Protein Science (1995), *4*:2129–2137. Cambridge University Press. Printed in the USA.

Thrombin-binding affinities of different disulfide-bonded isomers of the fifth EGF-like domain of thrombomodulin

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(RECEIVED December 29, 1994; ACCEPTED July 20, 1995)

Abstract

The fifth EGF-like domain of thrombomodulin (TM), both with and without the amino acids that connect the fifth domain to the sixth domain, has been synthesized and refolded to form several different disulfide-bonded isomers. The domain without the connecting region formed three disulfide-bonded isomers upon refolding under redox conditions. Of these three isomers, the (1-2,3-4,5-6) bonded isomer was the best inhibitor of fibrinogen clotting and also of the thrombin-TM interaction that results in protein C activation, but all the isomers were inhibitors in both assays. The isomer containing an EGF-like disulfide-bonding pattern (1-3,2-4,5-6) was not found among the oxidation products.

The domain with the connecting region amino acids (DIDE) at the C-terminus formed two isolable products upon refolding in redox buffer. These products had the same two disulfide-bonding patterns as the earliest and latest eluting isomers of the domain without the DIDE. In order to compare the thrombin-binding affinities of these isomers to the isomer with the EGF-like disulfide bonds, acetamidomethyl protection of the second and fourth cysteines was used to force the disulfide bonds into the EGF-like pattern. Thrombin-binding affinity, measured as inhibition of fibrinogen clotting and as inhibition of protein C activation correlated inversely with the number of crossed disulfide bonds. As was found for the domain without the connecting region, the isomer that was the best inhibitor of fibrinogen clotting and of protein C activation was the isomer with no crossing disulfide bonds (1-2,3-4,5-6). This isomer doubled the clotting time at a concentration of 200 nM and showed a K_i for protein C activation of 2 μM, both an order of magnitude better than the isomer with EGF-like disulfide bonds.

Keywords: anticoagulant; disulfide bond; fibrinogen; peptide synthesis; protein C

Thrombomodulin (TM) is an endothelial cell surface glycoprotein that regulates the function of thrombin by forming a 1:1 complex with it thus decreasing the activity of thrombin toward fibrinogen, while markedly increasing the activity of thrombin toward protein C (Esmon, 1989a, 1989b). 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.

The fragment of TM containing the fourth, fifth, and sixth EGF-like domains is equipotent to full-length TM for both binding to thrombin and activation of protein C (Stearns et al., 1989; Zushi et al., 1989; Hayashi et al., 1990; Tsiang et al., 1992). 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, and the fourth EGF-like domain is required for cofactor activity. Although the K_i for the EGF(5-6) fragment has been measured at 6 nM (Kurosawa et al., 1987), peptides from the C-terminal loop of the fifth domain and including the connecting region between the fifth and sixth domains bind much more weakly, with K_i's around 50 μM (Tsiang et al., 1992; Lougheed et al., 1995). This result would suggest that the sixth domain is essential for tight thrombin binding. On the other hand, TM from which the sixth domain was specifically deleted bound to thrombin only 10-fold less well than native TM (Tsiang et al., 1992) and the EGF(4-5) fragment of TM is only 10-fold less active than the EGF(4-5-6) fragment (Hayashi et al., 1990; White et al., 1995). The relative binding contributions of the

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fifth and sixth domains is thus still unresolved and it is possible that one domain influences binding of the other.

EGF-like domains are approximately 40 amino acids in length and have six cysteine residues that form three disulfide bonds. The structure of the EGF protein has been determined by two-dimensional NMR methods (Cooke et al., 1987; Makino et al., 1987; Montelione et al., 1987; Kohda et al., 1988; Hommel et al., 1992). The cysteine connectivities within EGF are the first to the third, the second to the fourth, and the fifth to the sixth or (1-3,2-4,5-6) (Savage et al., 1973), whereas the longer stretches of amino acids that form the loops are between the third and fourth cysteines and between the fifth and sixth cysteines. Structurally, EGF and EGF-like domains appear to have two subdomains each containing one large loop: the N-terminal subdomain contains two disulfide bonds between the first and third cysteines and between the second and fourth cysteines, and the C-terminal subdomain contains a single disulfide bond between the fifth and sixth cysteines and is a simple disulfide-bonded loop. To date, all of the EGF-like domains that have been studied have the same disulfide-bonding pattern as EGF protein. The disulfide-bonding patterns of the N-terminal domains of factors IX and X and E-selectin are all EGF-like (1-3,2-4,5-6) as is TGFα (Huang et al., 1989; Harvey et al., 1991; Moy et al., 1993; Graves et al., 1994). The disulfide-bonding pattern of the second EGF-like domain of factor X was partially determined, but an ambiguity between the (1-3,2-4,5-6) and (1-3,2-5,4-6) patterns could not be resolved (Hojrup & Magnusson, 1987). This domain was recently synthesized and upon refolding in a redox buffer, no peptide with the (1-3,2-4,5-6) disulfide-bonding pattern could be isolated (Yang et al., 1994). Thus, the disulfide-bonding patterns of very few native EGF-like domains are known. The disulfide-bonding patterns of the EGF-like domains of TM have not been determined due to a lack of availability of sufficient native protein.

The fifth EGF-like domain of TM is interesting because it is the domain within TM that binds to thrombin. We report here the synthesis of several disulfide-bonded isomers of this domain including the EGF-like disulfide-bonded isomer, which was prepared by the method of Yang et al. (1994). Two assays, one for direct inhibition of fibrin clot formation and the other for inhibition of protein C activation, were then employed to ascertain the thrombin-binding potential of each disulfide-bonded isomer of the domain.

