop6 library phage by digestion with *Ava*I and *Nae*I. This phage pool was selected against 5 nM biotin-TF_{1-218}. The recovered phage were propagated monovalently and selected at the fifth and sixth rounds against 500 pM biotin-TF_{1-218}. RF DNAs of individual phage clones were extracted (Miniprep kit, Qiagene, Chatsworth, CA) and sequenced via an automated thermal cycler (Perkin-Elmer, Foster City, CA) using a 5′-GGATAACAATTTCACA-CAGG-3′ primer.

Phage Binding Assay. Individual phage clones were selected from plated amplification and propagated multivalently at 25 °C with 1 mM IPTG, suspended and precipitated with $^1/_6$ volume of added 20% PEG and 1.6 M NaCl, and centrifuged, and the pellet was resuspended in PBS. The phage concentration was measured at OD_{280}. Phage were incubated with or without 100 nM biotin-TF_{1-218} in 100 μL of PBSA at 4 °C overnight. Immunolon2 plates (Dynex, Chantilly, VA) were coated with 4 μg of streptavidin/well (Sigma) at 4 °C overnight and blocked with 3% BSA at 25 °C for 2 h. The mixture of phage and biotin-TF_{1-218} was added to the wells and incubated at 25 °C for 30 min. After three PBST washes, bound phage were eluted with 100 μL of 0.2 M glycine (pH 2.2) and quantitated by a plaque assay.

Synthetic Peptides. Synthetic peptides were prepared (*35*) and purified to >95% purity by two cycles of reverse phase HPLC in 0.1% TFA followed by HPLC in triethylammonium phosphate. The peptides were characterized by

mass spectroscopy (*36*). The phage Hlp611 peptidyl sequence was modified by addition, for labeling purposes, of Lys-Tyr to the carboxyl aspect, resulting in NH_2-GE*CLRSVVTC*GGKY-NH_2 (the asterisk denotes a disulfide bond). The Hlp611SC scrambled control peptide NH_2-GR*CVSLETVC*GGKY-NH_2 was similarly prepared. Peptides were warmed to 65 °C for 1 h and then cooled to 30 °C to achieve reproducible solubility prior to use. A portion of each of the peptides was biotinylated.

Peptide Binding Assay. Peptide binding to TF_{1-218} was analyzed by an ELISA in which Immunolon2 plates were first coated with streptavidin at 4 mg/mL. Following washing of the wells with PBSA, either biotinylated Hlp611 or control biotinylated Hlp611SC at 30 μM in PBSA was added to each well and incubated for 1 h at room temperature. Then biotin at 10 μM in PBSA was added to saturate all unoccupied streptavidin binding sites for 10 min, and the mixture was washed with PBST three times. TF_{1-218} was added at indicated serial concentrations. After incubation at room temperature for 2 h followed by three washes with PBST, bound TF_{1-218} was quantitated with an anti-TF monoclonal antibody (500 nM) in PBSA. Plates were washed, and the reaction was developed with alkaline phosphatase (AP)-conjugated anti-mouse IgG antibody (Vector, Burlingame, CA). Following PBST washes, AP substrate (Bio-Rad, Hercules, CA) was added, the mixture was incubated overnight at room temperature, and the absorbance at an OD of 405 nm was determined in a 96-well plate reader (Molecular Devices, Sunnyvale, CA) and displayed (Figure 2A). For competitive peptidyl inhibition of TF_{1-218} binding, the concentration of TF_{1-218} present was 250 nM (Figure 2B).

