A Domain Composed of Epidermal Growth Factor-like Structures of Human Thrombomodulin Is Essential for Thrombin Binding and for Protein C Activation*

(Received for publication, July 22, 1988)

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Thrombomodulin, an endothelial thrombin receptor, acts as a cofactor for the thrombin-catalyzed activation of anticoagulant protein C. The extracellular region of human thrombomodulin consists of three tentative domains, a NH2-terminal domain (D1), a domain involving six consecutive epidermal growth factor-like structures (D2), and an O-glycosylation-rich domain (D3). To identify the domain onto which thrombin binds, a series of recombinant proteins corresponding to the entire protein, D1, D2, D1 + D2, D1 + D2 + D3, and D2 + D3 were expressed in simian COS-1 cells. The proteins were partially purified by rabbit anti-thrombomodulin-F(ab')2-agarose chromatography. Western blotting analysis showed the expression of the respective recombinant proteins. All proteins involving D2, as well as D2 alone, had cofactor activity that allowed binding directly to thrombin, but D1 did not. The cofactor activity of the entire protein but not the mutants is increased in the presence of phospholipids and this is the only protein that binds to the phospholipid layer. These results indicate that the domain involving the epidermal growth factor-like structures of thrombomodulin is essential for thrombin binding and expression of the cofactor activity for protein C activation and that none of the extracellular domains interact with phospholipids.

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Thrombomodulin, an high affinity thrombin receptor (Kd = 4.8 × 10^-18 M) on the vascular endothelial cell membrane (1, 2), accelerates the thrombin-catalyzed activation of plasma anticoagulant protein C (3-5), inhibits several procoagulant actions of thrombin (6, 7), and also may internalize the thrombin bound to thrombomodulin into the cells (8). Previously, we determined the nucleotide sequences of human thrombomodulin cDNA (91, 92), and genomic DNA (10). The amino acid sequence, as deduced from the nucleotide sequence, indicated that thrombomodulin is synthesized as a precursor consisting of 575 residues including an estimated 18-residue signal peptide. The mature protein is composed of a NH2-terminal extracellular region, a transmembrane region, and a COOH-terminal cytoplasmic region. The extracellular region, with which thrombin may interact, consists of a NH2-terminal domain (D1), a domain with six consecutive epidermal growth factor (EGF)-like structures (D2) and an O-glycosylation-rich domain (D3).

To clarify onto which domain in the extracellular region of thrombomodulin thrombin binds, we constructed six proteins with D1, D2, and/or D3, and then determined the cofactor activity of these proteins for the thrombin-catalyzed activation of protein C in the presence of absence of phospholipids, which are known to enhance the activation (11, 12). The binding of the recombinant proteins to thrombin and to the phospholipid layer was also studied.

EXPERIMENTAL PROCEDURES

Materials—All chemicals used were of the highest commercial grade available. Protein C (4), thrombin (13), and placent thrombomodulin (9) were purified from human materials as described previously. Peroxidase-conjugated anti-rabbit IgG-goat IgG, 4-chloro-1-naphthol, 4-methylumbelliferyl-β-D-galactoside, and prestandard marker proteins for sodium dodecyl sulfate-polyacrylamide gel electrophoresis were obtained from Bio-Rad. Boc-Leu-Ser-Thr-Arg-4-methyl-coumaryl-7-amide, a fluorescent substrate for activated protein C (14), was obtained from the Protein Research Foundation, Osaka. A synthetic thrombin inhibitor, (2R, 4R)-4-methyl-1-[N-(3-methyl-1,2,3,4-tetrahydro-8-quinoline sulfate)-L-arginyl]-2-piperidine carboxylic acid monochloride (MD-905) (15) was provided by Mitsubishi-Kasei, Tokyo. The restriction endonucleases, dNTPs, T4 DNA ligase, T4 polynucleotide kinase, M13mp19, M13 sequencing kit, and 7-deaza sequencing kit were purchased from Takara Shuzo, Kyoto. [32P]dCTP (3000 Ci/mmol) and [γ-32P]ATP (6000 Ci/ mmol) were obtained from Amersham Corp. pSV2-Dehydrofolate reductase, an expression vector containing a DHFR cDNA insert, was obtained from ATCC (ATCC 37146). Nitrocellulose membrane filters and nylon membrane filters were purchased from Schleicher & Schuell and Bio-Rad, respectively. The phosphoplipid suspension was prepared using bovine brain extract (type I1, Sigma) (16). Anti-human thrombomodulin-F(ab')2, was prepared (17) from rabbit IgG (9). F(ab')2-Sepharose 4B was prepared using BrCN-activated Sepharose 4B (Pharmacia LKB Biotechnology Inc.) according to the instructions of the manufacturer.