Results

Disulfide-bonded isomers of TM5

Initially, the fifth EGF-like domain of TM extending from M388 to T422 was prepared (Fig. 1). After purification of the fully reduced TM5 peptide, oxidative refolding was carried out either under kinetic control using air as the oxidant or under thermodynamic control using a mixture of reduced and oxidized glutathione. Both procedures consistently gave three products in approximately equal amounts. These were separated on reverse-phase HPLC by holding the gradient at 30% acetonitrile. A typical HPLC trace is shown in Figure 2. Confirmation that these three products were disulfide-bonded isomers and not different peptide sequences was obtained by electrospray mass spectrometry, which showed that each isomer had the same molecular weight (Table 1).

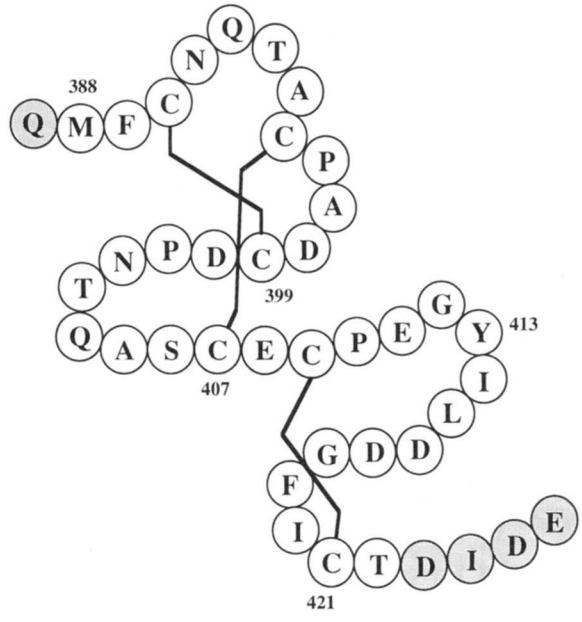

Fig. 1. Schematic diagram of the fifth EGF-like domain of TM. Shaded residues are those that are present in the TM5xt peptide but not in the TM5 peptide. Disulfide bonds shown between the cysteines are those expected from the disulfide-bonding pattern of EGF protein.

The partial reduction method reported recently by Gray (1993a,b) was used to determine the disulfide-bonding pattern of the earliest and latest eluting peaks. The disulfide-bonding pattern determined from partial reduction analysis of each iso-

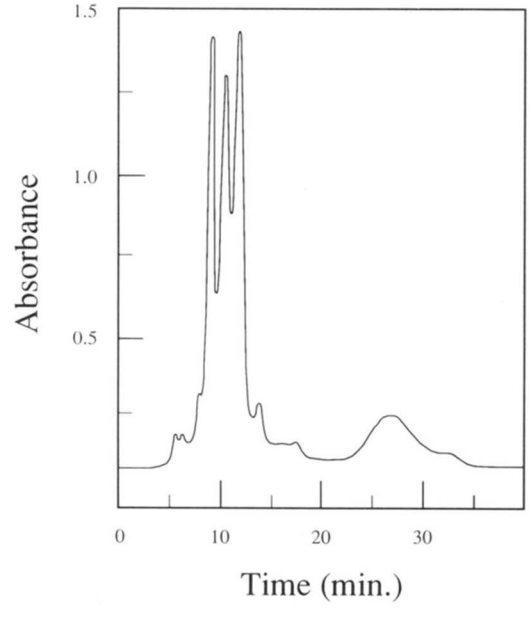

Fig. 2. Reverse-phase HPLC trace of the separation of disulfide-bonded isomers of TM5 after oxidation in the presence of reduced and oxidized glutathione. The isomers were detected at 226 nm and separated by reverse-phase HPLC on a Waters DeltaPak C18 column (19 mm × 300 mm) using a gradient of 0.1% TFA to 50% acetonitrile over 1 h, where the gradient was held at 30% acetonitrile to maximize separation of the isomers. The trace starts at 0 min, which corresponds to the start of the gradient being held at 30% acetonitrile.

Table 1. *Sequences and observed masses for each fifth domain peptide*[a]

Peptide	Sequence	Observed mass[b]
TM5	MFCNQTACPADCDPNTQASCECPEGYILDDGFICT	3,772
TM5xt	QMFCNQTACPADCDPNTQASCECPEGYILDDGFICTDIDE	4,372
TM5xt-Acm	QMFCNQTAC*PADCDPNTQASC*ECPEGYILDDGFICTDIDE[c]	4,516

[a] Peptides were purified on a Vydac C_{18} 4.6 × 250-mm analytical reverse-phase HPLC column using a step gradient consisting of 100% buffer A (0.1% TFA) for 10 min, 0–10% acetonitrile over 10 min, 10–40% acetonitrile over 90 min.
[b] Mass determined by electrospray mass spectrometry.
[c] C* denotes *S*-acetamidomethyl cysteine.

mer is shown in Table 2. Neither of the disulfide-bonded isomers had the disulfide-bonding pattern found in EGF protein (1–3,2–4, 5–6). The earliest eluting isomer had a (1–2,3–4,5–6) pattern so that the N-terminal subdomain disulfides were not crossed as they are in EGF protein. The latest eluting isomer had a (1–3, 2–5,4–6) pattern in which an additional crossing disulfide bond was present that is not found in EGF protein. Attempts to map the disulfide-bonding pattern of the isomer eluting between the earliest and latest peaks showed that it had a 5–6 disulfide bond, so it is possible that this isomer is EGF-like.

