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Thrombin inhibition by cyclic peptides from thrombomodulin

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Abstract

Peptides corresponding to the loop regions of the fourth, fifth, and sixth epidermal growth factor (EGF)-like domains of thrombomodulin (TM) have been synthesized and assayed for thrombin inhibition, as indicated by both inhibition of thrombin-mediated fibrinogen clotting and inhibition of the association of thrombin with TM that results in protein C activation. Peptides from the fifth EGF-like domain showed significant inhibition of fibrinogen clotting and protein C activation, whereas peptides from the fourth and sixth EGF-like domains were weak inhibitors in both assays.

Two structural features were important for inhibitory potency of the peptides from the fifth EGF-like domain: cyclization by a disulfide bond and attachment of the "tail" amino acids C-terminal to the disulfide loop. Linear control peptides did not significantly inhibit clotting or protein C activation. The C-terminal loop alone, the "tail" peptide, or a mixture of the two were at least 10-fold less potent inhibitors of clotting or protein C activation. A more constrained peptide analog was designed by deletion of an isoleucine within the C5–C6 disulfide loop, TM52-1+5_C. This analog was a better inhibitor in both assay systems, having a K_i for protein C activation of 26 μM.

Keywords: disulfide bonds; EGF-like domains; protein C; protein–protein interactions

Thrombomodulin (TM) is an endothelial cell surface glycoprotein that forms a 1:1 complex with thrombin and alters the function of thrombin both by decreasing the activity of thrombin toward fibrinogen and by markedly increasing thrombin's activity toward protein C (C.T. Esmon, 1989). The activation of protein C by the TM–thrombin complex is >1,000-fold faster than the activation of protein C by thrombin alone (Esmon et al., 1982). Following its activation, protein C cleaves factors Va and VIIIa, which are required for the formation of thrombin, and hence shuts down the coagulation cascade (N.L. Esmon, 1989; Dittman & Majerus, 1990). The significance of the protein C-mediated anticoagulation pathway is demonstrated by the observation that homozygous deficiency of protein C results in extensive thrombosis and is fatal in the first month after birth (Griffin et al., 1981). This observation suggests that the anticoagulation process begun by the formation of the TM–thrombin complex and resulting in activated protein C is essential for the maintenance of normal hemostasis. TM can thus be considered a dual-acting anticoagulant because it both inhibits thrombin cleavage of fibrinogen, leading to decreased clot formation, and activates thrombin to initiate the anticoagulant pathway.

The region of TM that is important for thrombin cofactor activity is comprised of epidermal growth factor (EGF)-like domains (Fig. 1). These domains are approximately 40 amino acids in length and have six cysteine residues that form three disulfide bonds (Campbell & Bork, 1993). The structure of the EGF protein, which has been determined by two-dimensional NMR methods, shows two larger loops of 9–15 amino acids (Cooke et al., 1987; Makino et al., 1987; Montelione et al., 1987; Kohda et al., 1988). The N-terminal loop is comprised of the amino acids between the third and fourth cysteines and is formed by two disulfide bonds between the first and third cysteines and between the second and fourth cysteines. The C-terminal loop is comprised of the amino acids between the fifth and sixth cysteines and is a simple disulfide-bonded loop. Models of the TM EGF-like domains based on the structure of EGF protein (Montelione et al., 1987) show that the third and fourth cysteines are actually close enough to form a disulfide bond (E. Komives, unpubl. data). The disulfide-bonding pattern of the EGF-like domains in TM has not been determined, so it is not known whether the disulfide bonds are the same as those in the EGF protein.

