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Monoclonal Antibodies to Human Thrombomodulin Whose Binding Is Calcium Dependent

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Four monoclonal antibodies to human thrombomodulin were characterized. Binding of two of these antibodies was dependent on the presence of calcium ions, and approximately 5 mM calcium was required for their maximum binding. These two antibodies inhibited the binding of thrombin to thrombomodulin, thereby inhibiting activation of protein C catalyzed by thrombin-thrombomodulin complex. These two antibodies bind to a major active fragment formed by limited proteolytic digestions of thrombomodulin with elastase and trypsin, suggesting that the antibodies bind to the thrombin-binding site (or its vicinity) located in the epidermal growth factor (EGF)-homology domain. One of the other calcium-independent antibodies also inhibited the binding of thrombin and the activation of protein C, but the inhibition was very weak and was observed only when the antibody was present in a molar excess over thrombomodulin. This antibody did not bind to the protease digests of thrombomodulin. Another calcium-independent antibody did not inhibit either thrombin binding or protein C activation, but bound to the active fragment of protease digests, suggesting that the antibody binds to a region other than the thrombin-binding site in the EGF-homology domain. These observations suggest that thrombomodulin undergoes a calcium-dependent conformational change which may occur in proximity to a thrombin-binding site located in the EGF-homology domain.

Protein C plays an important role in regulating the blood coagulation process *in vivo*, and its congenital deficiency results in thrombophilic states (*1*). Protein C must be activated to exert its physiological role as an anticoagulant, and its activation is catalyzed by thrombin bound to a cofactor called thrombomodulin in the presence of calcium ions (*2*). Thrombomodulin is present on the vascular endothelium as a membrane-bound protein (*3*). When thrombin is generated in the blood vessels, a part of the thrombin is bound to thrombomodulin to form thrombin-thrombomodulin complex. Once thrombin forms the complex, thrombin loses its procoagulant activities such as clotting fibrinogen and activation of blood coagulation factors V and VIII, but acquires an ability to activate protein C. Thus, thrombomodulin converts procoagulant thrombin to an anticoagulant and plays a central role in the protein C anticoagulant pathway.

Ca^{2+} is required for the activation of protein C by thrombin-thrombomodulin complex. Spectroscopic studies show that a Ca^{2+}-dependent conformational transition occurs in the protein C molecule, which correlates well with the Ca^{2+} dependence of the activation process (*4*). The Ca^{2+}-stabilized conformation of protein C may be necessary for protein C to interact effectively with thrombin-thrombomodulin.

In the present study, we have developed monoclonal antibodies to human thrombomodulin and have demonstrated that binding of some of the antibodies to thrombomodulin is Ca^{2+}-dependent, suggesting that thrombomodulin also undergoes Ca^{2+}-dependent conformational changes.

MATERIALS AND METHODS

Thrombomodulin—Thrombomodulin was prepared by our method previously described (*5*). The product was further purified by high-performance liquid chromatography (HPLC) (Fig. 1A). The molecular weight of the purified material estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was 71K or 105K before or after disulfide bond reduction, respectively. The value of 13.8 for the extinction coefficient $E_{1\%}^{1cm}$ of bovine thrombomodulin (*6*) was used tentatively for calculating protein concentrations of the purified thrombomodulin.

Enzyme-Treated Thrombomodulin—Thrombomodulin (1.2 mg/ml) was treated with trypsin (Type XIII, bovine pancreas, TPCK-treated; Sigma) (50 μg/ml) or elastase (Type IV, porcine pancreas; Sigma) (0.5 mg/ml) in 0.02 M Tris-HCl, 0.15 M NaCl, 0.01% Lubrol PX, pH 7.5 (TBS-Lubrol) at 37°C for 30 min. The reaction was terminated by adding *p*-aminodiphenylmethanesulfonyl fluoride-HCl (Wako) (17 mM final concentration). After incubation at 4°C for 30 min, the sample was dialyzed against TBS-Lubrol and was subjected to HPLC (Fig. 1, B and C). The fractions containing a major active fragment were col-

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Abbreviations: DIP, diisopropylphosphofluoridate; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; IRMA, immunoradiometric assay; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.