Each folded isomer of TM5 was assayed for its ability to inhibit clot formation in a purified solution containing human fibrinogen and human thrombin (Fig. 3A). Fragments of TM containing the fifth domain have been shown to inhibit fibrinogen clotting, presumably by binding to the fibrinogen binding site. Each isomer showed similar inhibition of clot formation but the earliest eluting isomer was a more potent inhibitor of clot formation than the other two isomers. None of the isomers were very potent inhibitors, presumably because they did not contain the connecting region between the fifth and sixth domains, which has been shown to be essential for binding of peptides from the C-terminal loop of the fifth domain (Tsiang et al., 1992; Lougheed et al., 1995).

Each folded isomer of TM5 was also assayed for its ability to inhibit TM cofactor activity (Fig. 3B). This assay measures the inhibition of the formation of the thrombin-TM complex that results in protein C activation. The ability of the thrombin-TM complex to activate protein C in the presence of each fifth domain isomer was determined by monitoring the amount of chromogenic substrate cleaved by the activated protein C that was generated during the incubation. All isomers showed measurable but weak thrombin inhibition, and there was not much difference in thrombin binding among the different isomers of the fifth domain that did not contain the connecting region (amino acids 423–426). The earliest eluting disulfide-bonded isomer was again the best inhibitor, with an IC_{50} of 370 μM.

Disulfide-bonded isomers of TM5xt

Because the fifth EGF-like domains of TM extending from M388 to T422 were poor inhibitors, the domain including the four additional amino acids connecting the fifth and sixth domains of TM (amino acids 423–426 in the TM sequence) was synthesized (Fig. 1). The peptide sequence extended from Q387 to E426 of TM and refolding of this peptide in a redox buffer formed two isolable products, but other minor products were produced as well (Fig. 4). The separation of these disulfide-bonded isomers was not as clean as for the domains without the DIDE, but repurification yielded single isomers that each eluted at different times and did not appear to interconvert (Fig. 5A).

In order to compare the thrombin-binding ability of TM5xt disulfide-bonded isomers with the isomer that had the EGF-like disulfide-bonding pattern, we utilized the method of Yang et al. (1994) to force the formation of the desired disulfide bonds. The

Table 2. *Analysis of disulfide-bonding patterns of TM peptides by partial reduction*[a]

Peptide	Peak #	Sequence	Result	Overall result
TM5 – earliest	2	MF**X**NQTA**X**PADCDPNTQASCECPEGYILDDGFICT	1-2	1-2,3-4,5-6
	3	MF**X**NQTA**X**PAD**X**DPNTQAS**X**ECPEGYILDDGFICT	1-2,3-4	
	4	MF**X**NQTA**X**PADCDPNTQASCE**X**PEGYILDDGFI**X**T	1-2,5-6	
TM5 – latest	2	MFCNQTA**X**PADCDPNTQASCE**X**PEGYILDDGFICT	2-5	1-3,2-5,4-6
	4	MFCNQTA**X**PADCDPNTQAS**X**E**X**PEGYILDDGFI**X**T	2-5,4-6	
TM5xt – earliest	2	QMF**X**NQTA**X**PADCDPNTQASCECPEGYILDDGFICTDIDE	1-2	1-2,3-4,5-6
	4	QMF**X**NQTA**X**PAD**X**DPNTQAS**X**ECPEGYILDDGFICTDIDE	1-2,3-4	
TM5xt – latest	1	QMFCNQTA**X**PADCDPNTQASCE**X**PEGYILDDGFICTDIDE	2-5	1-3,2-5,4-6
	3	QMF**X**NQTA**X**PAD**X**DPNTQASCE**X**PEGYILDDGFICTDIDE	1-3,2-5	
TM5xt – Acm	4	QMF**X**NQTA**X**PAD**X**DPNTQAS**X**ECPEGYILDDGFICTDIDE	1-3,2-4	1-3,2-4,5-6

[a] Results from partial reductive mapping of the disulfide bonds in the various synthetic fifth EGF-like domains of TM using the method of Gray (1993b). X denotes a cysteine that was reduced and alkylated by iodoacetamide, and C denotes those cysteines still in disulfide bonds after partial reduction.

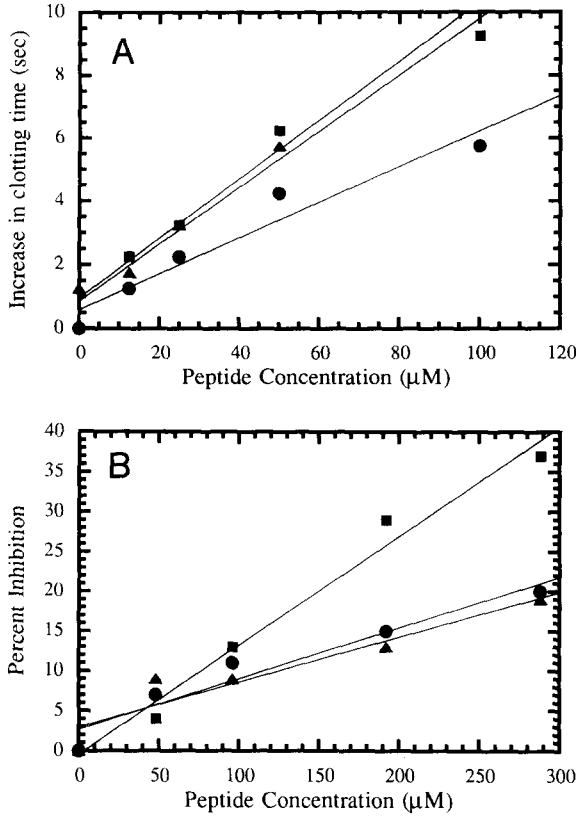

Fig. 3. **A:** Increase in clotting time caused by the various fifth EGF-like domains of the TM earliest eluting isomer (■), intermediate eluting isomer (▲), latest eluting isomer (●). **B:** Inhibition of thrombin-TM activation of protein C by the various TM5 domains. Percent inhibition of protein C activation was measured at a TM concentration of 0.29 nM. Earliest eluting isomer (■); intermediate eluting isomer (▲); latest eluting isomer (●).