Inhibition of TF-Initiated Generation of Xa and IXa. Specific limited proteolytic activation of X and IX by the functionally assembled TF_{1-218}−VIIa complex was analyzed using coupled chromogenic assays (*32*). TF_{1-218} at 250 nM and peptides at various concentrations were incubated together for 15 min at room temperature in HBSA buffer [20 mM Hepes, 150 mM NaCl, and 0.5% BSA (pH 7.4)] with 5 mM $CaCl_2$. VIIa was added and the mixture incubated at 37 °C for 5 min. X was added to start the reaction of 10 nM TF_{1-218}, 90 nM VIIa, and 300 nM X. After 10 min, the reaction was arrested by addition of EDTA to a final concentration of 100 mM. The generated Xa was quantitated with chromogenic Spectrozyme fXa substrate at 200 μM. The change at OD_{405} was quantitated in a kinetic microtiter plate reader. Peptidyl inhibtion of IXa generation was similarly analyzed with modification of the initial incubation to 20 min and reaction mixture concentrations of 100 nM TF_{1-218}, 200 nM VIIa, and 400 nM IX. These reactions were arrested with 30 mM EDTA and 60% ethylene glycol, and the amount of IXa that was generated was measured with Spectrozyme fIXa at 1 mM. There was no direct effect of the peptides on the function of Xa or IXa. Fractional inhibition was calculated using the formula $I_n = 1 - (A_n - A_0)/(A_1 - A_0)$, where I_n represents the fractional inhibition, A_n = Xa or IXa activity, A_1 = Xa or IXa activity in the absence of peptide (no inhibition), and A_0 = background with no Xa or IXa activity.

TF−VIIa Amidolytic Assay. Serial dilutions of TF_{1-218} were added to 5 nM VIIa in HBSA with 5 mM $CaCl_2$ for 20 min; substrate chromozyme t-PA was added to a final concentration of 1.33 mM and the mixture incubated for 20 min at 37 °C. The rate of OD_{405} increase was determined. For inhibition assays, the peptide was preincubated with TF_{1-218} prior to addition of VIIa.

Computational Analysis. The searchloop feature of Insight (Accelerys) was used to generate six backbone conformations of the disulfide loop of the peptide ECLRSVVTC. These were minimized by two different methods to obtain a total of 12 backbone conformations. Each of these was docked independently to tissue factor (PDB entry 1boy) using the computer program AutoDock (*37*). All side chains and the $\phi-\psi$ torsions of Glu1 (which is outside the disulfide loop) were flexible during the dockings, but the backbone torsions within the disulfide loop were fixed. The lowest-energy conformations from each of 100 docking calculations were gathered together and analyzed by clustering similar results together according to an rmsd (root-mean-square deviation) cutoff of 2.0 Å.

Statistics. Data displaying error bars represent the average and standard deviation for at least three measurements. Some raw data were converted into fractional numbers to combine data from independent experiments. Regression curves were constructed using Sigma Plot.

RESULTS

Identification of TF_{1-218} Binding Peptides from a Phage Display Library. TF_{1-218} was used as the target to select for binding peptidyl ligands using a fluid phase procedure. The combinatorial peptides are expressed on the surface of library phage. During selection, an equal number of irrelevant, nonbinding peptide-carrying control phage was included with the library. Selection of binding phage was demonstrated by a progressively increasing recovery of library phage, as well as a decreasing recovery of control phage. The latter were no longer found by the sixth round of selection (Figure 1A). In contrast, when this initial phage library was propagated repeatedly to the same extent without selection by TF_{1-218}, the control phage increased from 50% to more than 75% of the phage pool (data not shown). This is consistent with target selection rather than genetic selection during phage propagation.

Analysis of Selected TF_{1-218} Binding Phage. After six rounds of selection by TF_{1-218}, 59 individual phage clones were randomly selected for individual analysis. Their fusion peptide sequences, deduced from the DNA sequence, are listed in Table 1. The amino acid sequences were sorted by manual alignment and also using Clustal W 1.7 (*38*). Thirty-three peptide sequences were segregated into two alignment groups that contained either an S(H/R/K) or ER motif. Nine sequences were found multiple times, and eight of these shared a motif. The remaining 26 contained no recognizable motif.