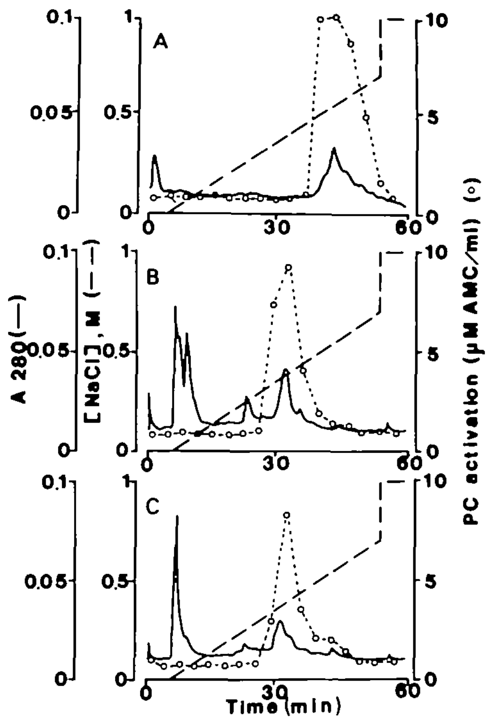

Fig. 1. **Purification of thrombomodulin and its enzyme digests by ion exchange chromatography.** Thrombomodulin (1.2 mg) (Panel A), or an elastase-digested (Panel B) or a trypsin-digested (Panel C) sample was subjected to HPLC with a TSK-DEAE-5PW column (Tosoh Co., Tokyo) using a linear NaCl gradient from 0.1 to 0.7 M in 0.02 M Tris-HCl, 0.01% Lubrol PX, pH 7.5. A_{280} (solid lines), the cofactor activity of thrombomodulin for protein C (PC) activation (○), NaCl gradient (broken lines).

lected. The molecular weight, estimated by non-reducing SDS-PAGE, of the major active fragment of elastase or trypsin digests of thrombomodulin was approximately 42,000 or 45,000, respectively.

Protein C—Protein C was purified from citrated fresh human plasma as described by Suzuki *et al.* (*7*).

Cofactor Activity of Thrombomodulin for Protein C Activation—Protein C (0.4 μM) was activated with thrombin (2.5 U/ml) and thrombomodulin in the same way as previously described (*5*). The activation was carried out in TBS containing 0.25 mM Ca^{2+} at 37°C for 30 min. Activation was terminated by the addition of an equal volume of antithrombin III (600 μg/ml) and incubation was carried out at 37°C for 10 min. The activated protein C present was then assayed for amidase activity on Boc-Leu-Ser-Thr-Arg-MCA (4-methylcoumaryl-7-amide) (Protein Research Foundation, Osaka) as previously described (*5*). AMC (7-amino-4-methylcoumarin) liberated was measured by a spectrofluorophotometer (Shimadzu RF-510, Kyoto) with excitation at 380 nm and emission at 460 nm, using a standard curve constructed with a standard AMC (Protein Research Foundation).

Thrombin and DIP-Thrombin—Thrombin was purified from a bovine thrombin preparation (Mochida Pharm. Co., Tokyo) by the method of Lundblad *et al.* (*8*). The purified thrombin (0.6 mg/ml) was incubated with diisopropylfluorophosphate (Sigma) (40 mM) in 0.05 M Tris-HCl, pH 7.4, 0.14 M NaCl (TBS) at 4°C for 30 min. DIP-thrombin thus formed was dialyzed against TBS and stored at −80°C.

Reagents for Monoclonal Antibody Production—The mouse myeloma cell line P3U1 was provided by Prof. Kumagai, Tohoku University. The following reagents were purchased: Freund's adjuvants, polyethylene glycol 1540, and 2,6,10,14-tetramethylpentadecane (pristane) from Wako Chemical Co. (Osaka); Dulbecco's modified Eagle's medium from GIBCO (Chagrin Falls, Ohio); fetal calf serum from Filtron (Victoria, Australia); and hypoxanthine, aminopterin, thymidine (HAT), gelatin, and bovine serum albumin from Sigma Chemical Co. (St. Louis, Mo.).

Monoclonal Antibody Production—Monoclonal antibodies against thrombomodulin were produced according to the method of Köhler and Milstein (*9*). Eight-week-old male BALB/c mice were immunized with human thrombomodulin. First, 20 μg in complete Freund's adjuvant was injected intradermally. Two and 4 wk later, 100 μg of thrombomodulin was injected intraperitoneally. A further 2 wk later, 100 μg in TBS was injected intravenously. Four days after the final booster injection, spleen cells were fused with the mouse myeloma cell line P3U1 by using 50% polyethylene glycol 1540. The fused cells were cultured in HAT media. Anti-thrombomodulin antibody-producing cell lines, screened by an enzyme-linked immunosorbent assay (ELISA), were recloned by limiting dilution. Finally, each clone was injected intraperitoneally into BALB/c mice that had been pretreated with pristane. Monoclonal antibodies were purified from the ascites fluid of the mice by affinity chromatography using protein A-Sepharose CL 4B (Pharmacia Fine Chemicals, Tokyo) according to the standard method.