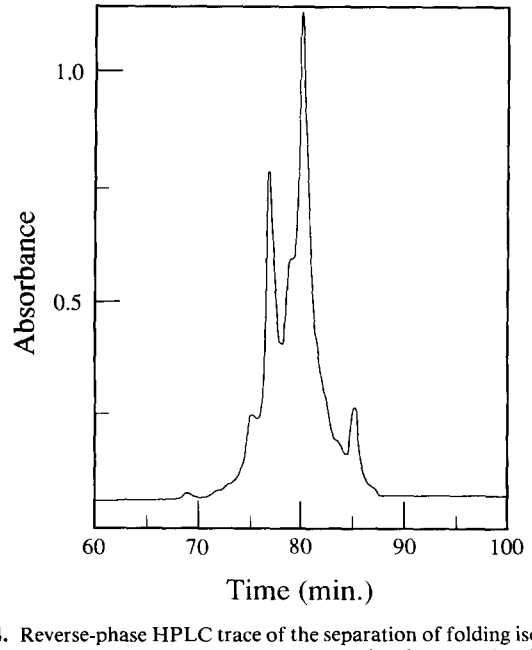

Fig. 4. Reverse-phase HPLC trace of the separation of folding isomers of TM5xt after oxidation in the presence of reduced and oxidized glutathione.

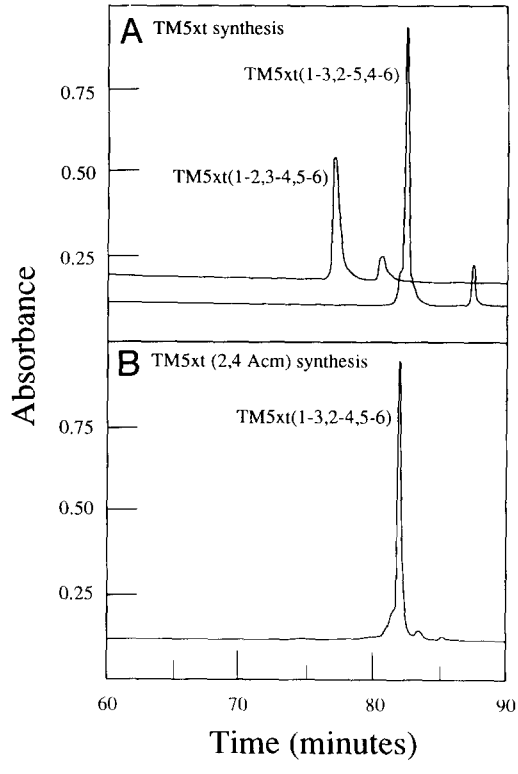

Fig. 5. Analytical reverse-phase HPLC analysis showing the purity and retention times of each of the disulfide-bonded isomers of TM5xt. Analysis was carried out with detection at 226 nm, on a Vydac C_{18} (4.6 × 250-mm) analytical reverse-phase HPLC column at a flow rate of 1 mL/min using an extended gradient of 100% buffer A (0.1% TFA) for 10 min, 0–10% acetonitrile over 10 min, 10–40% acetonitrile over 90 min. **A:** Products obtained from the synthesis in which all the cysteines were protected by trityl groups. The isomer with the (1–2,3–4, 5–6) disulfide bonds eluted at 77.3 min, whereas the isomer with the (1–3,2–5,4–6) disulfide bonds eluted at 83.0 min. **B:** Product obtained from the synthesis in which the second and fourth cysteine were protected by Acm groups. This product had the EGF-like (1–3,2–4,5–6) disulfide-bonding pattern and eluted at 82.0 min.

TM5xt peptide was therefore synthesized with Acm protecting groups on the second and fourth cysteines. The product containing the Acm groups was characterized by mass spectrometry and, after removal of the Acm groups and oxidation, the final product had the same mass as the other disulfide-bonded isomers of TM5xt (Table 1). The oxidized product was analyzed by reverse-phase HPLC and shown to elute slightly earlier than the isomer with the (1–3,2–5,4–6) disulfide bonds (Fig. 5B).

Each purified disulfide-bonded isomer of TM5xt was analyzed by the method of Gray (1993a) to determine the disulfide-bonding pattern. Upon treatment of each isomer with Tris-carboxyethylphosphine (TCEP), a reproducible distribution of partially reduced products was obtained, and the distribution obtained for each isomer was different (Fig. 6). After alkylation with *N*-methyliodoacetamide, the disulfide-bonding pattern was obtained by N-terminal sequence analysis of one or more peaks. The results from the N-terminal sequencing analysis after TCEP treatment and alkylation of each isomer are listed in Table 2. The order of elution of the disulfide-bonded isomers of TM5xt was the same as that for the domain without the DIDE sequence