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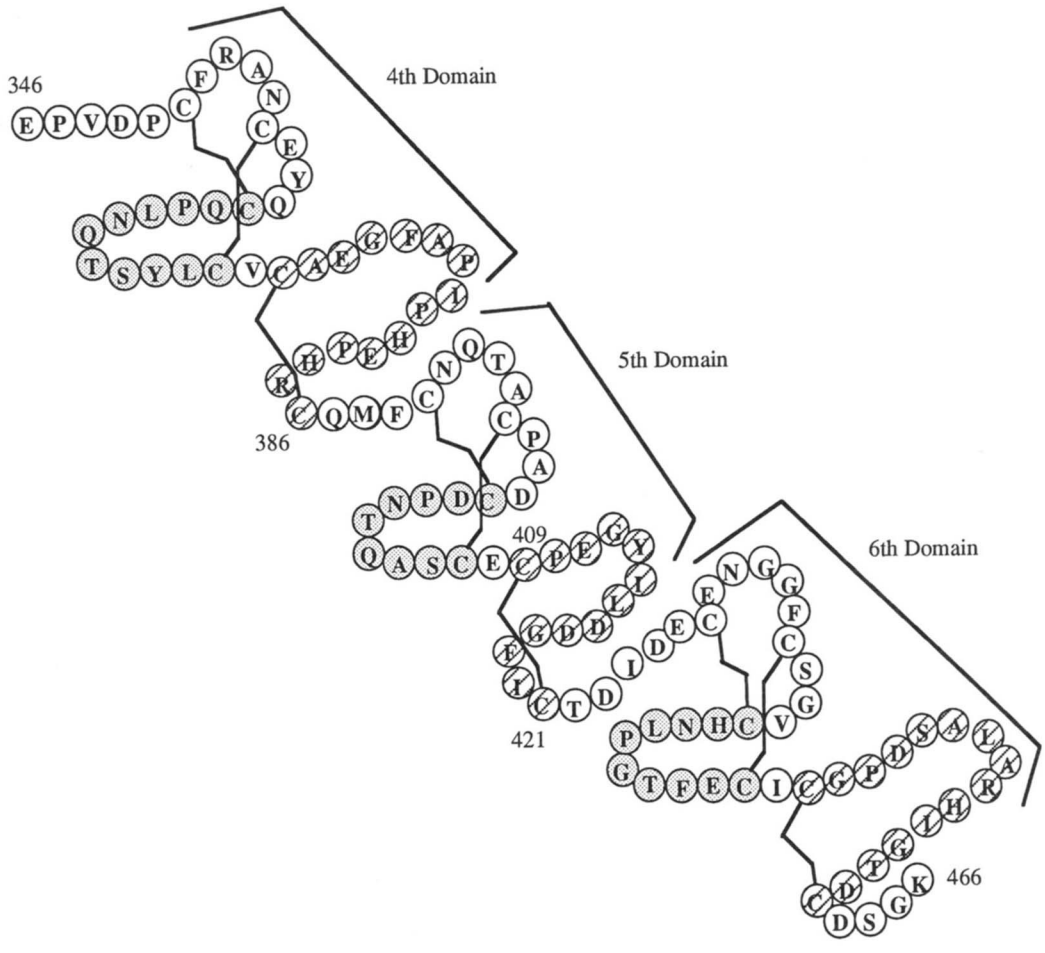

Fig. 1. Schematic diagram of the region of TM from which the peptides were derived. N-terminal loops are shaded and C-terminal loops are hatched. Numbering of the EGF-like domains is from the N-terminus and amino acids are numbered according to the entire protein sequence of human TM. Disulfide bonds shown in this schematic are based on the disulfide bonds found in EGF protein.

Experiments demonstrating that TM competes for thrombin binding with hirudin and fibrinogen show that TM binds to the anion-binding exosite of thrombin (Hofsteenge et al., 1986; Tsiang et al., 1990). The region of TM consisting of the fifth and sixth EGF-like domains inhibits fibrinogen cleavage and is a competitive inhibitor of protein C activation, but it possesses no cofactor activity (Kurosawa et al., 1988; Stearns et al., 1989; Ye et al., 1992). Thus, the thrombin-binding function can be ascribed to the fifth and sixth domains. The fourth EGF-like domain is required for cofactor activity (Stearns et al., 1989; Zushi et al., 1989; Hayashi et al., 1990; Tsiang et al., 1992) and a fragment of TM comprised of the fourth, fifth, and sixth EGF-like domains has been shown to elicit a conformational change 15 Å away from the active site of thrombin (Ye et al., 1992). Whether the fourth EGF-like domain can elicit these changes or can bind to thrombin on its own has yet to be determined.

Peptides corresponding to regions of the fifth and sixth EGF-like domains, especially the sequence from E408 to E426, have been shown to bind to thrombin (Hayashi et al., 1990; Tsiang et al., 1992). Alanine scanning mutagenesis has identified amino acids Y413–D417 within the C5–C6 loop and D423–E426 within the connecting region between the fifth and sixth domains as functionally important (Nagashima et al., 1993). Because the connecting region is highly acidic, it is possible that it provides a nonspecific negatively charged region that interacts with the anion-binding exosite. Questions that remain to be answered regarding this thrombin-binding region of TM include whether the C5–C6 disulfide bond is important, whether the connecting region between the fifth and sixth EGF-like domains interacts with the fifth, the sixth, or both of the domains, and how the connecting region functions in thrombin binding. The experiments presented here will decipher the thrombin binding contributions of each EGF-like subdomain of TM and determine the structural requirements for thrombin binding of the C-terminal part of the fifth EGF-like domain.

Results

Peptide synthesis and characterization

Peptides corresponding to the N-terminal and C-terminal loops of the fourth, fifth, and sixth EGF-like domains of human TM

have been synthesized. Each peptide, except the TMtail, contained two cysteine residues, which upon oxidation to the disulfide, form a cyclic loop. The peptides were designed assuming that the regions of the TM EGF-like domains between the third and fourth cysteines and between the fifth and sixth cysteines would be important for binding to thrombin. In order to determine the importance of the cyclic structure of the peptides, a portion of each peptide was reduced and alkylated with iodoacetamide to provide linear control peptides. The rest of the peptide was oxidized in dilute aqueous solution to produce the intramolecularly disulfide-bonded "cyclic" peptide. After HPLC purification, the identity of each peptide was confirmed by amino acid analysis, N-terminal sequencing, and fast atom bombardment (FAB) mass spectrometry. In addition, each peptide was tested with Ellman's reagent to confirm the absence of free thiol groups (Ellman, 1959). The peptide sequences and mass spectral data are presented in Table 1.