The binding to TF_{1-218} of each of the 59 individual selected phage clones was then further characterized following induction of multivalent expression of the gpVIII added by the peptide sequence. Phage (10^{11} pfu) were incubated with or without 120 nM biotin-TF_{1-218} and then recovered on streptavidin-coated wells. Recovered phage were quantitated, and the binding of 10 representative examples is shown in Figure 1B. Approximately 70% of selected phage clones exhibited significant and reproducible binding to biotin-

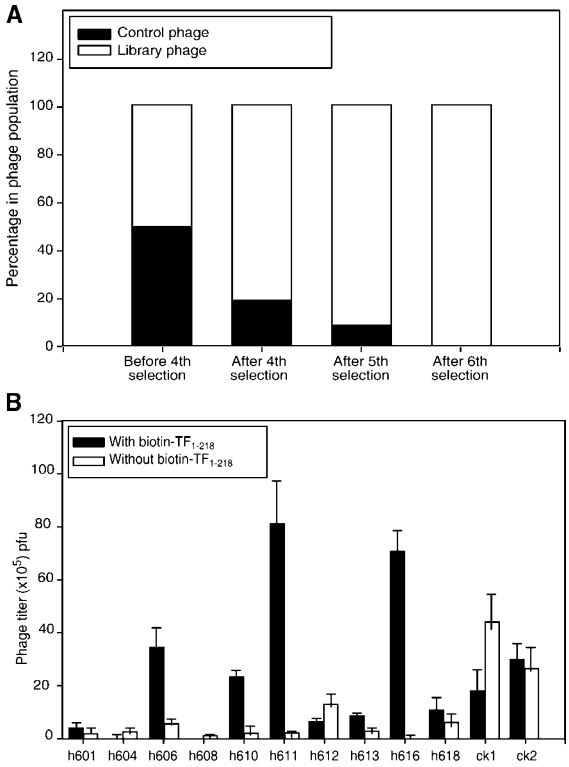

FIGURE 1: (A) Phage selection by monovalent panning. A control phage (distinguishable by *Ava*I and *Nae*I digestion) was included in the phage pool. The percentage of selected library phage increased through repeated panning, whereas the level of control phage decreased. (B) Binding of individual phage to TF_{1-218}. All 59 selected phage clones were analyzed for binding to biotin-TF_{1-218}, and the results of 10 representative phage are shown. Ck1 is a control phage, and ck2 is unselected original pJCM13-88-loop6 library phage. Phage h611 and h616 carry the identical peptide sequence (ECLRSVVTC).

TF_{1-218} compared to background in the absence of biotin-TF_{1-218}. Virtually all phage clones with either general motif were bound by TF_{1-218}, although there also were phage clones without either motif that bound TF_{1-218}. From the 59 selected and analyzed phage clones, h611 and h616 exhibited the most avid binding to TF_{1-218} (Figure 1B). These two phage clones had the same nucleotide sequence and encoded the novel peptide sequence ECLSRVVTC. Interestingly, this sequence shares both of the motifs and was selected for further characterization.

Synthetic Peptide Hlp611 Binds Specifically to TF_{1-218}. Peptide Hlp611 (NH_2-GE*CLSRVVTC*GGKY-NH_2) was synthesized, biotinylated on the added lysine, and cyclized by disulfide formation. Control scrambled peptide Hlp611SC (NH_2-GR*CVSLETVC*GGKY-NH_2) also was similarly synthesized, biotinylated, and cyclized. Binding to TF_{1-218} was analyzed in an ELISA (Figure 2A), and binding appeared to be specific with no binding when immobilized BSA or Hlp611SC was substituted for the Hlp611 target. Specificity was also confirmed by effective competitive inhibition by soluble nonbiotinylated Hlp611. Preincubation of TF_{1-218} at 250 nM with soluble Hlp611 for 1 h competitively inhibited binding of TF_{1-218} to immobilized Hlp611 with an IC_{50} of 500 nM (Figure 2B). Control scrambled peptide Hlp611SC was without effect.