Site-directed Deletion Mutagenesis—Plasmid pSV2TMJ2, which contains the entire sequence for human thrombomodulin (9), was digested with Ncol, filled in by DNA polymerase I (Klenow fragment), and then digested with HindIII. The obtained HindIII-Ncol fragment (1895 base pairs) was cloned into HindIII and HindII sites of plasmid M13mp19 using Escherichia coli JM106 as a host strain. This clone was designated M13mp19TM. Single strand recombinant phage DNA was used as a template for site-directed deletion mutagenesis was prepared (18). The oligonucleotides, which were complementary to the domain sequences immediately preceding and following the domain to be deleted, were synthesized. For construction of D1 + D2 + D3 (abre-
D4) and the cytoplasmic domain (designated D5). For construction used to delete the sequence of the transmembrane domain (designated D3 was used to delete the sequence of D2

CGGTGCCA3' was used to delete the sequence of D2

XbaI fragment (206 base pairs) from pSV2TMD12 were also ex-

0.2 M EDTA. Small aliquots of the reaction mixture were used to

pH 7.5,

were determined by the dideoxy chain termination method (19, 20).

DNA polymerase I (Klenow frag-

ment) (5 units), T4 DNA ligase (2 units), dNTPs (20 nmol each),

ATr (20 nmol), and 10 X Ht buffer (70 mM NaCl, 70 mM Tris-Cl,

pH 7.5, 70 mM Mg(CH3COO)2, 70 mM dithiothreitol) were added to

the annealed mixture to a final volume of 50 pL. After incubation at

37 °C for 30 min, the reaction was stopped by the addition of 5 pL of

0.2 M EDTA. Small aliquots of the reaction mixture were used to

transfect E. coli JM105. Plaque hybridization was performed using a

5'-32P-labeled deletion mutagenic oligonucleotide as a probe. Single-

stranded DNA and release factor DNA were prepared after purifica-

tion of the mutant phage plaques. DNA sequences of deletion mutants were determined by the dideoxy chain termination method (19, 20).

Mutant sequence were designated mp19TMD123, mpl9TMD1, and mp19TMD2345. The HindIII-BamHI fragments of

were determined for the construction of D2

KpnI-XbaI fragment (314 base pairs) from pSV2TMD123 and a KpnI-

inhibition of the mutant phage plaques. DNA sequences of deletion mutants were exchanged for the same fragment of

mutant sequence were designated mp19TMD123, mpl9TMD12, mpl9TMD1, and mp19TMD2345. The HindIII-BamHI fragments of

expression plasmid pSVTMJ2 that encodes the entire thrombomodulin precursor. The

Thin line represents the pBR322 sequence, hatched areas represent the SV40 early promoter and terminator sequences, the solid area represents the coding sequence of human thrombomodulin, and open areas represent the noncoding sequences of human thrombomodulin cDNA, respectively. B, structure of the coding region of various recombinant proteins involving the signal peptide. The numbering of the amino acid residues begins with the first residue of each domain in the mature thrombomodulin protein. TMindicates the precursor of the entire thrombomodulin protein. D123 encodes domains 1, 2, and 3, obtained by excluding domains 4 and 5 from the entire protein. D1 encodes domain 1, obtained by excluding domains 2, 3, 4, and 5 from the entire protein. D2 encodes domain 2, obtained by excluding domains 3, 4, and 5 from D2345. D23 encodes domains 2 and 3, obtained by excluding domains 4 and 5 from D2345. The cDNA fragments coding for the respective proteins were constructed by site-directed deletion mutagenesis as described under "Experimental Procedures." S, signal peptide; I, Dl (NH2-terminal domain); 2, D2 (a domain composed of six BGE-like structures); 3, D3 (O-glycosylation-rich domain); 4, D4 (transmembrane domain); 5, D5 (cytoplasmic domain).
Specific activity of recombinant proteins for protein C activation

Reaction mixture (130 μl) contained 350 nM protein C, 27.5 nM thrombin, and the indicated concentrations of recombinant protein. One unit of thrombomodulin activity corresponds to the amount of activated protein C formed (1 pmol/min/ml) under the conditions described under "Experimental Procedures." Concentration of protein antigen was determined by enzyme immunoassay. Each value represents the mean of triple assays. ND, not detected.