The fourth and sixth EGF-like domains

Peptides from the fourth and sixth EGF-like domains of TM were weak inhibitors of both fibrinogen clotting and protein C activation compared to the peptides from the fifth EGF-like domain (Table 2). None of the peptides from the fourth domain showed any inhibitory activity up to concentrations of 500 μM. Because the fourth EGF-like domain of TM has been shown to be required for TM cofactor activity, all the peptides from the fourth EGF-like domain were also tested for cofactor activity, but none had any effect on the activation of protein C in the absence of TM. Unfortunately, the TM42+3$_C$ peptide caused precipitation during protein C activation assays at concentrations higher than 150 μM, so it could not be determined whether this peptide had weak activity in this assay. Addition of the "tail" amino acids to the C-terminal loop peptide markedly improved the inhibition of clotting caused by the TM42+3$_C$ peptide relative to the TM42$_C$ peptide, suggesting that the weak binding observed in these experiments is real.

The peptide from the N-terminal loop of the sixth EGF-like domain was a weak inhibitor of protein C activation and clotting consistent with previous reports on a peptide from this region of TM (Tsiang et al., 1992). This peptide, TM61$_C$, showed twofold better inhibition in the cyclic form versus the linear form. The cyclic form had a C3–C4 disulfide bond that is not expected to be present if the domain has EGF-like disulfide bonds, so it is possible that this region of the sixth domain would bind more tightly if all four cysteines were present to form the C1–C3, C2–C4 crossing disulfide bonds.

Table 1. *Sequences and molecular weights of the TM peptides from mass spectrometry*[a]

Peptide	Sequence	Molecular weight[b]		
		Expected	M^+H^+	M^+Na^+
TM41$_C$	CQPLNQTSYLC	1,267.6	1,268	
TM41$_L$	aaCQPLNQTSYLCaa	1,383.6	1,384	
TM42$_C$	CAEGFAPIPHEPHRC	1,661.8	1,662	
TM42$_L$	aaCAEGFAPIPHEPHRCaa	1,777.8	1,778	
TM42+3$_C$	CAEGFAPIPHEPHRCQMF	2,068.5	2,069	
TM42+3$_L$	aaCAEGFAPIPHEPHRCaaQMF	2,184.5	2,185	
TM51$_C$	CDPNTQASC	936.3	936	958
TM51$_L$	aaCDPNTQASCaa	1,052.3	1,053	
TM52$_C$	CPEGYILDDGFIC	1,442.6	1,443	
TM52$_L$	aaCPEGYILDDGFICaa	1,558.6	1,559	
TM52+5$_C$	CPEGYILDDGFICTDIDE	2,015.9	2,016	
TM52+5$_L$	aaCPEGYILDDGFICaaTDIDE	2,131.9	2,132	
TM52A+5	APEGYILDDGFIATDIDE	1,952.9	1,953	
TM52-1$_C$	CPEGYILDDGFC	1,329.5	1,330	1,352
TM52-1$_L$	aaCPEGYILDDGFCaa	1,445.5	1,446	1,468
TM52-1+5$_C$	CPEGYILDDGFCTDIDE	1,902.8	1,903	1,925
TM52-1+5$_L$	aaCPEGYILDDGFCaaTDIDE	2,018.8	2,019	2,041
TM52-1A+5	APEGYILDDGFATDIDE	1,840.0	1,840	
TMtail	TDIDE	591.6	592	
TM61$_C$	CHNLPGTFEC	1,118.5	1,119	1,141
TM61$_L$	aaCHNLPGTFECaa	1,234.5	1,234	1,256
TM62$_C$	CGPDSALARHIGTDC	1,513.7	1,513	
TM62$_L$	aaCGPDSALARHIGTDCaa	1,629.7	1,630	

[a] Peptide nomenclature is such that the first number corresponds to the EGF-like domain from which it is derived, and the second number signifies the loop, where 1 denotes the N-terminal and 2 denotes the C-terminal loop in the domain. A "C" behind the peptide's name indicates the cyclic peptide, whereas an "L" indicates the linear peptide. The acetamido group is indicated by "aa" before or after the cysteine to which it is attached. Other cysteines are disulfide bonded.

[b] Molecular weight is that determined by mass spectrometry. The expected molecular weight for each peptide was determined from the calculated exact mass. In some cases, only the protonated form or the sodium adduct was observed in the FAB mass spectrum.

