Functional Domains of Membrane-bound Human Thrombomodulin

EGF-LIKE DOMAINS FOUR TO SIX AND THE SERINE/THREONINE-RICH DOMAIN ARE REQUIRED FOR COFACTOR ACTIVITY*

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Manuel Tsiang§§, Steven R. Lentz§§, and J. Evan Sadler‡‡§§

From the lHoward Hughes Medical Institute and §Division of Hematology-Oncology, Departments of Medicine and of Biochemistry and Molecular Biophysics, The Jewish Hospital of Saint Louis, Washington University School of Medicine, Saint Louis, Missouri 63110

Thrombomodulin is an endothelial cell thrombin receptor that serves as a cofactor for thrombin-catalyzed activation of protein C. Structural requirements for thrombin binding and cofactor activity were studied by mutagenesis of recombinant human thrombomodulin expressed on COS-7 and CV-1 cells. Deletion of the fourth epidermal growth factor (EGF)-like domain abolished cofactor activity but did not affect thrombin binding. Deletion of either the fifth or the sixth EGF-like domain markedly reduced both thrombin binding affinity and cofactor activity. Thrombin binding sequences were also localized by assaying the ability of synthetic peptides derived from thrombomodulin to compete with diisopropyl fluorophosphate-inactivated 125I-thrombin binding to thrombomodulin. The two most active peptides corresponded to (a) the entire third loop of the fifth EGF-like domain \( K_d = 85 \pm 6 \) \( \mu \)M and (b) parts of the second and third loops of the sixth EGF-like domain \( K_d = 117 \pm 9 \) \( \mu \)M. These data suggest that thrombin interacts with two discrete elements in thrombomodulin. Deletion of the Ser/Thr-rich domain dramatically decreased both thrombin binding affinity and cofactor activity and also prevented the formation of a high molecular weight thrombomodulin species containing chondroitin sulfate. Substitutions of this domain with polypeptide segments of decreasing length and devoid of glycosylation sites progressively decreased both cofactor activity and thrombin binding affinity. This correlation suggests that increased proximity of the membrane surface to the thrombin binding site may hinder efficient thrombin binding and the subsequent activation of protein C. Membrane-bound thrombomodulin therefore requires the Ser/Thr-rich domain as an important spacer, in addition to EGF-like domains 4-6, for efficient protein C activation.

Thrombomodulin is an endothelial cell integral membrane glycoprotein. By binding to thrombin in a 1:1 stoichiometric complex (1), thrombomodulin inhibits the procoagulant functions of thrombin (2, 3) and serves as a cofactor for thrombin-catalyzed activation of the anticoagulant protein C. These activities make thrombomodulin an important physiological anticoagulant (4).

Thrombomodulin contains a large amino-terminal extracellular region, a single transmembrane segment, and a short cytoplasmic tail (5-7). The extracellular region is composed of a lectin-like domain, six tandemly repeated EGF-like domains, and a Ser/Thr-rich domain. An elastase-digested rabbit thrombomodulin fragment containing the six EGF-like domains was shown to have both thrombin binding capacity and cofactor activity (8). Further cleavage of this fragment with cyanogen bromide generated a fragment containing only the fifth and sixth EGF-like domains that lacked cofactor activity but retained the ability to bind thrombin (9). Deletion studies on secretable recombinant human thrombomodulin confirmed the localization of thrombin binding to the fifth and sixth EGF-like domains and reduced the minimal essential region for cofactor activity to the last three EGF-like domains (10, 11). These authors suggested that the fourth EGF-like domain may bind protein C through calcium ions (12).

In vivo, thrombomodulin is thought to function on the endothelial cell surface. Free thrombomodulin can be detected in plasma but only at a low concentration (13), and its physiological significance is unknown (14). To identify structural elements important for the function of thrombomodulin on the cell surface, we have expressed a series of NH2-terminal and internal deletion mutants of membrane-bound recombinant human thrombomodulin. These studies were complemented with competition binding experiments using synthetic peptides spanning the fifth and sixth EGF-like domains of human thrombomodulin. The results suggest that in addition to EGF-like domains 4-6, the Ser/Thr-rich domain is required for optimal cofactor activity. Moreover, the thrombin binding site of thrombomodulin appears to be composed of discrete regions within EGF-like domains 5 and 6.