<table>
<thead>
<tr>
<th>Recombinant protein</th>
<th>Activity</th>
<th>Antibody</th>
<th>Specific activity</th>
</tr>
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<tbody>
<tr>
<td>Thrombomodulin</td>
<td>16.5</td>
<td>2.1</td>
<td>7.9</td>
</tr>
<tr>
<td>D123</td>
<td>13.7</td>
<td>1.8</td>
<td>7.6</td>
</tr>
<tr>
<td>D12</td>
<td>7.7</td>
<td>3.5</td>
<td>2.2</td>
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<tr>
<td>D1</td>
<td>ND</td>
<td>0.3</td>
<td>ND</td>
</tr>
<tr>
<td>D2</td>
<td>5.8</td>
<td>0.5</td>
<td>11.6</td>
</tr>
<tr>
<td>D23</td>
<td>3.5</td>
<td>0.1</td>
<td>35.0</td>
</tr>
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</table>

TABLE I

Specific activity of recombinant proteins for protein C activation

As a control, the wells were coated with 100 μl of gelatin (10 mg/ml) in the same buffer. The wells were washed with Hepes buffer and blocked with 200 μl of 10% bovine serum albumin for 2 h. After the wells were washed with Hepes buffer, 100 μl of various concentrations of the recombinant protein in Hepes buffer containing 0.1% bovine serum albumin were added and incubated for 2 h. After three washings with Hepes buffer, the bound protein was determined using anti-thrombomodulin-F(ab')2 coupled with β-d-galactosidase as described in the binding assay of proteins to thrombin.

Partial Purification of the Recombinant Proteins—The recombinant proteins secreted in the culture medium were partially purified by affinity chromatography using anti-thrombomodulin-F(ab')2 fragment-Sepharose 4B. Briefly, the culture medium harvested was concentrated using a Millipore Immersible CX-10. Then it was applied to a column of F(ab')2-Sepharose 4B equilibrated with 0.05 M Tris-HCl, 0.1 M NaCl, pH 7.5. After the column was washed with Tris buffer, the bound protein was eluted with 8 M urea. The eluate was dialyzed against Tris buffer and stored at -80 °C until use.

Western Blotting Analysis—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Blobel and Dobberstein (23) using 5-15% gradient slab gels. The sample was electrophoresed with and without 2-mercaptoethanol. Western blotting analysis was performed by the method of Burnette (24). The recombinant protein which had been electrophoretically transferred onto a nitrocellulose membrane was detected by treatment first with anti-thrombomodulin-F(ab')2, next with peroxidase-conjugated anti-rabbit IgG-goat IgG, and then with the peroxidase substrate, 4-chloro-1-naphthol.

RESULTS

Expression of the Recombinant Proteins—Fig. 1 shows the plasmid containing the cDNA encoding the entire thrombomodulin protein and the schematic models of the recombinant proteins composed of the various domains. These plasmids were transfected into COS-1 cells to achieve transient expression of the cloned genes. The amount of immunoreactive proteins expressed in the serum-free medium was determined.
Effect thrombin, unreduced predominant recombinant proteins composed of binant protein. Each value represents the mean of triple assays. ND, bomodulin activity (unit) and antigenic concentration of each recombinant protein, nor detected.

The apparent modulin-I. Fig. 2 shows the results followed by Western blotting analysis using anti-thrombomodulin (A). Details are described under “Experimental Procedures.”

TABLE I
Interaction of recombinant proteins with thrombin fixed to microplate wells

<table>
<thead>
<tr>
<th>Recombinant protein</th>
<th>Apparent turnover</th>
<th>Apparent</th>
<th>Apparent interaction turnover ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{max}$ units $\times 10^6$</td>
<td>$K_a$ mol $\times 10^{-4}$</td>
<td>$V_{max}/K_a$ (fluorescence $\times$ mol $^{-1} \times 10^5$)</td>
</tr>
<tr>
<td>Thrombomodulin</td>
<td>7.79</td>
<td>5.85</td>
<td>1.33</td>
</tr>
<tr>
<td>D123</td>
<td>2.94</td>
<td>1.02</td>
<td>2.88</td>
</tr>
<tr>
<td>D12</td>
<td>17.30</td>
<td>99.97</td>
<td>0.18</td>
</tr>
<tr>
<td>D1</td>
<td>ND</td>
<td>&gt;500</td>
<td>0.09</td>
</tr>
<tr>
<td>D2</td>
<td>0.05</td>
<td>2.10</td>
<td>0.38</td>
</tr>
<tr>
<td>D23</td>
<td>0.79</td>
<td>0.09</td>
<td>8.56</td>
</tr>
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</table>

TABLE II
Effect of phospholipids on the activation of protein C by thrombin and recombinant proteins

<table>
<thead>
<tr>
<th>Recombinant protein</th>
<th>Specific activity</th>
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</thead>
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<tr>
<td></td>
<td>Phospholipids</td>
</tr>
<tr>
<td>Thrombomodulin</td>
<td>7.6</td>
</tr>
<tr>
<td>D123</td>
<td>7.5</td>
</tr>
<tr>
<td>D12</td>
<td>2.2</td>
</tr>
<tr>
<td>D1</td>
<td>ND</td>
</tr>
<tr>
<td>D2</td>
<td>11.8</td>
</tr>
<tr>
<td>D23</td>
<td>31.2</td>
</tr>
</tbody>
</table>