Table 2. *Data from assays for inhibition of clot formation and inhibition of thrombin-induced protein C activation for each peptide*[a]

Peptide	Amount of peptide (μM) required to double clotting time	Protein C activation IC_{50} (μM)
$TM41_C$	ND[b]	ND[c]
$TM41_L$	ND[b]	ND[c]
$TM42_C$	ND[b]	ND[c]
$TM42_L$	ND[b]	ND[c]
$TM42+3_C$	550 ± 70	ND[d]
$TM42+3_L$	1,270 ± 130	ND[d]
$TM51_C$	710 ± 30	1,400 ± 90
$TM51_L$	1,420 ± 100	3700 ± 500
$TM52_C$	1,500 ± 130	1,110 ± 60
$TM52_L$	ND[b]	1,900 ± 60
$TM52+5_C$	230 ± 14	50 ± 3
$TM52+5_L$	ND[b]	740 ± 50
TM52A+5	–	704 ± 160
$TM52\text{-}1_C$	1,100 ± 150	710 ± 12
$TM52\text{-}1_L$	ND[b]	2,800 ± 300
$TM52\text{-}1+5_C$	24 ± 2	23 ± 3
$TM52\text{-}1+5_L$	530 ± 110	ND[c]
TM52-1A+5	–	735 ± 160
TMtail	ND[b]	1,000 ± 200
$TM61_C$	790 ± 80	510 ± 16
$TM61_L$	ND[b]	1,200 ± 90
$TM62_C$	ND[b]	1,400 ± 120
$TM62_L$	ND[b]	1,600 ± 160

[a] Experiments were carried out as described in the Materials and methods and errors were calculated from the slope of the line as determined by least-squares analysis.

[b] ND, no significant increase in clotting time was observed at peptide concentrations up to 500 μM.

[c] ND, no significant inhibition of protein C activation was observed at peptide concentrations up to 500 μM.

[d] ND, no significant inhibition of protein C activation was observed at peptide concentrations up to 150 μM. Due to solubility problems, this peptide could not be studied at higher concentration.

The fifth EGF-like domain

Both the N-terminal and C-terminal loops of the fifth EGF-like domain were weak inhibitors of fibrinogen clotting and protein C activation and the cyclic peptides were twofold more potent inhibitors than the linear peptides. The cyclic peptide with the "tail," $TM52+5_C$ (IC_{50} = 50 μM), inhibited protein C activation 15-fold better than its linear counterpart $TM52+5_L$ (IC_{50} = 740 μM). Similar large differences were observed in the clotting assays in which the linear peptides did not measurably increase clotting time. As a further test for the importance of the cyclic structure, and to obviate any possibility that the acetamido groups were inhibiting binding of the linear peptides, peptides corresponding to the TM52+5 and TM52-1+5 sequences in which the two cysteines were replaced by alanines were also synthesized and tested. These peptides showed similar IC_{50}'s of approximately 700 μM as were observed for the acetamidylated linear peptides.

A large difference in inhibitory potency was observed upon addition of the "tail" amino acids to the C-terminal loop of the fifth domain. The peptide containing the loop and the "tail," $TM52+5_C$ (IC_{50} = 50 μM), was a 20-fold more potent inhibitor of protein C activation than the loop alone ($TM52_C$, IC_{50} = 1,110 μM). The peptide corresponding to just the "tail" amino acids, or TMtail, was also tested to determine if it was an inhibitor, but this peptide did not significantly inhibit clot formation and was a weak inhibitor of protein C activation (IC_{50} = 1,000 μM). An equimolar mixture of the fifth-domain C-terminal loop and the TMtail peptides together was also assayed to determine if these peptides would show synergistic inhibitory activity. This experiment yielded the identical IC_{50} as that reported for the loop peptide alone. Both clotting and protein C inhibition data indicated that the "tail" region is very important for binding of the C-terminal loop peptide. Furthermore, the loop must be attached to the "tail."

Design of a TM-based thrombin inhibitor

The data on the native sequence peptides clearly showed that the peptides from the C-terminal loop of the fifth EGF-like domain were the best thrombin inhibitors. For these peptides, two structural features seemed essential for thrombin inhibition, the cyclic loop structure and attachment of the "tail" amino acids. Using this knowledge, a cyclic peptide that would have a more constrained cyclic loop was designed. The design was based on the hypothesis that, because the loop structure is essential for thrombin binding, a more constrained loop would involve less of a loss in entropy upon binding and thus should have an improved K_i. The isoleucine at position 12 in the peptide was chosen for deletion in order to preserve the N-terminal sequence of the loop, PEGY, which is a region of the loop that is conserved in many EGF-like domains. The ILDD sequence was also preserved because it was deemed the most likely thrombin-binding sequence because it is both hydrophobic and acidic and would be expected to bind in the anion-binding exosite of thrombin. Furthermore, these residues were shown to be important by alanine scanning mutagenesis (Nagashima et al., 1993). All of the designed peptides fortuitously had improved solubility compared to the native sequence peptides. The designed peptide consisting of the loop alone was 1.5-fold more potent than the corresponding native sequence peptide. The designed peptide consisting of the loop and the "tail," $TM52\text{-}1+5_C$, was a ninefold better clotting inhibitor and a twofold better protein C activation inhibitor than the native sequence peptide, $TM52+5_C$. Figure 2 shows the inhibition of clotting by $TM52+5_C$ and $TM52_C$ versus $TM52\text{-}1+5_C$ and $TM52\text{-}1_C$. The K_i was determined for $TM52\text{-}1+5_C$ by measuring the amount of inhibition of activated protein C production at several different concentrations of peptide and at several different concentrations of TM to obtain a Dixon plot (Fig. 3). The measured K_i of 26.5 μM agrees well with the IC_{50} of 23 μM obtained for this peptide. The linearity of the Dixon plot suggests that the peptide is a purely competitive inhibitor of TM binding to thrombin in this assay system.