MATERIALS AND METHODS

Plasmids—The previously described thrombomodulin expression vector pRSVTM (20) was modified by the insertion of a 151-base pair SV40 origin fragment into the SphI site of the vector sequence; this construct, pRSVSVTM, is suitable for both stable expression in CV-1 cells and transient expression in COS-7 cells. Plasmid pTMCN was derived from pRSVSVTM by the introduction of unique Sac1 and NolI recognition sites in the thrombomodulin cDNA sequence to facilitate subsequent constructions. Both sites were introduced by oligonucleotide-directed mutagenesis using the polymer-
Recombinant Membrane-bound Thrombomodulin

The amino acid residues of thrombomodulin are referred to by codon number, and the amino terminus of native thrombomodulin is alanine 19 (6). Construction of the SacI site changes proline 23 into leucine. The Nol site is adjacent to the termination codon, outside of the coding region.

All deletions were made by oligonucleotide-directed mutagenesis using the PCR. The N-terminal deletions were constructed using SacI site-containing 5'-oligonucleotide primers that matched different deletion end points, and either Nol or NotI site-containing oligonucleotides as 3'-primers (Fig. 1). The deletion fragments (SacI/NolI) or (SacI/NotI) were synthesized using PCR as templates and cloned into vector fragments (Nol/SacI) or (NotI/SacI) derived from pTMNC. The internal deletions used BclI site-containing oligonucleotides that matched the different deletion end points as either 5'- or 3'-primers. Each internal deletion necessitated the synthesis of two fragments (SacI/BclI) and (BclI/NotI) which were then cloned into the vector fragment (Nol/SacI) derived from pTMNC in a three-member ligation reaction. The deletion mutants derived from pTMNC were named pTMNCΔx/y, where x refers to the first deleted codon number and y refers to the last deleted codon number. To increase the level of expression, some mutant inserts were subcloned into pCMV (16) which makes use of a stronger CMV promoter. The corresponding deletion subclones were named pCMVNCΔx/y.

Plasmids pOsO(+), pOsO(−) (where "O" refers to the Ser/Thr-rich domain and the lower case "a" signifies "is substituted by") were constructed by reinsertion in positive and negative orientations, respectively, of the PCR-synthesized Ser/Thr-rich domain into the BclI deletion junction of pTMNCΔ481/513. Plasmids pOsH(1/2) and pOsH(1/1) were derived by insertion of synthetic oligonucleotides encoding 17 and 34 amino acid peptides (referred to as "H" for their presence of the BclI site). The underlined amino acids represent the terminal cap residues predicted to favor the formation of an α-helix.

The sequences of all synthetic inserts and amplified constructs were confirmed by dideoxy sequencing (19).

Cell Culture and Transfections—COS-7 and CV-1 cells were used for transient and stable transfections, respectively. Cell culture and transfection conditions were described previously (20). The sequences of all synthetic inserts and amplified constructs were confirmed by dideoxy sequencing (19).

Cell culture media, After reaching confluency, the cells were washed once with FACS buffer (per cent human thrombomodulin.

The four clones corresponding to the five EGF-like domains (NdL, NdE1, NdE2, NdE3, and NdE4). The four clones corresponding to the five EGF-like domains (NdL, NdE1, NdE2, NdE3, and NdE4).

TABLE I

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Sequencea</th>
<th>Predicted secondary structureb</th>
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<tbody>
<tr>
<td>pTMNC</td>
<td>DSGKVDGDSGSGEEPPSPPTGSLTPPAVGLVH</td>
<td>Turns and β-sheets</td>
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<tr>
<td>pOsO(−)</td>
<td>DQRRPPEEERWSASAGAQRSGCHRPCRD</td>
<td>Turns and β-sheets</td>
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<tr>
<td>pOsH(1/1)</td>
<td>PDEAQAAAQAAQAQAAAQAAQQAAQAAQOKRD</td>
<td>α-Helix</td>
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<tr>
<td>pOsH(2/1)</td>
<td>PDEAQAAAQAA</td>
<td>KRHD</td>
</tr>
<tr>
<td>pOsH(1/2)</td>
<td>PDEE</td>
<td>KRHD</td>
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<tr>
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<td>PDEE</td>
<td>KRHD</td>
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<tr>
<td>pOsH(1/5)</td>
<td>DQ</td>
<td>ID</td>
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<tr>
<td>pTMNCΔ481/513</td>
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</table>

a The bold type represents an amino acid required by the presence of the BclI site. The underlined amino acids represent the terminal cap residues predicted to favor the formation of an α-helix.