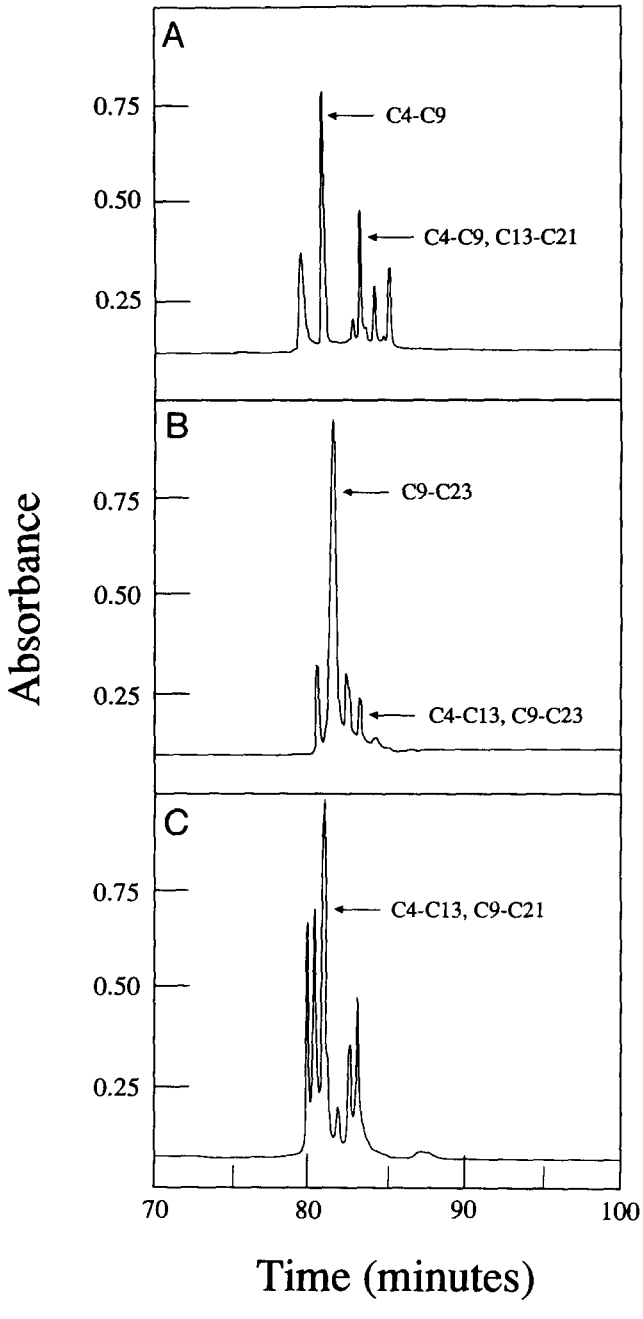

Fig. 6. HPLC separation of the partially reduced products of each disulfide-bonded isomer of TM5xt after treatment with TCEP. Separation was carried out using the gradient described in Figure 5. **A:** Earliest eluting isomer, for which the disulfide-bonding pattern was determined to be (1-2,3-4,5-6). **B:** Latest eluting isomer, for which the disulfide-bonding pattern was determined to be (1-3,2-5,4-6). **C:** Domain in which the disulfide bonds were forced to be EGF-like and for which the disulfide bonds were determined to be (1-3,2-4,5-6).

at the C-terminus. The earliest eluting isomer was shown to have the (1-2,3-4,5-6) disulfide-bonding pattern and the latest eluting isomer had the (1-3,2-5,4-6) disulfide-bonding pattern. The disulfide-bonding pattern of the isomer that was forced to be EGF-like was determined to be (1-3,2-4,5-6), as expected (Table 2).

All three isomers of the TM5xt peptides were much more potent inhibitors of fibrinogen clot formation and of protein C activation than the TM5 peptides without the DIDE sequence. The isomer with all crossed disulfide bonds (1-3,2-5,4-6) was the least potent inhibitor in both assays, doubling the clotting time at a concentration of 24 μM (Fig. 7A) and having a K_i for protein C activation of 35 μM (Fig. 7B). The EGF-like disulfide-bonded isomer, which had the N-terminal disulfide bonds crossed, showed intermediate inhibitory potency, doubling the clotting time at a concentration of 9 μM and having a K_i for protein C activation of 13 μM. The non-EGF-like isomer in which none of the disulfide bonds was crossed (1-2,3-4,5-6) was approximately two orders of magnitude more potent an inhibitor of clot formation and one order of magnitude more potent an inhibitor of protein C activation. This isomer doubled the clotting time at a concentration of 200 nM and had a K_i for protein C activation of 2 μM. The data for inhibitory potency of all of the fifth domain isomers are summarized in Table 3. To check whether all three of these isomers were binding to the same site on thrombin, the affect of each isomeric fifth domain on the cleavage of chromogenic substrates by thrombin was determined. All three isomeric domains stimulated thrombin cleavage of SpectrozymeTH and had no effect on the cleavage of S2222. These same effects were observed previously for the EGF(5-6) fragment of TM (Ye et al., 1992).