Discussion

Synthetic peptides corresponding to the loop regions of the fourth, fifth, and sixth EGF-like domains of TM have been used to probe the region of TM that binds to thrombin in more detail. The experiments have deciphered the contribution of each

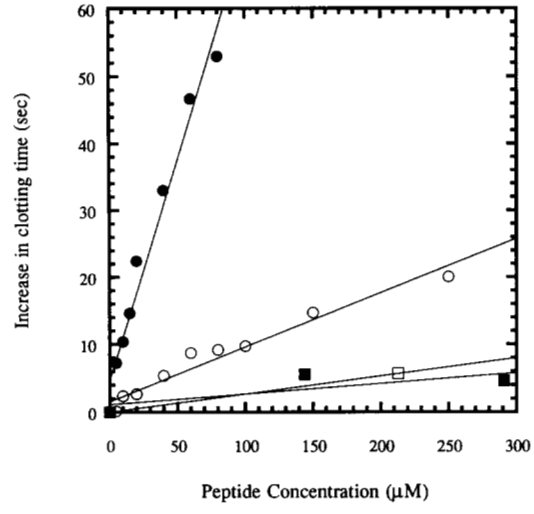

Fig. 2. Plot of the increase in clotting time caused by $TM52_C$ (□) and $TM52+5_C$ (○), compared with $TM52\text{-}1_C$ (■) and $TM52\text{-}1+5_C$ (●). The experiment was carried out as described in the Materials and methods.

of the individual EGF-like subdomains of TM to thrombin binding. More importantly, the experiments have clearly shown that, within the major thrombin-binding region of TM, two structural features are required: the disulfide bond that constrains the C-terminal loop into a cyclic structure, and the attachment of the "tail" amino acids to the C-terminal loop region that precedes them in the TM sequence.

Two different assays were used to assess whether the peptides interacted with thrombin in the same manner as TM. The peptides directly inhibited fibrin clot formation as well as the activation of protein C by the thrombin–TM complex and the two different assays gave similar results – peptides were either good

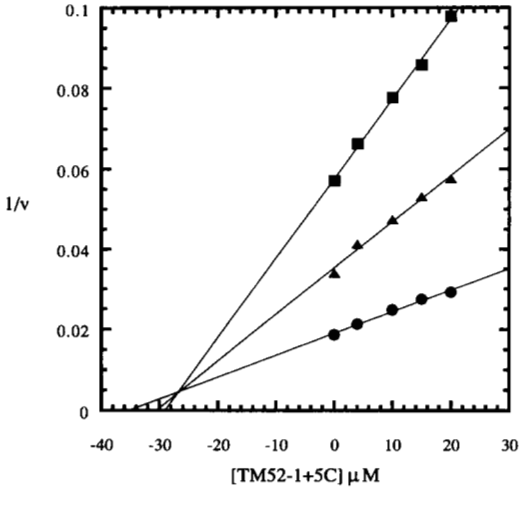

Fig. 3. Dixon plot of $1/v$ versus concentration of $TM52\text{-}1+5_C$ at three different concentrations of TM: 0.14 nM (■), 0.21 nM (▲), 0.29 nM (●). The K_i for the peptide is the concentration at which the lines intersect, 26.5 μM.

inhibitors in both assays or poor inhibitors in both assays. Thus, it is most likely that the peptides are interacting directly with thrombin rather than interacting with protein C or with fibrinogen. More importantly, the results suggest that the same region of TM inhibits both clot formation and protein C activation by binding to thrombin. Previous studies have shown that the fifth and sixth EGF-like domains of TM are potent inhibitors in both these assays. The results presented here show that of all the loop regions within this fragment, only the region of the C-terminal loop of the fifth domain and the attached "tail" inhibits well when it is isolated from the context of the larger fragment. Furthermore, this small region has specific structural requirements.

The peptides bind more weakly than larger TM fragments

Most of the peptides in these experiments bound to thrombin weakly, with binding constants on the order of hundreds of micromolar. Only the region of TM consisting of the C-terminal loop of the fifth EGF-like domain and including the "tail" amino acids bound in the tens of micromolar concentration range. The peptide binding constants are approximately 2,000–4,000-fold weaker than full-length TM devoid of chondroitin sulfate (K_m approximately 6 nM for rabbit and 15 nM for human TM) and the rabbit EGF(5-6) fragment (K_d approximately 9 nM) (Kurosawa et al., 1987, 1988; Parkinson et al., 1990). This difference in binding can be rationalized in several ways. First, it is known from recent NMR studies that even the designed peptide with the constrained loop, $TM52\text{-}1+5_C$ (K_i = 26.5 μM) is completely unstructured in solution. In contrast, the thrombin-bound form of the peptide has a well-defined structure (Srinivasan et al., 1994). Thus, a likely explanation for the difference in binding is that the rest of the EGF-like domain imparts structure to the loop and hence decreases the entropy loss required for thrombin binding. An alternative explanation is that although regions of the fourth and sixth EGF-like domains bind only weakly to thrombin on their own, when they are attached to the rest of the molecule, they also bind, and the overall binding constant is due to a combination of these many weak binding forces. Although the peptides bind more weakly than larger fragments of TM consisting of several EGF-like domains, significant differences in inhibition were observed for the different peptides and can be interpreted to indicate which regions of TM contribute most to thrombin binding and what are the structural requirements of these regions.