Horseradish Peroxidase (HRP)-Conjugated Antibody—Conjugation of the antibodies with HRP (Sigma) was performed by the method of Nakane and Kawaoi (*10*).

ELISA—ELISA was used to measure the antibodies. Polyvinyl chloride microtiter plates (Titertek, Zwanenburg, The Netherlands) were coated with thrombomodulin at 2.4 μg/ml in 0.05 M carbonate buffer, pH 9.6, for 18 h at 4°C. The plates were washed and incubated with TBS containing 1% gelatin for 2 h at 25°C to accomplish the coating. After washing three times with TBS containing 0.05% Tween 20 (TBS-Tween), aliquots of 100 μl of the antibody (3 ng/ml) in TBS or TBS containing various concentrations of Ca^{2+} were added to the coated wells and incubated for 2 h at 25°C. The wells were then washed three times with TBS-Tween in the absence or in the presence of Ca^{2+}. Then, aliquots of 100 μl of horseradish peroxidase-conjugated goat anti-mouse IgG (Cooper Biomedical Inc., Pa.) (0.1 μg/ml) in TBS-Tween were added to the wells and incubated for 2 h at 25°C. After washing in the same way, 0.01% H_2O_2 and ortho-phenylenediamine (Sigma) (0.4 mg/ml), dissolved in 0.075 M citrate-sodium phosphate buffer pH 5.0, were placed in each well and incubated for 30 min at 25°C. The reaction was stopped by addition of 50 μl of 4.5 M H_2SO_4 to each well, and the absorbance at 492 nm was measured using a Titertek Multiskan MC. ELISA was also used for measurement of thrombomodulin. Microtiter plates were coated with a monoclonal antibody KA-3 (5 μg/ml) in the same way as described above. After washing, the plates were incubated with thrombomodulin (0–10 ng/ml) for 4 h at 37°C in the presence of 5 mM Ca^{2+}. The wells were washed. Then, aliquots of 100 μl of HRP-conjugated monoclonal antibody KA-4 in the presence of 5 mM Ca^{2+} were added and incubated for 2 h at 37°C. The following procedures were performed in the same way as described above.

Competition between Antibodies for the Same Epitope on Thrombomodulin—Polyvinyl microtiter plates were coated with thrombomodulin in the same way as for ELISA. Prior to competition experiments, HRP-conjugated monoclonal antibody in TBS containing 5 mM Ca^{2+} was added to each well and a subsaturating level of monoclonal antibody determined for each antibody. Each non-conjugated monoclonal antibody (330 nM final concentration) and a constant subsaturating level of each HRP-conjugated monoclonal antibody in TBS-Ca^{2+} were added simultaneously to the wells coated with thrombomodulin and incubated for 2 h at 25°C. After washing, the enzymatic activity of each well was measured as described for ELISA. The results are shown as a percentage of HRP-conjugated monoclonal antibody bound in the absence of competing antibodies.

Polyacrylamide Gel Isoelectric Focusing—Isoelectric focusing was performed on LKB ampholine PAG plates (LKB, Bromma, Sweden) at 2,000 V (5 mA) over a pH range of 3.5 to 9.0 using 1 M NaOH as a cathode and 1 M H_3PO_4 as an anode. After electrofocusing, the gel was fixed, dried, and stained with Coomassie blue. A broad pI calibration kit (pH 3-10) (Pharmacia Fine Chemicals, Tokyo) was used as standards.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)—SDS-PAGE on a slab gel was carried out using a gradient separating gel of acrylamide (7.5 to 15%) containing 0.1% SDS and a stacking gel of 4% acrylamide containing 0.1% SDS according to the method of Laemmli (*11*). Molecular weight standards (carbonic anhydrase [31K], ovalbumin [45K], bovine serum albumin [66.2K], phosphorylase B [92.5K], β-galactosidase [116.25K], and myosin [200K]) were obtained from Bio-Rad Laboratories.

