The Last Three Consecutive Epidermal Growth Factor-like Structures of Human Thrombomodulin Comprise the Minimum Functional Domain for Protein C-activating Cofactor Activity and Anticoagulant Activity*

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We have identified a minimum functional domain of human thrombomodulin for anticoagulant activity using deletion analysis. Four mutants were constructed by site-directed deletion mutagenesis to delete one or more epidermal growth factor (EGF)-like structures from the domain of human thrombomodulin containing six repeated EGF-like structures. These deletion mutants were expressed transiently in COS-1 cells, and their protein C-activating cofactor activities in the culture medium were examined. One mutant protein, E456, which contains the fourth, fifth, and sixth EGF-like structures expresses apparent cofactor activity. However, neither E456-N24 (24 NH2-terminal-residue deletion) nor E456-C16 (16 COOH-terminal-residue deletion) have cofactor activity. E456 was partially purified and its anticoagulant effects on plasma clotting time and platelet aggregation examined. E456 expressed almost the same anticoagulant activities as thrombomodulin.

It was concluded that E456 is the minimum functional domain for both protein C-activating cofactor activity and anticoagulant activity.

Thrombomodulin is an endothelial cell surface protein that forms a 1:1 complex with thrombin. In this complex thrombin activates protein C approximately 1000-fold faster than thrombin alone (1) and loses its procoagulant activities which include fibrinogen clotting, Factor V activation (2), and platelet activation (3). Activated protein C is a vitamin K-dependent serine protease which inactivates Factor Va and factor VIIIa (4–6) and neutralizes an inhibitor of tissue-type plasminogen activator (7, 8). Thus, thrombomodulin plays a role as a physiologically important anticoagulant on the endothelial cell surface. Thrombomodulin has been purified from rabbit (2), bovine (9), and human placenta (10) and human lung (11).

We have previously isolated a human thrombomodulin cDNA clone (11). The amino acid sequence deduced from the nucleotide sequence indicated that human thrombomodulin is synthesized as a precursor consisting of 575 residues and contains an 18-residue signal peptide. Mature human thrombomodulin is composed of five domains: an NH2-terminal domain (D1), a domain with six epidermal growth factor (EGF)-like structures (D2), an O-glycosylation site-rich domain (D3), a transmembrane domain (D4), and a cytoplasmic domain (D5). We have also inserted thrombomodulin cDNA into the eukaryotic expression vector PSV2 and have expressed immunoreactive and functionally active thrombomodulin in COS-1 cells (11). The gene coding for human thrombomodulin was isolated (12, 13) and localized to chromosome 20 (14). There are no intervening sequences in this thrombomodulin chromosomal gene (12, 13).

Recently Kurosawa et al. (15) reported that the thrombin-binding domain of rabbit thrombomodulin is located in the fifth and sixth EGF-like structures, but cofactor activity could not be detected in this region. In our previous paper (20), by using gene technology, we have identified a domain consisting of six consecutive EGF-like structures (D2) that have protein C-activating cofactor activity.

In this paper we demonstrate that the region including the fourth, fifth, and sixth EGF-like structures is the minimum domain for protein C-activating cofactor activity and anticoagulant activity.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases, T4 polynucleotide kinase, Escherichia coli DNA polymerase 1 (Klenow fragment), T4 DNA ligase, dNTPs, M13mp19, and the 7-deaza sequencing kit were purchased from Takara Shuzo, Kyoto. [γ-32P]ATP (7000 Ci/mmol) and [α-32P]CTP (3000 Ci/mmol) were purchased from Amersham Corp. Bovine protein C (16) and bovine antithrombin III (17) were purified as previously described. Human thrombin was purchased from Sigma. Boc-Leu-Ser-Thr-Arg-MCA, a fluorogenic substrate for activated protein C (18), was purchased from the Protein Research Foundation, Osaka. Platelet-rich plasma was prepared as described previously (19). Control plasma (Ci-TROL I Coagulation Control Level I) was obtained from Dade. All other chemicals and reagents used were the best commercial grades available.

Site-directed Deletion Mutagenesis—A 944-bp base pair HindIII-XbaI fragment from pSV2TMD2 (20) carrying the coding sequence for D2 of human thrombomodulin was subcloned into M13mp19. This clone was designated M13mp19TMD2. Single-stranded recombinant phage DNA was used as a template for site-directed deletion mutagenesis and was prepared as previously described (21). Deletions of one or more EGF-like structures were generated by the "loop-out" mutagenesis technique using 25-mer oligonucleotides. The oligonucleotides were complementary to the sequences immediately preceding and following the EGF-like structures to be deleted. D123, which contained domain D1, domain D2, and domain D3 was constructed and expressed as a positive control (20). Annealing, second strand synthesis, and plaque hybridization were performed as previously described (20). The HindIII-XbaI fragment of pSV2TMD2 was exchanged with the same fragments from M13 clones verified to have