b Chou-Pasman prediction (18).
FIG. 1. Schematic representation of deletion mutants of human thrombomodulin. The different domains of thrombomodulin are represented to scale from codon 19 to codon 576, the signal peptide being omitted. The numbers in the name of the plasmids indicate the inclusive codon boundaries of the deletion. The restriction sites SacI, NotI, Nhel, and BclI are indicated. L, lectin-like domain; 1-6, EGF-like domains 1-6; O, Ser/Thr-rich domain; T, transmembrane domain; C, cytoplasmic domain.

![Schematic representation of deletion mutants of thrombomodulin.](image_url)

FIG. 2. Transient expression of mutant thrombomodulins in COS-7 cells. A, deletion mutants of thrombomodulin. B, substitution mutants of thrombomodulin. The names of the mutant constructs of plasmid pTMNC used for transfection are indicated. pJC119 is a vector control. Ten micrograms of DNA were used to transfect 1 x 10⁷ COS-7 cells. Two days post-transfection, the cells were metabolically labeled with [35S]cysteine for 5 h. Thrombomodulin was immunoprecipitated from the cell lysate by the polyclonal antiserum (see "Materials and Methods") and analyzed on 10% polyacrylamide gels under reducing conditions. In A, lanes 0-7 were exposed for 5 days and lanes 8-11 are from a 14-day exposure of the same gel.

EGF-like, both the fifth and sixth EGF-like, and the Ser/Thr-rich domains are: IdE5, IdE6, IdE56, and ID0, respectively. Clone TM, transfected with pRSVTM (the parent construct of pTMNC), was also selected. All of the thrombomodulin species expressed by these clones were correct in size by polyacrylamide gel electrophoresis (data not shown). There was no difference in either thrombin binding (Table II) or cofactor activity (data not shown) between TMnc and TM, indicating that the proline 23 to leucine substitution in TMnc did not alter thrombomodulin cofactor function. Cell surface thrombomodulin expression was demonstrated for all clones except IdE5 and IdE56, with a ²⁻⁻⁺ for thrombin was estimated using the

<p>| Table II: Surface expression level of thrombomodulin mutants in stably transfected CV-1 cell lines and their affinity for thrombin. |
|---------------------------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Cell line</th>
<th>Kᵣ (nM)</th>
<th>Rₐ₇₅ (fmol/10⁶ cells)</th>
<th>Rₐ₇₅ (fmol/10⁶ cells)</th>
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<tbody>
<tr>
<td>Full-length thrombomodulins</td>
<td></td>
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<tr>
<td>CV-1(18A)</td>
<td>3.14 ± 0.80</td>
<td>360 ± 23</td>
<td>ND</td>
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<tr>
<td>TM</td>
<td>1.37 ± 0.37</td>
<td>197 ± 14</td>
<td>196 ± 21</td>
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<td>TMnc</td>
<td>2.63 ± 0.64</td>
<td>504 ± 34</td>
<td>488 ± 21</td>
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<td>Deletion mutants</td>
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<td>NdL</td>
<td>2.60 ± 0.71</td>
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<td>533 ± 48</td>
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<tr>
<td>NdE1</td>
<td>1.55 ± 0.31</td>
<td>291 ± 17</td>
<td>321 ± 10</td>
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<td>NdE2</td>
<td>2.97 ± 1.16</td>
<td>288 ± 40</td>
<td>133 ± 15</td>
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<tr>
<td>NdE3</td>
<td>2.34 ± 1.68</td>
<td>145 ± 24</td>
<td>122 ± 10</td>
</tr>
<tr>
<td>NdE4</td>
<td>1.52 ± 0.41</td>
<td>116 ± 9</td>
<td>169 ± 9</td>
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<tr>
<td>IdE5</td>
<td>No binding</td>
<td>No binding</td>
<td>No binding</td>
</tr>
<tr>
<td>IdE6</td>
<td>27.5 ± 11.9</td>
<td>301 ± 56</td>
<td>314 ± 50</td>
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<tr>
<td>IdE56</td>
<td>No binding</td>
<td>No binding</td>
<td>ND</td>
</tr>
<tr>
<td>Id0</td>
<td>&gt;700</td>
<td>&lt;100</td>
<td>77 ± 50</td>
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<tr>
<td>Substitution mutants</td>
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<tr>
<td>OsO(–)</td>
<td>&gt;18</td>
<td>&lt;100</td>
<td>24 ± 07</td>
</tr>
<tr>
<td>OsH(1/1)</td>
<td>4.02 ± 3.48</td>
<td>61 ± 19</td>
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<td>OsH(1/2)</td>
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<td>&lt;100</td>
<td>62 ± 12</td>
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<td>OsH(1/4)</td>
<td>&gt;70</td>
<td>&lt;100</td>
<td>192 ± 12</td>
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<tr>
<td>OsH(1/8)</td>
<td>&gt;100</td>
<td>&lt;100</td>
<td>28 ± 14</td>
</tr>
</tbody>
</table>