Selective Inhibition of TF Functions by Hlp611 Peptides. We analyzed Hlp611 inhibition of proteolytic activation of

Table 1: Analysis and Sequences of 59 Peptides Selected by tTF Affinity Panning[a]

With **S(H/R/K)** motif	With **ER** motif	No apparent motif
AC**SKSH**AVC[h613]	**ECR**FLLLIC	TCWILGVGC
DC**SH**DMAAC[h606]	**ECR**FLLLIC	YCRLWCLAC
IC**SH**GYACC	**ECLR**SVVTC[h611 b]	LCPPIRGGC
IC**SH**GYACC	**ECLR**SVVTC[h616 v]	QCWWVRIGC
RC**SH**ASSDC	**ECTFWR**YVC	PCAWFRIAC
GCA**SR**GVGC	WCLWC**ER**HC	PCVCLEGAC
SCV**SR**GWCC[h610]	WCLWC**ER**HC	RCPVGGRRC
LCSVM**SR**SC	RCRVY**ER**VC[h601]	VCIMCCVRC
LCWIP**SLK**C	RCRVY**ER**VC	LCWEVVLVC
WCSC**HS**SLC	ICVW**RVGE**C	WCGWAFVGC
WCSC**HS**SLC	ICVW**RVGE**C	MCGEAWPGC[h608]
LC**KT**ADLSC	ICVW**RVGE**C	LCWPDWIIC
LC**KT**ADLSC	GCRG**RE**LVC	VCTCWRIIC
YCL**KS**WIDC	CC**RE**GLVGC	ICWSLCTNC
ECL**RS**VVTC[h611 b]		ICWSLCTNC
ECL**RS**VVTC[h616 v]		ICWSLCTNC
RCCNI**RLS**C		NCGSAETYC
GC**R**DAAP**T**C[h618]		GCQTGCTTC[h604]
RCVGVQ**S**CC		VCSYSTEEC
GCV**R**MLP**S**C		LCALMFSNC
LC**H**MD**S**YCC		FCARGYTWC
		VCWWGMPSC
		SCWWFVFGC
		SCCCWMVFC
		TCYAMKGYC[h612]
		GCVMVFLMC

[a] Amino acid sequences were deduced from the DNA sequences of selected phage. These peptide sequences are listed under both S(H/R/K) and ER groups. All peptides have an extra G at the N-terminus upon gVIII protein leader sequence processing and an additional GGGGS linker sequence at the C-terminus. [b] Phage included in Figure 2 are marked by their clone numbers.

X to Xa by TF−VIIa. As illustrated in Figure 3, Hlp611 inhibits TF_{1-218}−VIIa functional activity in the Xa generation assay with a relative inhibitory concentration (IC_{50}) of 30 nM, though inhibition was partial at saturation with a maximal level of inhibition of ~40%. A VIIa amidolytic assay was used to analyze the effect of Hlp611 on binding of TF_{1-218} to VIIa and also to exclude a direct effect on VIIa catalytic function. We observed no effect of Hlp611 on the VIIa amidolytic assay which requires binding to TF as well as integrity of the catalytic structure. Hlp611 also exhibited no direct inhibition of Xa catalytic activity (not shown), supporting the effect through direct binding to TF_{1-218} and thereby interfering with association of X with the TF_{1-218}−VIIa complex. Elimination of the cyclic structure of the peptide by reduction of the disulfide bond of Hlp611 completely abolished inhibitory properties. These results support the possibility that the Hlp611 peptide interferes with TF_{1-218} presentation of substrate X to the functional TF_{1-218}−VIIa complex. In contrast to functional inhibition of activation of X by TF_{1-218}−VIIa, there was no detectable inhibition of IX activation (Figure 3). Thus, we conclude that the peptide Hlp611 quite selectively interferes with formation of the transient TF_{1-218}−VIIa−X complex without interfering with the TF_{1-218}−VIIa−IX complex and thus preserves the intrinsic limb of the coagulation pathway.