1 The abbreviations used are: EGF, epidermal growth factor; MCA, 4-methyl-coumaryl-7-amide; BOC, tert-butoxycarbonyl; ELISA, enzyme-linked immunosorbent assay.
Functional Domain of Human Thrombomodulin

RESULTS

Identification of the Minimum Region for Cofactor Activity—To identify the minimum region for thrombin-catalyzed pro-

Table I

Protein C-activating cofactor activity and concentration of mutant protein in the conditioned medium

| Mutant protein | Activity in conditioned medium | Concentration of mutant protein |
|----------------|--------------------------------|--------------------------------|
| D123           | 51.9                           | 5000                           |
| E12345         | ND                             | 160                            |
| E1234          | ND                             | 150                            |
| E456           | 19.1                           | 1700                           |
| E456-C16       | ND                             | 550                            |
| E456-N24       | ND                             | 250                            |

Fig. 1. Schematic representation of deletion mutants of human thrombomodulin. Deletion mutants were constructed by site-directed deletion mutagenesis, as described in the text. The numbering corresponds to the sequences deduced from the CDNA of human thrombomodulin (11). Two deletion mutants were constructed from E456. E456-N24 began in the fourth EGF-like structure at Cys347. E456-C16 terminates in the sixth EGF-like structure at Cys446.

Fig. 2. E456 containing fourth, fifth, and sixth EGF-like structures. The numbering corresponds to the sequences deduced from the CDNA of human thrombomodulin (11). Two deletion mutants were constructed from E456. E456-N24 began in the fourth EGF-like structure at Cys347. E456-C16 terminates in the sixth EGF-like structure at Cys446.

COS-1 cells were transfected with various plasmid DNAs coding for deletion mutants, cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum for 24 h, washed with phosphate-buffered saline, and cultured in serum-free Dulbecco's modified Eagle's medium for 48 h. The amount of mutant protein expressed in the conditioned medium was determined by sandwich-type ELISA using anti-human thrombomodulin antiserum. Protein C-activating cofactor activity in the conditioned medium was determined as described under "Experimental Procedures." One unit of thrombomodulin activity was defined as 1 nmol of activated protein C formed/ml/min. ND indicates nondetectable value.

The correct deletion mutations by dyeoxy sequencing (22, 23). These clones were designated pSv2TME12345 (used to express mutant protein E12345 which contains EGF structures 1-5), pSv2TME1234 (used to express mutant protein E1234 which contains EGF structures 1-4), pSv2TME456 (used to express mutant protein E456 which contains EGF structures 4-6), pSv2TME56 (used to express mutant protein E56 which contains EGF structures 5 and 6), pSv2TME456-N24 (used to express mutant protein E456-N24 which is missing 24 residues from the NH2-terminal end of E456), and pSv2TME456-C16 (used to express mutant protein E456-C16 which is missing 16 residues from the COOH-terminal end of E456), respectively. Plasmid DNAs used for transfection experiments by electroporation were purified twice via CacCl/ethidium bromide equilibrium centrifugation.

Cells and Transfection—Twenty μg of plasmid DNA were transfected into COS-1 cells (4 × 10⁵ cells) in phosphate-buffered sucrose (7 mM sodium phosphate, pH 7.4, 1 mM MgCl₂, 272 mM sucrose) by electroporation (3 microfarads, 450 V, twice). The conditioned medium containing secreted recombinant proteins was harvested after 72 h of cultivation.

Purification of Recombinant Proteins—Mutant proteins D123 and E456 were partially purified from the COS-1 cell culture medium by a modification of a previously described method using QAE-cellulose (LKR, ZETA-PREP QA 15) and DIP-TB (diisopropylphosphoryl-thrombin-agarose).

Immunological Detection of Mutant Proteins—The amount of mutant protein was measured by a sandwich-type enzyme immunoassay (ELISA) system using rabbit anti-human thrombomodulin IgG (Fab'), and murine monoclonal anti-human thrombomodulin IgG which blocks protein C-activating cofactor activity.

Protein C Activation Assay—The ability of thrombomodulin to accelerate thrombin-catalyzed protein C activation was measured as previously described (20). One unit of thrombomodulin activity was defined as 1 nmol of activated protein C formed/ml/min.

Coagulation Assay—Plasma clotting time was measured using control plasma (Citrol I) by a coagulometer (KC10; Amelung).

Platelet Aggregation Assay—Platelet-rich plasma adjusted to a concentration of 3 × 10⁵ platelets/μl was used for the measurement of platelet aggregation inhibition using a platelet aggregometer (NBS Hema Tracer IV). Thrombin and mutant protein were mixed in the following concentrations: thrombin, 15 nM; D123, 36 nM; E456, 28 nM.