For all mutants involving the Ser/Thr-rich domain, except OsH(1/1), the Kᵣ for thrombin was estimated using the Rₐ₇₅ determined by IgG-TM binding.

The mouse monoclonal antibody IgG-TM, specific for the fifth EGF-like domain, bound to all clones, except IdE5 and IdE56, with a Kᵣ of 1-3 nM. ND, not done.

CV-1(18A) is a previously characterized pool of 18 CV-1 clones stably transfected with pRSVTM (20).
thrombomodulin per cell \((R_{max})\) determined by each ligand correlated with a slope of 0.95 and a correlation coefficient of 0.93. IgG-TM bound with similar affinities to all mutant thrombomodulins containing the fifth EGF-like domain. DIP-thrombin bound to all of the NH2-terminal deletion mutants and to the parent protein (TMnc) with similar \(K_d\) values, but bound to IdE6, the internal deletion mutant of the sixth EGF-like domain, with lower affinity \((K_d = 27.5 \pm 11.0 \text{ nM})\). Clones IdE5 and IdE56, with internal deletions of the fifth EGF-like domain, showed no detectable binding of DIP-thrombin. In addition, clone Id0, the internal deletion of the Ser/Thr-rich domain, bound DIP-thrombin with very low affinity \((K_d > 700 \text{ nM})\).

Cell surface cofactor activity for protein C activation was determined for each clone (Fig. 3A). Full activity was retained when the NH2-terminal deletion extended up to and included the third EGF-like domain. However, when the fourth EGF-like domain was also deleted, there was an abrupt loss of all activity. Total loss of activity was also observed with the internal deletion mutant of the fifth EGF-like domain. There was a residual activity of <0.5% when the sixth EGF-like domain alone was deleted. Deletion of the Ser/Thr-rich domain also dramatically reduced cofactor activity to <3%.

**Competition of Synthetic Thrombomodulin Peptides with DIP-thrombin**—The site on thrombomodulin that interacts with thrombin was also localized by competition binding studies. Eight overlapping synthetic thrombomodulin peptides spanning the fifth and sixth EGF-like domains were initially tested for inhibition of \(^{125}\text{I}-\text{DIP}-\text{thrombin} (Fig. 4). Peptide Tm\text{426-444} which was described previously (20) corresponded to the third loop of the fifth EGF-like domain and was the best competitor \((K_p = 85 \pm 6 \text{ M})\) among the eight peptides. The second best competitor was Tm\text{460-474} which overlapped the second and third loops of the sixth EGF-like domain \((K_p = 117 \pm 9 \text{ M})\). Peptides Tm\text{418-436} and Tm\text{451-466} from the second and third loops of the fifth EGF-like domain \((K_p = 1317 \pm 113 \text{ M})\) and Tm\text{460-466} from the second loop of the sixth EGF-like domain \((K_p = 796 \pm 48 \text{ M})\), had much lower affinities for thrombin. The remaining four peptides all had values for \(K_p > 5000 \text{ M}\).

Based on these initial results, additional peptides from the third loop of the fifth EGF-like domain were tested. Peptides Tm\text{436-440} and Tm\text{426-436} competed with lower affinity \((K_p = 1009 \pm 91 \text{ and } 2370 \pm 334 \text{ M}, \text{respectively})\) than Tm\text{426-444} (Fig. 4). These results provide independent evidence that elements in both the fifth and sixth EGF-like domains are important for high affinity binding of thrombin to thrombomodulin.