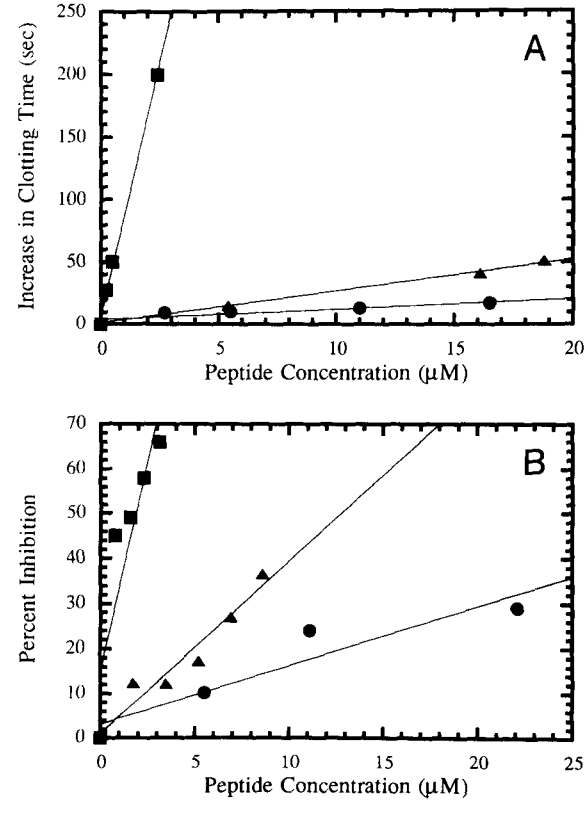

Fig. 7. A: Increase in clotting time caused by the isomeric TM5xt fifth EGF-like domains of TM: ■, (1-2,3-4,5-6); ▲, (1-3,2-4,5-6); ●, (1-3,2-5,4-6). **B:** Inhibition of thrombin-TM activation of protein C by the isomeric TM5xt fifth EGF-like domains of TM: ■, (1-2,3-4,5-6); ▲, (1-3,2-4,5-6); ●, (1-3,2-5,4-6). Percent inhibition of protein C activation was measured at a TM concentration of 0.29 nM.

Table 3. *Summary of thrombin inhibition by the various TM fifth domains*[a]

Peptide	Amount of peptide to double clotting time (μM)	K_i for protein C activation (μM)
TM5(1-2,3-4,5-6)	210 ± 50	370 ± 50
TM5 (N.D.)	200 ± 50	740 ± 50
TM5(1-3,2-5,4-6)	340 ± 50	830 ± 50
TM5xt(1-2,3-4,5-6)	0.2 ± 0.02	1.9 ± 0.2
TM5xt(1-3,2-5,4-6)	24 ± 2	35 ± 3
TM5xt(1-3,2-4,5-6)	9 ± 1	13 ± 1

[a] Peptides correspond to those listed in Table 1. The amount of peptide required to double the clotting time was determined as described in the Materials and methods. K_i's for the TM5 peptides were determined from the IC_{50} values. K_i's for the TM5xt peptides were determined from Dixon plots as described in the Materials and methods. All concentrations are in μM.

Discussion

Comparison of the whole fifth domain to C-terminal loop peptides

The fifth EGF-like domain of TM is one of few EGF-like domains that has a known binding function. The C-terminal loop of this domain has been shown to bind to thrombin (Tsiang et al., 1992; Lougheed et al., 1995). The experiments presented here show that for the whole domain, as was true for the C-terminal loop peptide, the amino acids, DIDE, C-terminal to the last cysteine of the fifth domain are very important for thrombin binding, improving the K_i for protein C activation by almost two orders of magnitude. The peptide studies suggested that the major site of thrombin binding was located within the C-terminal loop of the fifth domain; however, the experiments presented here show that the entire fifth domain is a much more potent inhibitor of thrombin than the C-terminal loop peptide alone. The disulfide-bonded isomer that was the best inhibitor of thrombin was approximately a 25-fold more potent inhibitor of protein C activation and approximately 1,000-fold more potent inhibitor of fibrinogen clotting. In fact, the isolated fifth domain was equipotent to the TMEGF(4-5) fragment for inhibition of fibrinogen clotting and only fivefold less potent than full-length TM. This result shows definitively that an isolated EGF-like domain can indeed be largely responsible for the binding required for a protein-protein interaction. It also shows that the entire fifth domain is required for tight thrombin binding.

Thermodynamically stable products were not EGF-like

Refolding of the fifth domain peptides with or without the "tail" amino acids, resulted in a very similar distribution of disulfide-bonded isomers. In each case, the earliest eluting isomer was that in which none of the disulfide bonds was crossed (1-2,3-4, 5-6), and the latest eluting isomer was that in which all the disulfide bonds were crossed (1-3,2-5,4-6). Neither domain formed any observable product with EGF-like disulfide bonds (1-3,2-4,5-6). It is interesting that the second EGF-like domain of factor X also does not form any isolable EGF-like disulfide-bonded product upon refolding in a redox buffer (Yang et al., 1994). One possible explanation is that the rest of the protein influences the folding of these EGF-like domains so that in the native protein they are (1-3,2-4,5-6), even though this is not a thermodynamically stable form of the isolated domain. The other possibility is that not all EGF-like domains have the EGF-like disulfide-bonding pattern in the native state.

A non-EGF-like disulfide-bonded isomer binds best to thrombin

Two independent assays have been used to assess the thrombin-binding potency of the synthetic isomeric fifth domains from TM. One measures the direct inhibition of fibrinogen clot formation, and the other measures the competitive inhibition of the formation of the thrombin-TM complex that results in protein C activation. Thus, in the first assay, the synthetic domains compete with fibrinogen for binding to thrombin, and in the second they compete with TM. Similar trends in both assays should indicate the degree to which the synthetic domains bind to thrombin. In both assays, the non-EGF-like disulfide-bonded isomer that had no crossing disulfide bonds was an order of magnitude more potent than either the EGF-like disulfide-bonded isomer or the completely crossed disulfide-bonded isomer. This result also supports the possibility that the fifth EGF-like domain of TM does not have EGF-like disulfide bonds in the native protein. It is impossible to isolate enough native TM to map the disulfide bonds in the native protein, but we have now expressed a fragment of TM containing only the fourth and fifth EGF-like domains, which has almost full TM activity (White et al., 1995). This fragment is a single, sharp peak on reverse-phase HPLC so it is likely that this protein is not heterogeneous in its disulfide-bonding pattern. Experiments to map the disulfide bonds in this expressed, active protein are in progress.