The other five deletion mutants were constructed on D2 involving the amino acid residues starts with the first residue of each domain (NH2-terminal domain); TM indicates the precursor of the entire human thrombomodulin. D123 encodes for a thrombomodulin protein, terminated at residue 498, which is devoid of domains 4 and 5. The other five deletion mutants were constructed on D2 involving six repeated EGF-like structures. S, signal peptide; D1, domain 1 (NH2-terminal domain); D2, domain 2 (a domain containing six EGF-like structures); D3, domain 3 (O-glycosylation site-rich domain); D4, domain 4 (transmembrane domain); D5, domain 5 (cytoplasmic domain); E1, first EGF-like structure; E2, second EGF-like structure; E3, third EGF-like structure; E4, fourth EGF-like structure; E5, fifth EGF-like structure; E6, sixth EGF-like structure.

Protein C activation in the EGF-like domain of human thrombomodulin (D2), we constructed four deletion mutants which lack one or more of the regions containing EGF-like structures (Fig. 1). As shown in Table I, all conditioned medium contained levels of immunoreactive protein which could be detected by ELISA. Cofactor activity was detected only in the conditioned medium derived from COS-1 cells transfected with plasmid pSv2TME456, which codes for the fourth, fifth, and sixth EGF-like structures.

We also constructed two other deletion mutants to determine the minimum essential region for thrombin-catalyzed protein C activation on E456. pSv2TME456-N24, which lacks the coding sequence for the 24 NH2-terminal amino
TABLE II

Anticoagulant effect of mutant protein on plasma clotting time

The amount of mutant protein partially purified was determined by sandwich-type ELISA assay using rabbit anti-human thrombomodulin IgG F(ab')2 and murine monoclonal anti-human thrombomodulin IgG. Thrombin was mixed with mutant protein at the molar ratio indicated. Clotting time was measured as described under "Experimental Procedures." The control experiment was performed using only the buffer solution used for dissolving mutant protein.

| Concentration of mutant protein | Mutant protein:thrombin | Clotting time |
|---------------------------------|-------------------------|---------------|
| nM                              |                         | D123 | E456 | Control |
| 25.0                            | 1.0:1                   | 61.0 | 73.0 | 38.3    |
| 57.5                            | 1.5:1                   | 90.0 | 95.0 | 39.0    |
| 50.0                            | 2.0:1                   | 130.0| 115.0| 36.0    |

Endogenous thrombin was further modified. Following clotting and platelet activation were determined. Table II shows the effect of mutant proteins on clotting time. Both D123 and E456 prolonged the clotting time, and this prolongation was proportional to the ratio of mutant protein to thrombin. E456 activity was almost the same as that of D123. The effect of mutant proteins on thrombin-induced platelet aggregation of human platelet-rich plasma was determined. In the absence of mutant proteins, platelets aggregated in response to thrombin stimulation. Platelet aggregation was inhibited with increasing mutant protein concentrations and was completely inhibited by the addition of a 2-fold molar excess of mutant protein to thrombin (data not shown).

DISCUSSION
We have constructed a series of thrombomodulin deletion mutants which are missing one or more EGF-like structures from the domain which contains six EGF-like structures (D2) in the native molecule. The expression vector for one mutant protein found to have activity was further modified. Following transient expression of these deletion mutants in COS-1 cells, protein C-activating cofactor activity was measured. Only mutant E456 was found to have cofactor activity. In addition, E456 has inhibitory activity on thrombin-induced plasma clotting and platelet activation. A cyanogen bromide digestion study by Kurosawa et al. (15) indicated that a 10-kDa fragment including the fifth and sixth EGF-like structures contains the primary thrombin binding site of rabbit thrombomodulin, but it had no cofactor activity at all. In this study we have demonstrated that the minimum functional domain of human thrombomodulin for thrombin-induced protein C activation is a 115-residue segment which contains the fourth, fifth, and sixth EGF-like structures of domain D2.

According to structural predictions by Cooke et al. (24) with human EGF, we predict that each EGF-like structure in human thrombomodulin has a similar secondary structure to human EGF, with three disulfide bridges corresponding to those of human EGF, as shown in Fig. 2. When either two loops formed with disulfide bonds from the NH2 terminus of E456 or one loop from the COOH terminus was deleted, cofactor activity was lost. Treatment of thrombomodulin from rabbit and bovine lung with 2-mercaptoethanol has been shown to inactivate its cofactor activity (2, 9). These results suggest that a complete structure consisting of the fourth, fifth, and sixth EGF-like structures is essential for maintaining protein C-activating cofactor activity.

An EGF-like structure of urokinase has been reported to be a binding region for the urokinase receptor on hepatocyte surfaces (25), but the functions of other proteins which contain EGF-like structures are still unknown. This study demonstrates a role for the EGF-like structures of human thrombomodulin: the binding to thrombin and the changing of its substrate specificity.

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