To determine whether disulfide bond formation between cysteines 427 and 439 was contributing to binding, the first cysteine residue in peptide Tm\text{426-444C/A} from the second loop of the fifth EGF-like domain \((K_p = 117 \pm 9 \text{ M})\) was changed to an alanine. This peptide, otherwise identical to Tm\text{426-444} but incapable of intrapeptide disulfide bond formation, competed with thrombomodulin for \(^{125}\text{I}-\text{DIP}-\text{thrombin} binding with an affinity similar to Tm\text{426-444} \((K_p = 42 \pm 4 \text{ M})\) (Fig. 4).

**Substitution of the Ser/Thr-Rich Domain**—Deletion of the Ser/Thr-rich domain essentially inactivated the mutant thrombomodulin expressed on cell line Id0 (Table II; Fig. 3B), and this was unexpected. To show that this result was not due to the introduction of a BclI site at the deletion junction, changing serine 515 to glutamine, a separate construct deleting the Ser/Thr-rich domain was made using a PCR strategy that does not introduce a new restriction site (17). The new deletion mutant displayed the same low cofactor activity as Id0 (data not shown).

To determine whether full activity could be recovered from plasmid pTMNC\text{A481}5/13, the Ser/Thr-rich domain was reinserted into the BclI site at the deletion junction. Reinsertion in either the original orientation (plasmid pOsO(+) or in the reverse orientation (plasmid pOsO(-)) (Table I) restored cofactor activity in transiently transfected COS-7 cells (data not shown). The product of pOsO(-) had a higher mobility than that of pOsO(+) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 2B, lanes 3 and 4), suggesting differential glycosylation.

To determine the effect of insert size, independent of glycosylation, synthetic oligonucleotides of different lengths were
substituted for the original sequence encoding the Ser/Thr-rich domain (Table I). The sequences encoded an Ala-Gln copolymer flanked by tetrapeptide caps favoring an α-helical conformation (25) and did not contain potential glycosylation sites. These substitution constructs were expressed efficiently in transiently transfected COS-7 cells (Fig. 2B). The effects of these substitutions on cofactor activity were compared in four stably transfected CV-1 clones: OsH(1/1), OsH(1/2), OsH(1/4), and OsH(1/8). The level of surface thrombomodulin expression was determined by equilibrium binding with $^{125}$I-labeled IgG-TM (Table II). $^{125}$I-DIP-thrombin bound to OsH(1/1) and to the parent construct, TMnc, with similar affinities, but bound with decreasing affinities to constructs with shorter spacers (Table II). Except for cell line OsH(1/1), the value for $K_D$ of thrombin binding to the cell lines expressing mutations of the Ser/Thr-rich domain was approximate because of either a low level of thrombomodulin surface expression or low affinity for thrombin. This condition prevented the simultaneous solution of the $R_{max}$ and $K_D$ for thrombin binding by curve fitting. To estimate the value of $K_D$ for thrombin, binding equations using the $R_{max}$ predetermined from IgG-TM binding were employed to fit the thrombin binding data. Reinsertion of the Ser/Thr-rich domain in the reverse orientation, as in clone OsO(-), essentially restored full cofactor activity (Fig. 3B). Substitution of this domain with an Ala-Gln copolymer of 34 amino acids (the same number of residues as the original domain) restored $75 \pm 22\%$ of activity in OsH(1/1) (Fig. 3B). An Ala-Gln copolymer half as long restored $50 \pm 13\%$ of activity in OsH(1/2) (Fig. 3B). The ability to restore cofactor activity continued to decrease as the spacer was shortened further. When the spacer was only four amino acids long, no activity was restored.