Computational Docking. The existence of common sequence motifs in a high percentage of selected TF binding peptides suggests that these peptidyl structures may recognize a common surface locus on TF. The ECLRSVVTC sequence was used to computationally map the most likely docking site on TF. Multiple backbone conformations of this peptide loop were explored. The computational result of peptide

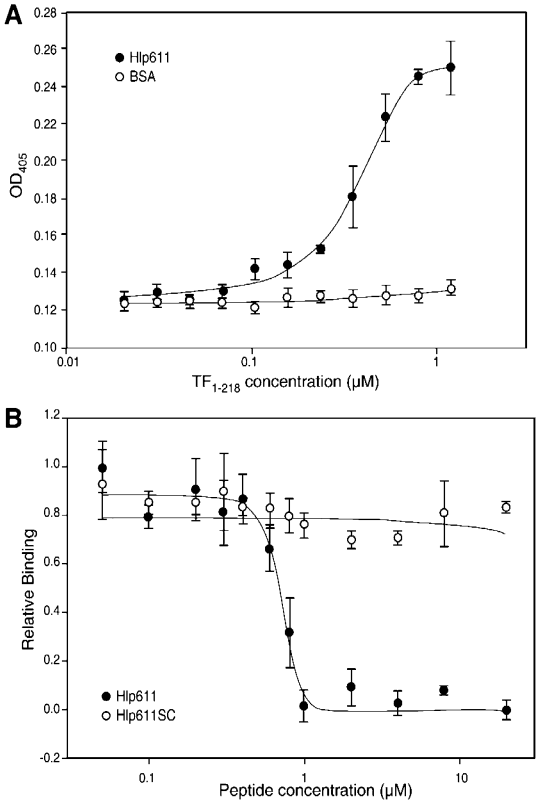

FIGURE 2: (A) Direct binding of TF_{1-218} to the immobilized Hlp611 peptide. Dilutions of TF_{1-218} were added to Hlp611 peptide-immobilized or control BSA-coated wells. The level of binding of TF_{1-218} to the Hlp611 peptide increased as does the TF_{1-218} concentration, whereas no TF_{1-218} binding was evident in BSA-coated controls at comparable concentrations. (B) Competitive inhibition by soluble Hlp611 peptides. The binding of TF_{1-218} to immobilized Hlp611 peptides can be specifically challenged by soluble Hlp611 peptides, but not a scrambled control peptide Hlp611SC with the same amino acid composition as Hlp611. Data displaying error bars represent the average and standard deviation for at least three experiments.

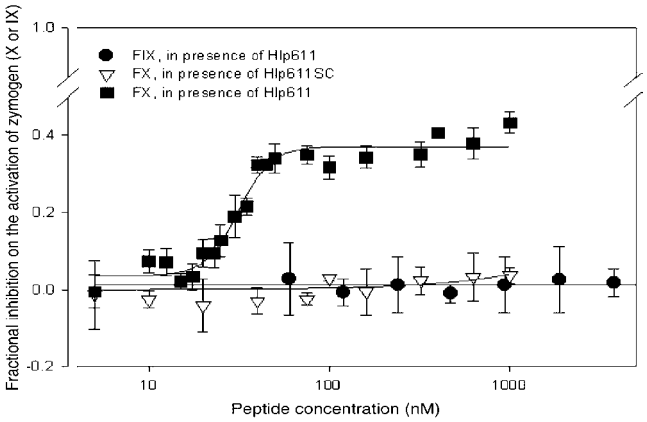

FIGURE 3: Selective inhibition of TF_{1-218}−VIIa-catalyzed substrate zymogen activation by Hlp611 peptides. The inhibition of Xa generation in the absence of detectable inhibition of IXa generation is observed with an IC_{50} of ∼30 nM for Xa generation. TF-driven Xa and IXa generation through limited proteolytic activation of X or IX by the TF_{1-218}−VIIa complex was analyzed by a coupled chromogenic assay (*34*).

docking to TF is shown in Figure 4. Significantly, a recurring mode of binding has the glutamate residue interacting with the basic patch on TF formed by Arg200 and Lys201, an area that was previously implicated in binding of factor X