Comparison of the thrombin-binding potencies for the three disulfide-bonded isomers reveals that for the isomers with crossed disulfide bonds the amount of peptide required to double the clotting time is similar to the K_i value. The amount of the isomer, with no crossed disulfide bonds that was required to double the clotting time was only 1/10th the K_i value. This result correlates well with studies on loop peptides from this region of TM, which showed that the disulfide-bonded loop peptide corresponding to the sequence between the third and fourth cysteines ($TM51_C$) was a good inhibitor of clotting but was a weaker inhibitor of protein C activation (Lougheed et al., 1995). Thus, it appears that the N-terminal subdomain of the fifth domain of TM may contribute more to inhibition of clotting than to inhibition of protein C activation.

Inhibitory potency of the fifth domain isomers correlates with flexibility

Inhibitory potency for the fifth domain isomers both with and without the connecting region amino acids was inversely related to the number of crossing disulfide bonds. This is a surprising result in light of the fact that all of the EGF-like domains that have been studied to date are structurally similar. Even though they have very different sequences, the cysteines are highly conserved and are thought to determine the structure. All of the EGF-like domains that have been studied to date have the EGF-like disulfide-bonding pattern, although the list of EGF-like do-

mains for which the disulfide bonds have been determined is quite short.

Preliminary NMR spectra of TM5xt(1-3,2-5,4-6), TM5xt(1-3, 2-4,5-6), and TM5xt(1-2,3-4,5-6) indicate that the degree of disulfide crossing influences the flexibility of the N-terminal subdomain with respect to the C-terminal subdomain (M. Hunter, unpubl. data). These spectra also show that many of the resonances are exchange broadened, suggesting that all of the disulfide-bonded isomers of this domain are conformationally flexible. A peptide analog of the C-terminal loop of the fifth domain, which is missing I420, has no structure when free in solution but becomes a tristranded β-sheet upon binding to thrombin (Srinivasan et al., 1994). It is therefore possible that the whole fifth EGF-like domain of TM binds to thrombin by an induced fit mechanism, and that the more flexible domain, the one with no crossing disulfide bonds, can therefore bind most tightly. Future structural studies on the different disulfide-bonded domains in the presence and absence of thrombin should shed light on this interesting phenomenon.

Materials and methods

Peptide synthesis

All peptides were synthesized using amino acids that were protected with a fluorenylmethoxycarbonyl group on the amine and were preactivated as the pentafluorophenyl esters on the carboxylic acid. Standard solid-phase peptide synthesis was carried out on a Milligen 9050 peptide synthesizer. The amino acids, PEG-PS-threonine, and PEG-PS-glutamate solid support and hydroxyazabenzotriazole were from Milligen/Biosearch (Bedford, Massachusetts), and dimethylformamide (glass distilled) was from EM Sciences (distributed by VWR, Los Angeles, California). Coupling efficiency was monitored during the synthesis and double coupling was performed as necessary.

After synthesis was complete, the resin was washed extensively with dichloromethane and dried overnight under vacuum. Peptides were cleaved from the resin and protecting groups were removed by treatment of the resin with a mixture of trifluoroacetic acid (TFA; 90%), thioanisole (5%), ethanedithiol (3%), and anisole (2%) for 2-4 h with gentle rocking. After removal of the resin by filtration through glass wool, the peptides were isolated by ether precipitation overnight at −20 °C. The precipitate was collected on a cintered glass funnel, dissolved in 5% ammonium acetate buffer, pH 5.0, and freeze-dried. Yield of crude peptides was 100% of the theoretical yield for a 0.2 mmol synthesis scale.

Portions of the crude peptides (100 mg) were dissolved in 10 mL Tris-HCl buffer (100 mM, pH 8.2) and reduced under nitrogen with dithiothreitol (78.4 mM) for 2 h. The fully reduced peptides were then purified by reverse-phase HPLC on a Waters DeltaPak C_{18} column (19 mm × 300 mm) in argon-sparged buffers (gradient of 0.1% trifluoroacetic acid to 50% acetonitrile over 1 h). The HPLC trace showed that greater than 60% of the product eluted as a single peak. Peptides were shown by mass spectrometry and amino acid analysis to have the correct amino acid composition.

Peptide oxidation by glutathione exchange

Purified, reduced, lyophilized peptides were refolded by air oxidation in 0.5% NH_4CH_3COOH buffer, pH 8.3. The peptides (25 mg) were first dissolved in 100 mL of 5% NH_4CH_3COOH buffer, pH 5, and then diluted to 1 L in a redox buffer containing 1 mM reduced/0.1 mM oxidized glutathione in 0.5% NH_4CH_3COOH buffer, pH 8.3, at a peptide concentration of 0.025 mg/mL. The solution was stirred, and the pH was readjusted daily to 8.3 until the peptide was fully oxidized (4 days). The TM5 products were purified by reverse-phase HPLC as described for the reduced peptide except that the buffers were not sparged with argon, and the gradient was held at 30% acetonitrile for better separation of the isomers. The TM5xt products were purified by reverse-phase HPLC on a Vydac C_{18} (10 mm × 250 mm) analytical reverse-phase HPLC column at a flow rate of 3 mL/min using an extended gradient (100% buffer A [0.1% TFA] for 10 min, 0-10% acetonitrile over 10 min, 10-40% acetonitrile over 90 min) with detection at 226 nm. The three isolable products from the TM5 oxidation were shown to have the expected mass by electrospray mass spectrometry. For the TM5xt peptides, the two isolable products from this synthesis, in which all the cysteines were protected with trityl groups, were shown to have the correct mass (Table 1).