Presence and Localization of Chondroitin Sulfate in Mutant Thrombomodulins—In a previous study, the CV-1(18A) cell line was shown to express two forms of recombinant thrombomodulin: a minor species of 160,000 daltons that contains a chondroitin sulfate moiety and a major species of 100,000 daltons that does not contain chondroitin sulfate (22). To determine whether chondroitin sulfate was also associated with recombinant thrombomodulin lacking the Ser/Thr-rich domain, cell lines were incubated with either $[^{35}]$S$-$cysteine or $[^{35}]$S$-$sulfate, and radiolabeled thrombomodulin was immunoprecipitated and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. When labeled with $[^{35}]$S$-$cysteine, cell lines TMnc, IdO, OsO(-), and NdL showed major thrombomodulin bands consistent with the absence of chondroitin sulfate (Fig. 5A). After labeling with $[^{35}]$S$-$sulfate (Fig. 5B), TMnc and NdL cells showed an additional high molecular weight chondroitinase ABC-sensitive band and also incorporated $[^{35}]$S$-$sulfate into a chondroitinase-resistant species having the same electrophoretic mobility as the major species labeled with $[^{35}]$S$-$cysteine. The chondroitinase-sensitive species of the deletion mutant, NdL (Fig. 5B, lane 7), has greater electrophoretic mobility than that of the full-length construct TMnc (Fig. 5B, lane 1). This correspondence suggests that the chondroitin sulfate moiety is specifically associated with the thrombomodulin polypeptide, probably by a covalent linkage. No $[^{35}]$S$-$sulfate was incorporated into either chondroitinase-sensitive or chondroitinase-resistant species of recombinant thrombomodulins expressed by cell lines IdO and OsO(-). Both of these mutant proteins lack the thrombomodulin Ser/Thr-rich domain, and this result suggests that sulfate may be incorporated into both chondroitin sulfate and other Ser/Thr-linked sulfated oligosaccharides attached to this domain. In mutant thrombomodulin OsO(-), the Ser/Thr-rich domain is replaced by a sequence that contains 4 serine residues, but this mutant does not incorporate $[^{35}]$S$-$sulfate. Therefore the addition of both chondroitin sulfate and other O-linked sulfated oligosaccharides to thrombomodulin may require specific hydroxyamino acid-containing sequences.

**DISCUSSION**

The present study analyzes the structural requirements for thrombin binding and thrombomodulin cofactor activity by deletion and substitution mutagenesis of recombinant membrane-bound thrombomodulin. Thrombin binding sequences were also localized by assaying the ability of synthetic peptides, derived from thrombomodulin, to inhibit $^{125}$I-DIP-thrombin binding to thrombomodulin.

NH$_2$-terminal deletions of consecutive structural elements of thrombomodulin showed that the lectin-like domain and EGF-like domains 1–3 are dispensable for cofactor activity (Fig. 3A); EGF-like domain 4 is required for cofactor activity (Fig. 3A) but not for thrombin binding (Table II). Deletions of EGF-like domains 5 or 6 dramatically reduced both thrombin binding and cofactor activity. With one potentially significant difference, these results with membrane-bound forms of thrombomodulin are consistent with previous studies of soluble forms of thrombomodulin, produced either by proteolytic digestion (9) or by recombinant DNA methods (11, 12). A soluble recombinant human thrombomodulin fragment (EGF45), consisting of EGF-like domains 4 and 5, retained ~10% of the cofactor activity of a fragment that in addition contained EGF-like domain 6 (EGF456) (12). In contrast, membrane-bound recombinant thrombomodulin lacking EGF-like domain 6 (IdE6), retained only <0.5% of the cofactor activity of full-length thrombomodulin (TMnc) (Fig. 3A). This difference in the effect of deleting a single EGF-like domain suggests that the constraints on thrombomodulin structure-function relationships are not identical for soluble and membrane-bound thrombomodulin.

Competition binding experiments with synthetic thrombomodulin peptides provide additional support for the participation of both EGF-like domains 5 and 6 in thrombin binding (Fig. 4). A peptide corresponding to the third disulfide loop...
of EGF-like domain 5 (Tm426-444) inhibited thrombin-thrombomodulin binding (Fig. 4) as reported previously (20, 12); this peptide binds directly to thrombin (28). Peptides corresponding to other regions of EGF-like domain 5 did not have inhibitory activity (Fig. 4) (12). A peptide corresponding to part of EGF-like domain 6 (Tm460-474) also inhibited thrombin-thrombomodulin binding. In addition, the acidic tetrapeptide Asp-Ile-Asp-Glu that links EGF-like domains 5 and 6 contributed to the inhibitory activity of peptide Tm426-444, since removal of this sequence in peptide Tm426-440 markedly increased the Kc (Fig. 4). Thrombomodulin interacts with an anion-binding exosite on thrombin (20), and mutation of basic amino acids within this exosite inhibits binding of thrombin to thrombomodulin (16). The acidic character of the inhibitory thrombomodulin peptides suggests that electrostatic interactions account for part of the free energy of thrombin-thrombomodulin binding.