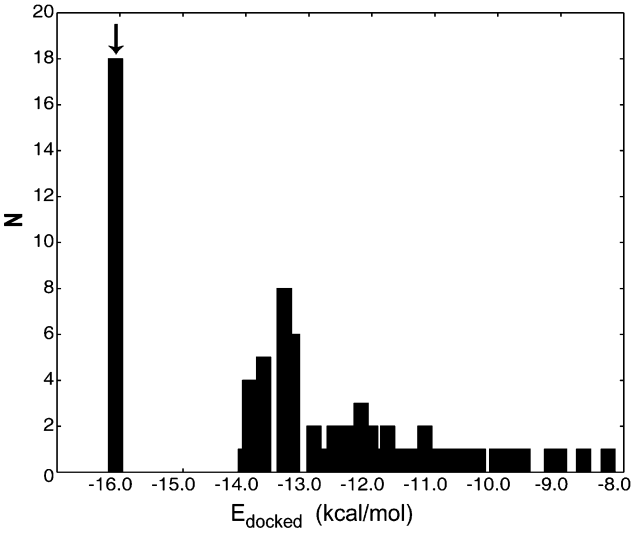

FIGURE 4: Clustering histogram of the docking results. The docked energy (E_{docked}) of the lowest-energy conformation in the cluster is shown on the horizontal axis, and the number of members of each cluster (N) is shown on the vertical axis. Conformations in a cluster are within 2.0 Å (rmsd) of the lowest-energy member of the cluster. The best docked cluster that represents the most probable mode of peptide binding is marked with an arrow.

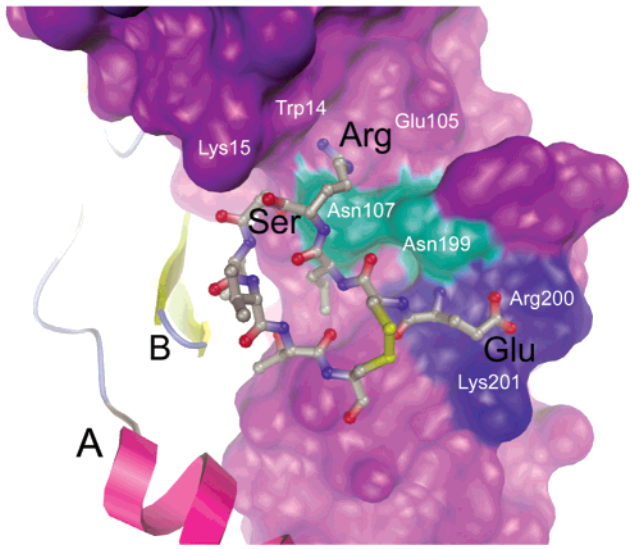

FIGURE 5: Molecular docking of the Hlp611 peptide to the TF−VIIa complex. The lowest-energy docked conformation is shown. The surface of tissue factor is colored magenta, with basic patch Arg200 and Lys201 colored blue and Asn107 and Asn109 cyan. The peptide is shown in a ball-and-stick representation, with the Ser, Glu, and Arg residues labeled. For reference, factor VIIa from the TF−VIIa complex superimposed on TF is shown as a ribbon. A marks the C-terminal helix of the Gla domain and B the Ca^{2+}-binding loop of the first EGF domain.

to the TF−VIIa complex (*39*). This region of the TF surface has also recently been implicated in the binding of the Xa−TFPI complex (*40*). The arginine and serine of the peptide interact near Asn109 and Asn199, the latter of which was also observed to affect binding to factor X (Figure 5). In addition to the interaction with side chains of the two asparagines, the arginine side chain has the potential to interact with the carbonyl oxygens of Glu105 and Trp14. The peptide serine residue also has inferred interactions with Asn107, and with the Lys15 side chain. The binding site for the arginine and serine residues is in fact a pocket formed

in the elbow between the N- and C-terminal domains of TF. This mode of binding has the peptide docked very close to the interface between TF and VIIa; therefore, the computational dockings were further advanced with the TF−VIIa complex (*13*). The mode of peptide binding characterized in docking to TF is also permitted in docking to the TF−VIIa complex, and the conformation of the docked peptide is comparable to that found for docking to TF.