Contribution of the fourth and sixth EGF-like domains

Cofactor activity has been shown to depend on the presence of the fourth EGF-like domain of TM, but no evidence that the fourth domain alone binds to thrombin has been generated. The peptides from the fourth EGF-like domain were only weak inhibitors of thrombin clotting of fibrinogen and had no effect on protein C activation at concentrations that did not cause precipitation in the assay. The inhibition of fibrinogen clotting by $TM42+3_C$ was strongly dependent on the presence of the "tail" amino acids as well as on the loop structure providing evidence that the interaction of this peptide with thrombin is specific. Further evidence for the specificity of this peptide was gained from an experiment in which equal volumes of $TM42+3_C$ and

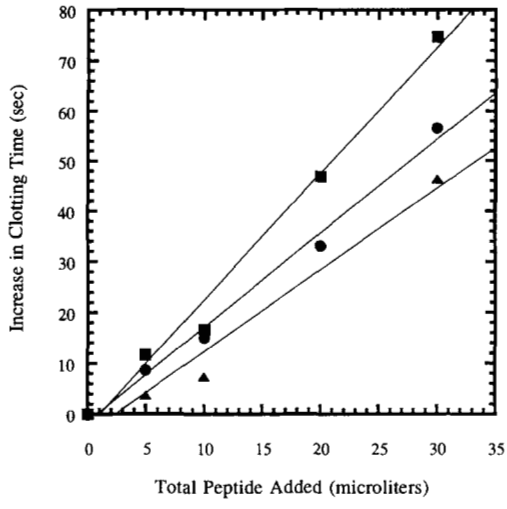

Fig. 4. Plot of the increase in clotting time caused by TM42+3C (▲), TM52+5C (●), and a mixture of the two peptides together (■). Increase in clotting time was measured as described in the Materials and methods.

$TM52+5_C$ were mixed and compared for inhibition of clot formation to test whether the inhibition of the two peptides was additive. Figure 4 shows the results of this experiment, which showed that the inhibition observed for each of the peptides was indeed additive. This result can be interpreted that the peptides are not binding at the same site, which implies that the $TM42+3_C$ peptide is binding specifically. Despite the specificity of binding, the $TM42+3_C$ peptide bound weakly compared to the fifth domain peptides. The fact that the fourth domain peptides did not bind well to thrombin suggests that although the fourth EGF-like domain is essential for protein C activation, it is unable to perform its function if it is not connected to the fifth EGF-like domain because it requires the fifth domain in order to bind to thrombin. Alternatively, the conformation of the fourth domain might change when it is attached to the fifth domain.

Structural features of the fifth domain required for thrombin binding

The results presented here confirm that the major thrombin-binding region of TM is located within the fifth EGF-like domain and the connecting region between the fifth and sixth EGF-like domains, as had been previously suggested (Hayashi et al., 1990; Tsiang et al., 1992). The structural features of this region, comprised of amino acids C409–E426, consist of the cyclic loop structure formed by a disulfide bond between C409 and C421 and of the attachment of the "tail" amino acids to this loop.

The results from both the clotting and protein C activation assays showed convincingly that the disulfide bond is essential for thrombin binding of the peptides corresponding to the C-terminal loop of the fifth EGF-like domain of TM. The cyclic peptides were more than 10-fold better inhibitors than the linear peptides. This result is different from that obtained for a peptide similar to TM52+5 by Tsiang et al. (1992) in which the cyclic peptide, ECPEGYILDDFICTDIDE, and its "linear" counterpart, EAPEGYILDDGFICTDIDE had K_d's of 85 μM and 42 μM, respectively. The most likely explanation for the observation that the "linear" peptide was a better inhibitor is that the "linear" peptide contained a free thiol on the single cysteine. In this study, the ability of peptides to release ^{125}I-thrombin bound to TM on cell surfaces was ascertained. If the peptide containing a free thiol group disrupted the structure of the TM, ^{125}I-thrombin would be released. The sensitivity of TM to free thiol has been noted (Esmon et al., 1982; Lentz & Sadler, 1991), and we have observed inhibition of TM cofactor activity with peptides that were not fully alkylated or cyclized. To test the hypothesis that the free thiol was responsible for the observed inhibition by the "linear" peptide, peptides in which both cysteines were changed to alanines were tested and found to be very weak inhibitors of protein C activation.