**FIG. 4.** Binding of recombinant proteins to the phospholipid layer fixed to the microplate wells. Entire thrombomodulin (A); D123 protein (Δ); D12 protein (B); D23 protein (C); D2 protein (E); D1 protein (A). Details are described under “Experimental Procedures.”

by enzyme immunoassay. To concentrate and partially purify the recombinant protein, the medium was subjected to a column of anti-thrombomodulin-F(ab')2-Sepharose 4B. The bound protein was eluted with 8 M urea, and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Western blotting analysis using anti-thrombomodulin-IgG. Fig. 2 shows the results of the Western blotting analysis. The $M_r$ of the recombinant thrombomodulin ($M_r$ 78,000) was identical to that of the purified placental protein. The apparent $M_r$ values of the predominant bands of the unreduced predominant recombinant proteins composed of D12, D123, D23, D2, and D1 were 56,000, 77,000, 50,000, 42,000, and 40,000, respectively. The proteins that were reduced with 2-mercaptoethanol showed higher $M_r$ values than the unreduced proteins except for the D1 protein (data not shown).

**Thrombomodulin Activity of the Recombinant Proteins—** From the cofactor activity and the concentration of protein in the eluates from the affinity column, specific activities of the respective proteins were determined (Table I). As described previously (9), the recombinant, entire thrombomodulin protein had the same specific activity as the purified placental protein. The recombinant proteins containing D2, as well as D2 alone, possessed cofactor activity, as did the entire thrombomodulin protein, with the D23 protein showing the highest activity.

**Binding of the Recombinant Proteins to Thrombin—** Fig. 3A shows that the recombinant proteins containing D2 and D2 alone, as well as the entire thrombomodulin protein, bound to thrombin, but the D1 protein alone did not. The binding activity of the D23 protein reached a plateau at relatively low concentrations for thrombin binding. Fig. 3B shows a double reciprocal plot analysis of the data in A used to obtain the apparent maximum interaction velocity constant ($V_{max}$) and the apparent dissociation constant ($K_a$) of each recombinant protein with fixed thrombin. Of the proteins that bound to thrombin, the D23 protein and the D123 protein showed a higher affinity for thrombin than the entire protein (Table II). These findings indicate that the D2 protein is essential for thrombin-binding and protein C activation.

**Binding of the Recombinant Proteins to Phospholipids—** As shown in Table III, phospholipids only enhanced activation of protein C induced by the entire thrombomodulin protein. Next, we examined whether the extracellular region of thrombomodulin interacts with the phospholipid layer. The binding of the entire or partial recombinant proteins to phospholipids, which had been fixed to the microplate wells, was determined. Before the protein was added to the wells, Lubrol PX in the protein sample was replaced with octyl glucoside, according to Kurosawa et al. (25). As shown in Fig. 4, only the entire thrombomodulin protein bound to the phospholipids.

**DISCUSSION**

The present study demonstrates that thrombin bound to the domain consisting of six consecutive EGF-like structures in the extracellular region of thrombomodulin causes the activation of protein C. These findings are consistent with the biochemical properties of thrombomodulin cofactor activity, which have been studied using native thrombomodulin purified from lung or placenta. The cofactor activity is markedly resistant to heat and treatment with various detergents but loses stability and disappears after treatment with reducing reagents. The EGF-like structure contains three disulfide bonds. The disappearance of the cofactor activity after cleaving the disulfide bonds suggests that the structural conformation is indispensable for thrombin binding and cofactor activity.
After completing this study, Kurosawa et al. (26) performed cyanogen bromide digestion of a fragment with cofactor activity obtained by elastase digestion of rabbit thrombomodulin and isolated a peptide fragment that binds to thrombin. The amino acid sequence of this fragment suggested that the fifth and sixth EGF-like structures contain the thrombin-binding site, but no cofactor activity for protein C activation was observed in this peptide. Other EGF-like structures, in addition to those for thrombin binding, might be necessary for cofactor activity. Kurosawa et al. (25) also suggested the presence of one or more phospholipid-binding sites in the thrombomodulin molecule. In our study, only the entire protein containing the transmembrane domain bound to the phospholipid layer. This finding suggests the absence of sites for interactions with phospholipids in the extracellular region.

The EGF-like structures in the light chain of activated thrombomodulin seems to be the first membrane receptor protein in which the EGF-like structures are involved in binding to its ligand as well as in having ligands with alternate functions.

REFERENCES