DISCUSSION

In vivo initiation of the coagulation protease cascade results from cellular expression of TF followed by formation of the functional binary TF−VIIa complex on a cell surface membrane (*3*, *16*). This binary protease TF−VIIa complex assembles with substrate proteins through extensive and spatially extended exosite interactions that result in selective hydrolysis of the target scissile peptide bond(s), thereby activating factor X to factor Xa or factor IX to factor IXa (*1*−*4*, *10*). As a result, the coagulation protease cascade is initiated and propelled to generate downstream products, including thrombin and fibrin (*4*, *11*), to activate platelets and other cells (*5*). It is also involved in diverse pathobiology not only with respect to the thrombotic diseases but also with respect to cellular immune responses (*17*−*19*), to contribute critically to the pathology and lethality of sepsis syndrome (*14*, *26*, *41*), to ischemia reperfusion injury (*15*), and to facilitate hematogenous metastasis of neoplastic cells (*42*−*44*). Thus, direct inhibition of TF function may be more effective than attempts to attenuate one derivative downstream response or another. To this end, monoclonal antibodies have been developed for potential therapeutic intervention (*14*, *16*, *20*−*22*, *45*). The most characterized epitope (*3*, *9*, *21*, *22*, *45*−*47*) docks antibody to mask a major substrate association locus on TF and thus successfully competes with the low-affinity interactions of substrates X and IX rather than the high-affinity interaction of TF with VIIa (*32*).

Interactions with TF−VIIa to inhibit the protease function of the binary TF−VIIa complex, or substrate docking functions, have been viewed as alternatives to interrupting the very high affinity assembly and slow the K_{off} of VIIa with TF. Dennis *et al.* (*27*, *28*) have identified a peptidyl ligand for a functional exosite on factor VIIa that interferes with the interaction of VIIa with substrate and thus activation of X even when assembled in the transient TF−VIIa−X ternary complex.

In this attempt to discover a form of molecular intervention of only a selected TF function, namely, presentation of substrates to the TF−VIIa complex, we sought to identify a peptidyl entity that might permit selective inhibition of only X and not IX. This would introduce the novel possibility of attenuating the extrinsic limb of the thrombogenic pathway with preservation of the TF−VIIa-driven intrinsic limb implicated selectively for effective hemostasis. We thus targeted the functional extracellular domain TF_{1-218} with an M13 phage-displayed large peptidyl library in which the expressed peptides are constrained by a single disulfide bond and expressed at low density by a second incorporated gene encoding the surface gpVIII. It is known that TF undergoes little conformational change upon binding VIIa (*13*, *21*, *46*, *48*). Because of their smaller size in comparison to a protein, such as an antibody, peptidyl ligands selected for binding to free TF_{1-218} may be capable of selective inhibition of substrate protein association and proper presentation to the catalytic site.

To achieve initial selective reduction of diversity of the peptidyl library in favor of candidate peptide-expressing phage, the initial rounds of selection were conducted with multivalent expression (approximately four to seven copies of peptide per phage surface) of the library gene VIII and selection with a high concentration of target TF_{1-218}. The TF_{1-218} target concentration was then progressively reduced in the two following selection cycles to favor higher-affinity ligand enrichment. Initial multivalent phage selection greatly increased the level of tryptophan-containing peptides (>90%). Collectively, more than 21% of initially selected peptide amino acids were tryptophan compared to the theoretical 1.6%. Many previous studies using multivalent phage display have similarly documented this undesirable bias (*49*, *50*). Phage bearing these tryptophan-rich peptides have an increased level of binding to TF_{1-218} as well as to a variety of other proteins such as BSA (not shown), indicative of low-affinity nonspecific enrichment. Most tryptophan-rich peptides were excluded in the subsequent rounds of high-affinity monovalent selection (Table 1), supporting the superiority of monovalent selection. Thus, the three subsequent cycles of selection adopted monovalent phage expression.