The amino acids (C-terminal to the last cysteine) that form the connecting regions between both the fourth and fifth and the fifth and sixth EGF-like domains have been shown to be important by alanine-scanning experiments, but their role was not determined (Clarke et al., 1993; Nagashima et al., 1993). The experiments presented here show that these "tail" amino acids markedly increase thrombin binding of the preceding C-terminal loop and in the case of the fifth domain, the requirement that the "tail" be attached to the domain was demonstrated. Thus, the loop may interact with the "tail" to generate binding specificity. The fact that the "TMtail" peptide is highly negatively charged and yet shows little inhibitory potency indicates that the thrombin binding of the C409–E426 region of TM is not primarily due to nonspecific binding of anionic residues to the anion-binding exosite of thrombin.

Design of a TM-based thrombin inhibitor

A TM-derived cyclic peptide that contained a more constrained loop was designed to test the importance of the cyclic structure and to try to improve the thrombin-binding potency of the TM-derived peptides. The designed peptide, $TM52\text{-}1+5_C$, was a ninefold better inhibitor of thrombin-induced clotting of fibrinogen and a twofold better inhibitor of thrombin-induced protein C activation. The designed peptide also required the cyclic structure and the attachment of the "tail" amino acids, and it appeared to be more sensitive to these requirements than the native sequence peptides. The difference in inhibitory potency in the protein C assay between the cyclic and linear peptides was 32-fold for the designed peptides and only 15-fold for the native sequence peptide. Similarly, the attachment of the "tail" amino acids improved the inhibitory potency in the designed analog by 30-fold and only 22-fold for the native sequence peptide. The design strategy appears to have been successful in that constraining of the loop resulted in a peptide that was a better thrombin inhibitor. The fact that the designed peptide was a better thrombin inhibitor strongly suggests that entropy is lost upon binding. This result is consistent with the recent transferred NOE experiments showing that the peptide does indeed become structured upon binding to thrombin (Srinivasan et al., 1994). These NMR experiments also show that the "tail" interacts with the loop in the bound structure, confirming the data presented here that indicate that the "tail" must be attached to the loop. This analog represents the first attempt at rational design of a TM-

based thrombin inhibitor with improved thrombin-inhibitory potential over the native TM sequence.

Materials and methods

Peptide synthesis

Peptides were synthesized by standard solid-phase Fmoc chemistry on a Milligen 9050 peptide synthesizer employing the pentafluorophenyl esters of the amino acids with further activation by hydroxybenzotriazole. The peptides were cleaved from the resin using trifluoroacetic acid (TFA) containing 5% thioanisole, 2.5% ethanedithiol, and 1.25% anisole. The cleaved, deprotected peptides were isolated by ether precipitation overnight at −20 °C and the precipitated peptides were collected on a sintered glass funnel and lyophilized. Typical yields were 80–90% of the theoretical yield or between 200 and 500 mg, depending on the molecular weight of the peptide.

The crude cysteine-containing peptides were reduced at a concentration of 10 mg/mL in 20 mM dithiothreitol, 100 mM potassium phosphate buffer, 1 mM EDTA, pH 8.2, for 30 min at 37 °C under nitrogen. Occasionally, even after complete reduction, an insoluble residue remained, and this was removed by centrifugation at 10,000 × *g* for 10 min prior to alkylation or oxidation of the peptide. A portion of the peptide solution was diluted to 0.1 mg/mL in MilliQ H_2O, the pH was adjusted to 8.1, and the peptide was air-oxidized by stirring for 2–4 days at room temperature to afford the intramolecular disulfide-bonded product. The rest of the peptide was alkylated with a 20-fold molar excess of iodoacetamide for 30 min at 37 °C. The peptides were purified using standard reverse-phase HPLC on a Waters DeltaPak 300-Å semi-preparative column with a 0–50% 0.1% TFA–CH_3CN gradient over 1 h at a flow rate of 10 mL/min. Because the oxidized peptide was in a large volume (approximately 1–2 L), the entire solution was pumped onto the HPLC through the solvent pump at a flow rate of 10 mL/min. Typical yields of the purified peptides after oxidation or alkylation were 30–70 mg each of the alkylated and oxidized peptides. The identity of each peptide was confirmed by mass spectrometry, amino acid analysis, and N-terminal sequencing. The molecular weights were within 1 mass unit of the calculated molecular weight. Ellman's Reagent was used to confirm the absence of free thiols in both the cyclic and linear peptides (Ellman, 1959). The TMtail peptide, which did not contain any cysteines, was isolated from the TFA mixture by completely evaporating the solvent under a stream of nitrogen, dissolving the peptide in 5% NH_4OAc, and then lyophilizing the resulting mixture. The peptide was then purified by HPLC as described above.

The peptides were dissolved in MilliQ H_2O at a nominal concentration of 3.6 mg/mL and stored in small portions at −20 °C. The concentration of each peptide in these stock solutions was determined by amino acid analysis using a known amount of norleucine as a standard. Before each assay, portions of the peptide solution were thawed and an appropriate amount of a 10× solution of Tris-buffered saline (TBS) was added so that the final concentration of Tris-HCl and NaCl was the same as that of the TBS (20 mM Tris-HCl, 100 mM NaCl, pH 7.4) used in the assays described below. Occasionally, it was necessary to adjust the pH of the resulting peptide solution, and this was accomplished by adding a few microliters of 0.1 N NaOH and matching the pH of the peptide solution to that of the TBS as assessed by pH paper.