The most potent inhibitory peptide (Tm426-444) is mono-meric and has >75% of its cysteines in an oxidized state, consistent with the intrapeptide disulfide bond; the same disulfide bond is predicted to be present in native thrombomodulin, based upon the structures determined for the homologous proteins EGF (27) and transforming growth factor-α (28, 29). Substitution of alanine for cysteine in peptide Tm426-444C4A, to prevent cyclization, did not reduce the inhibitory activity of the peptide. Thus, the predicted disulfide bond between cysteine 427 and cysteine 439 may not be required for thrombomodulin cofactor activity (Fig. 3) or thrombin binding affinity (Table II).

Secretable derivatives of thrombomodulin that lack the Ser/Thr-rich domain were reported to have cofactor activity similar to that of full-length thrombomodulin (10). Unexpectedly, deletion of the Ser/Thr-rich domain from membrane-bound thrombomodulin (Id0) markedly reduced cofactor activity (Fig. 3) and thrombin binding affinity (Table II), suggesting that this domain performs an important spacer function. Fluorescence energy transfer measurements showed that the thrombin binding site on thrombomodulin was positioned more than 45 Å above the membrane. To test the necessity of a spacer between the thrombin binding site and the membrane surface, a predicted α-helical structure was chosen to replace the Ser/Thr-rich domain. This α-helical structure contains 34 residues and would provide a rather stable rod to raise the sixth EGF-like domain to a height of ~52 Å above the membrane. This structure does not contain any potential glycosylation sites. Recombinant thrombomodulin with this substitution (OsH(1/1)) had cofactor activity similar to that of the parent construct (TMnc). Both the affinity for thrombin and cofactor activity decreased as the length of this structure was decreased. When the segment between EGF-like domain 6 and the membrane surface was shortened to only 4 residues, the cofactor activity level was similar to that of the Ser/Thr-rich domain deletion mutant, Id0 (Fig. 3B). These results suggest that the proximity of the membrane surface to the thrombin binding site may hinder efficient thrombin binding and subsequent interaction of thrombin with protein C and that the Ser/Thr-rich domain acts as a spacer to correctly position the thrombin binding site above the membrane.

These results also suggest an explanation for the relatively small effect on cofactor activity of deleting EGF-like domain 6 from soluble forms of thrombomodulin. For membrane-bound thrombomodulin, deletion of EGF-like domain 6 in IdE6 may place the thrombin binding site of EGF-like domain 5 unfavorably close to the cell membrane; this constraint would not apply to the soluble mutant thrombomodulin EGF45.

The Ser/Thr-rich domain of human thrombomodulin contains one potential Ser-Gly-X-Gly glycossaminoglycan attachment site consensus sequence (31). However, glycosaminoglycans are found at sites that do not conform to this consensus (32). Recent studies show that secretable recombinant human thrombomodulin can be modified by a chondroitin sulfate side chain, and this modification markedly affects both the direct and the antithrombin III-dependent anticoagulant activities of the mutant thrombomodulins (33–35). Our data show that the Ser/Thr-rich domain is necessary for the addition of chondroitin sulfate and probably of other Ser/Thr-linked oligosaccharides, at least some of which are sulfated (Fig. 5).

However, substitution of this domain with sequences devoid of glycosaminoglycan attachment or other glycosylation sites (OsO(−) and OsH(1/1)) did not significantly impair thrombomodulin cofactor activity, indicating that neither chondroitin sulfate nor Ser/Thr-linked glycosylation is essential for efficient protein C activation (Fig. 3). This result is consistent with the modest effect on protein C activation of the chondroitin sulfate attached to secretable recombinant human thrombomodulin (33, 34). When lysates of CV-1 expressing recombinant thrombomodulin were assayed for their ability to prolong the thrombin time, no significant differences were detected between TMnc, IdO, OsO(−), and untransformed CV-1 cells (data not shown), suggesting that none of these recombinant thrombomodulins displayed a significant direct anticoagulant activity. This observation may be accounted for by the fact that >90% of the thrombomodulin expressed in CV-1 cells lacks chondroitin sulfate (22) (Fig. 5).

In conclusion, proximity to the cell surface influences the properties of membrane-bound thrombomodulin, and the Ser/Thr-rich domain is necessary for optimal function of the thrombin-binding site. The requirement for such a spacer is not apparent for soluble thrombomodulin derivatives. In addition, discrete segments of both EGF-like domains 5 and 6 may interact directly with thrombin.

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REFERENCES
Recombinant Membrane-bound Thrombomodulin