After six cycles of increasingly stringent affinity selection, 59 phage clones were selected and sequenced. On the basis of phage binding assays, two phage clones expressing the peptidyl addition GECLRSVVTC demonstrated excellent binding to TF_{1-218}. The biotinylated synthetic cyclic peptide Hlp611 bound TF_{1-218} with high affinity both in fluid phase and when immobilized. This peptidyl sequence shares no recognizable sequence similarity or motifs with known TF interactive proteins such as factors VII, X, and IX, TFPI, other serine proteases, or other molecules in the protein databases. Peptide Hlp611 inhibits the proteolytic activation of factor X by TF_{1-218}−VIIa, but it does not inhibit TF_{1-218}−VIIa amidolytic activity or Xa catalytic activity. Therefore, the inhibition of TF_{1-218}−VIIa proteolytic activation of X by Hlp611 is not mediated by disruption of assembly of TF_{1-218} with VIIa which would deprive VIIa of its requisite cofactor and allosteric induction of the catalytic site. Also, since the peptide binds TF rather than VIIa, it does not function by exosite inhibition of the VIIa interactive surface with X as is the case for peptide E-76 inhibition of X activation (*51*). Rather, Hlp611 interferes with TF_{1-218}−VIIa assembly with X to form the transient TF_{1-218}−VIIa−X ternary complex necessary for proper presentation of the subject scissile bond of X to the catalytic site of VIIa. Notable is the lack of any detectable inhibition of the activation of IX. As such, this is the first selective inhibitor of the initiation of the extrinsic coagulation protease cascade of which we are aware. Other described forms of intervention inhibit the overall initiation of both intrinsic and extrinsic limbs of the coagulation protease cascade with the attendant effects on both thrombogenesis and hemostasis.

Factor IX and factor X are highly homologous members of the γ-carboxylated serine protease family. They have four distinct domains, and it is suggested that they evolved by gene duplication and subsequent divergence (*52*). Like factor X, factor IX is also activated by forming a transient ternary

complex with TF−VIIa. A patch of seven to fifteen amino acid residues of the carboxyl module of the extracellular domain of TF, which include Lys165 and Lys166, critically facilitates substrate recognition and presentation (*3*, *6*). The contact areas for IX are partially identical to those for factor X (*9*, *53*). Previous study has shown that monoclonal anti-TF antibody TF8-5G9 inhibited association and activation of both IX and X (*45*, *47*), and from the crystal structure of this antibody in complex with TF, the characteristics of this locus in substrate presentation have been elucidated (*21*). However, in the current study, Hlp611 did not inhibit activation of factor IX by TF_{1-218}−VIIa. This indicates that the TF site occupied by Hlp611 is sufficiently distant from the IX contact locus to avoid an effect on IX association while occupying a site sufficient to attenuate but not entirely block association of X with the TF−VIIa complex. This is consistent with the evidence that residues Lys159 and Gly164 or Asn204 appear to interact with factor X rather than factor IX. Such mutations in this region of TF impair activation of factor X more severely than of factor IX (*6*, *53*).

The computational docking of the peptide to TF suggests a binding site between the binding sites for the EGF-1 domains of VIIa and X (*54*, *55*), which does not interfere with binding of factor VIIa to TF. The interaction of the Glu and Arg residues of the peptide with Asn199, Arg200, and Lys201 suggests a possible mode of inhibition of factor X activation, as these residues have been shown to be involved in the interaction with factor X. However, the same exosite is believed to be involved in interactions between TF and IX (*53*, *56*), the activation of which is not inhibited by this peptide.

Attenuation of the extrinsic thrombogenic pathway by a structural entity such as this peptide provides a guide to the design of a novel class of peptidyl derivatives for selectively inhibiting the thrombogenic cascade, which could provide a therapeutically beneficial impact on the pathobiology of one of the most common molecular pathways resulting in severe morbidity and mortality. In contrast to other approaches, such advanced derivatives may lend themselves to long-term therapeutic attenuation of thrombotic risk rather than an acute interruption of thrombotic pathology.

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