Preparation of TM5xt(1-3,2-4,5-6)

The procedure described by Yang et al. (1994) was used to force the disulfide bonds in TM5xt to be EGF-like. This procedure was modified to avoid the oxidation of M388 by replacing the final oxidation step with that described by Tamamura et al. (1993) (Hunter & Komives, 1995). The peptide containing two disulfide bonds and two S-Acm cysteines (10 mg) was treated with TFA (500 μL), anisole (10 μL), and silver trifluoromethanesulfonate (AgOTf) (27 mg) at 4 °C for 1.5 h. Ice-cold dry ether (1 mL) was added to the reaction mixture and the precipitate was isolated by centrifugation. The precipitate was then washed twice with ice-cold dry ether (2 mL) and the resulting peptide with S-Ag cysteine residues was isolated by centrifugation and subjected to mild oxidation in an aqueous solution containing 2 mL dimethylsulfoxide (DMSO) and 2 mL of 1 M HCl (4 mL total volume) for 7-24 h. The solution was then filtered and the filtrate diluted to 20 mL with deionized H_2O. The product was purified by reverse-phase HPLC as described above. Mass spectrometry indicated that the major product had no oxidized methionine and had the expected molecular weight (Table 1). The disulfide bonds were determined by the Gray procedure (Gray, 1993a) to be (1-3,2-4,5-6) as are found in EGF protein.

Determination of disulfide bonds

The disulfide-bonding patterns of the peptides were determined by a partial reduction method (Gray, 1993b). Typically, 100-500 μg of pure peptide was dissolved in 700 μL of buffer A (0.1% TFA in H_2O) to which 700 μL of an aqueous solution containing 20 mM (Tris) TCEP and 0.17 M citric acid, pH 3.0, was added. The mixture was allowed to incubate at room temperature for 10-30 min and then injected onto the HPLC. The resulting partially reduced peaks were isolated on a Vydac C_{18} (4.6 mm × 250 mm) analytical reverse-phase HPLC column at a flow rate of 1 mL/min using an extended gradient (100% buffer A [0.1% TFA] for 10 min, 0-10% acetonitrile over 10 min, 10-40% acetonitrile over 90 min) with detection at 280 nm. A portion of each elution peak (500 μL) was immediately injected into

a saturated solution of *N*-methyliodoacetamide (2.2 M) to alkylate the cysteines that had been reduced. After 30 s, the reaction was quenched by acidification with 800 μL of 0.5 M citric acid. Each resulting mixture was frozen until purification by reverse-phase HPLC. Finally, the purified, alkylated products were characterized by N-terminal sequencing.

The original Gray (1993a) procedure utilized iodoacetamide as the alkylating agent, but the phenylthiohydantoin (PTH) derivative of *S*-carboxamidomethylcysteine elutes at the same time as that of glutamic acid. This was problematic for determination of the disulfide bonding of the fourth and fifth cysteines in the fifth domain peptides because these cysteines are separated by a single glutamic acid. To circumvent this problem, we utilized *N*-methyliodoacetamide instead. This compound was prepared by the method of Krutzsch and Inman (1993) and purified by flash chromatography on silica gel in 50% dichloromethane/50% ethylacetate. The PTH derivative of *N*-methyl-*S*-carboxamidomethylcysteine elutes approximately 1 min before the PTH derivative of histidine in a standard N-terminal sequencer. This derivative of iodoacetamide was even more soluble in Tris-acetate buffer than iodoacetamide, and we did not observe any evidence for disulfide bond exchange. Repurification of the alkylated products showed a single major product for each original partially reduced peak.

Determination of peptide concentration

Peptides were dissolved in H_2O at a concentration of 1–2 mM and the pH was adjusted to pH 5–6 with 1 N NaOH. The peptide solutions were divided into small portions and stored at −20 °C. The concentration of each peptide solution was determined by quantitative amino acid analysis using norleucine as the standard.

Assay for inhibition of clot formation

The direct inhibition of thrombin-induced clot formation by TM was assayed as described previously (Lougheed et al., 1995). The fifth domain peptides were used in place of TM in this assay. The increase in clotting time was linear over a range of peptide concentrations 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. For ease of comparison of the data, the results are also tabulated as the peptide concentration required to double the clotting time.

Assay for inhibition of protein C activation

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 (Lougheed et al., 1995). For the TM5 peptides, only the IC_{50} was determined but we have previously demonstrated that under the conditions of the assay in which the concentration of TM is much lower than its K_m, the IC_{50} should be similar to the K_i (Lougheed et al., 1995). For the other fifth domains, the K_i was measured by performing the protein C assay described above at several different TM concentrations (0.14, 0.21, 0.29, and 0.35 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 $-K_i$. In order to show the comparison of the results for the different disulfide-bonded isomers on the same plot, the percent inhibition was also measured. As for the TM5 peptides, the K_i was also be determined from the concentration that inhibited protein C activation by 50%. In every case, the K_i obtained from the Dixon plots agreed with that obtained from the IC_{50} to within 15%.

Assay for chromogenic substrate cleavage by thrombin

The assays for cleavage of S2222 (Chromogenix) and SpTH (American Diagnostica) by thrombin in the presence of each of the isomeric fifth domains of TM were performed exactly as described by Ye et al. (1992).

Acknowledgments

We thank Dr. John Fenton for the generous gift of human α-thrombin. We thank Dr. Jack Kyte for use of his amino acid analyzer; Drs. Susan Taylor and Siv Garrod for N-terminal sequencing; Drs. A.L. Burlingame and Kati Medzihradszky at the University of California San Francisco Mass Spectrometry Facility and Gary Siusdak at the Scripps Research Institute for electrospray mass spectrometry analysis; and Craig Dobbs for advice on peptide synthesis. This work was supported by NIH grant HL47463, by the Rita Allen Foundation, and by the Searle Scholars program. This work was done during the tenure of a graduate research fellowship from the American Heart Association, California Affiliate to M.J.H.

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