Clotting assays

The direct inhibition of thrombin-induced clot formation by TM has been previously demonstrated by measuring the time it takes for thrombin to induce clot formation at different concentrations of TM in a solution containing fibrinogen (Bourin et al., 1988). For the experiments presented here, rabbit TM was prepared by the method of Esmon et al. (1982) but appeared to be devoid of the chondroitin sulfate region as assessed by calcium titration experiments (data not shown). The amount of rabbit TM required to cause a doubling of the clotting time from 20 s to 40 s was 40 ± 3 nM. Increase in clotting time was linear with increasing TM and the TM standard curve was the same as that reported by Clarke et al. (1993). Because the TM employed in these studies was devoid of the chondroitin sulfate region, it is expected to inhibit clotting primarily by interacting with thrombin in the fibrinogen (anion-binding exosite I) and not in the heparin-binding (anion-binding exosite II) site (Ye et al., 1993).

Peptides corresponding to regions of TM were used in place of TM in this assay at concentrations up to 1 mM, but for ease of comparison of the data, the results are tabulated as the peptide concentration required to double the clotting time. The assays were carried out by incubating several different concentrations of each peptide with 6.6 U of human thrombin (a generous gift of Dr. John Fenton) in 200 μL TBS containing 5 mM $CaCl_2$ and 1.76 mg/mL PEG 8000 for easier clot visibility. After 10 min, 200 μL of a solution of 2 mg/mL purified human fibrinogen (Calbiochem) in TBS was then added and the time for the clot to form was measured using a stop watch. The time for clot formation in the absence of peptides was 20 ± 1 s. For each measurement, clotting time was measured in the absence and then in the presence of peptide to determine the increase in clotting time caused by addition of the peptide. The increase in clotting time was linear over a range of peptide concentrations from 10 to 1,000 μM and experiments were repeated at least twice. The amount of peptide required to double the clotting time was calculated from the equation of each line, and the errors were determined from the standard error of the slope of the line generated by least-squares analysis of the data.

Protein C activation assays

TM cofactor activity, which results in the thrombin-dependent production of activated protein C, was measured using an assay in which thrombin and TM were incubated with protein C and the resulting activated protein C was then assayed with a chromogenic substrate (Stearns et al., 1989). This same assay has been used successfully to measure the K_d of fragments of TM for thrombin (Kurosawa et al., 1988). Under conditions in which the concentration of TM was 0.1–0.3 nM, which is well below K_m for rabbit TM that does not contain the chondroitin sulfate region (6 nM), the amount of activated protein C generated was linearly dependent on the amount of TM present and the kinetics of the reaction could be simply described by treating TM as the substrate, activated protein C as the product of the reaction, and the peptides as inhibitors. Under these conditions, the K_i should be similar to the IC_{50} because the kinetic expression: $\%\text{inhibition} = 100I/[K_i(1 + S/K_m) + I]$ reduces to

%inhibition = $100I/(K_i + I)$ when $S \ll K_m$ (Segel, 1976). Therefore, at 50% inhibition, $K_i = I$. We have tested this simple assumption for the case of the TM52-1+5_C peptide and found it to hold. To save reagents, we only measured the IC_{50} for each of the other peptides, but we expect the IC_{50} values to be in good agreement with the K_i values.

Inhibition of protein C activation was measured by incubating various concentrations of each peptide with human thrombin (0.4 μg/mL, 0.875 U/mL, 11 nM) and rabbit TM (20 ng/mL, 0.29 nM) in TBS containing bovine serum albumin (1 mg/mL) and $CaCl_2$ (5 mM). After 10 min, protein C (7.25 μg/mL, 120 nM, Hematologic Technologies) was added and the mixture was incubated for 20 min. The thrombin activity was quenched with heparin-antithrombin III (80 ng/mL and 220 ng/mL, respectively) and assayed by monitoring the release of *p*-nitroaniline at 405 nm from a chromogenic substrate, ChromozymPCa (Boehringer-Mannheim) or S-2366 (Chromogenix) at a concentration of 0.74 mg/mL. The assays were performed with either bovine or human protein C (Hematologic Technologies) with similar results. Correlation coefficients for each line were at least 0.95, and experiments were repeated at least twice. The data are presented as the amount of peptide that inhibits the thrombin–TM activation of protein C by 50% (IC_{50}), which was calculated from the equation for the line, and the errors were determined in the same manner as for the clotting assays. For the TM52-1+5_C peptide, the K_i was measured by performing the protein C assay described above at several different TM concentrations (0.14, 0.21, and 0.29 nM) and the data were plotted as $1/v$ versus the concentration of peptide in a Dixon plot. The K_i was determined from this plot as the lines intersect at the value of $(-K_i)$.

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