Immunoblotting Technique—After separation by SDS-PAGE, the proteins were electrophoretically (50 V for 1.5 h) transferred to nitrocellulose membranes (Bio-Rad) essentially as described by Towbin *et al.* (*12*). After the transfer, the nitrocellulose sheets were blocked by being incubated in TBS containing 1% gelatin for 1 h at 25°C, washed with the washing buffer (0.05 M Tris-HCl, 0.5 M NaCl, 0.05% Tween, 5 mM $CaCl_2$; pH 7.5), and incubated with monoclonal antibodies (1 μg/ml) dissolved in the washing buffer at 25°C for 3 h. The nitrocellulose sheets were then washed with the washing buffer, incubated with horseradish peroxidase-conjugated antimouse IgG goat antibody (Bio-Rad) dissolved in the washing buffer, and subjected to color development using 4-chloro-1-naphthol (Bio-Rad).

Radioiodination of Protein—Thrombomodulin, DIP-thrombin, monoclonal antibodies, and non-immunized mouse IgG were radioiodinated using immobilized lactoperoxidase glucose oxidase (Enzymobead, Bio-Rad) and $Na^{125}I$ (17.4 Ci/mg, New England Nuclear, Boston). Free unconjugated radiolabeled compounds were removed by dialysis. The labeled thrombomodulin, DIP-thrombin, monoclonal antibodies KA-1, -2, -3, -4, and non-immunized IgG had radioactivities of 4.6×10^3, 5.0×10^3, 4.0×10^3, 8.6×10^3, 7.1×10^3, 4.4×10^3, and 3.6×10^3 cpm/ng, respectively.

Immunoradiometric Assay (IRMA) for Binding of Thrombin to Thrombomodulin—Polyvinyl chloride microtiter plates were coated with thrombomodulin (2.4 μg/ml) in the same way as described for ELISA. After washing, the plates were coated with 1% gelatin in 0.05 M carbonate buffer, pH 9.6. After 1 h at 25°C, the wells were washed with 1% gelatin in TBS-Tween (TBS-Tween-gelatin). Samples containing ^{125}I-DIP-thrombin, diluted with TBS containing 1% gelatin and 5 mM Ca^{2+}, were added to the coated wells and incubated for 2 h at 25°C. After this time, the wells were washed with TBS-Tween-gelatin, then the radioactivity in each well was measured.

Binding Study of Monoclonal Antibodies—The dissociation constants (K_d) of ^{125}I-labeled monoclonal antibodies for thrombomodulin, elastase-treated thrombomodulin, and trypsin-treated thrombomodulin were estimated by a solid-phase assay previously described (*13*). The microtiter plates were coated with thrombomodulin (2.4 μg/ml), elastase-treated thrombomodulin (1.8 μg/ml), and trypsin-treated thrombomodulin (2 μg/mg) as described above. Various concentrations of the ^{125}I-labeled antibodies or non-immunized mouse IgG dissolved in TBS-gelatin containing 5 mM Ca^{2+}, were added to the coated wells and incubated for 3 h at 25°C. After washing, the wells were individually removed and subjected to γ counting. For each antibody concentration, the results of four replicate wells were averaged. The level of radioactivity found in each well was converted to the concentration of antibody bound, and the dissociation constants were calculated as described (*13*).

Influence of Thrombomodulin and Monoclonal Antibodies on Thrombin Clotting Time—Thrombomodulin (2 μg/ml) and monoclonal antibody (2 μ/g/ml) were incubated in TBS-5 mM Ca^{2+} at 37°C for 30 min. Fifty microliters of this mixture was added to 50 μl of thrombin solution (2 U/ml) in TBS-5 mM Ca^{2+} and the mixture was incubated at 37°C for 20 min. To the mixture was then added 100 μl of fibrinogen (2 mg/ml) in TBS, and the clotting time was observed at 37°C. Controls were run replacing monoclonal antibody with non-immunized IgG with or without thrombomodulin.

RESULTS

Hybridomas producing monoclonal antibodies against human thrombomodulin were isolated. After limiting dilution, 23 clones were shown to produce antibodies capable of binding to thrombomodulin. Four clones were selected for further investigation and were named KA-1, KA-2, KA-3, and KA-4. The purified monoclonal antibodies from these clones were all classified as γ1K. The isoelectric points of the antibodies KA-1, -2, -3, and -4, determined by isoelectric focusing, were 7.5 ± 0.3, 8.5 ± 0.7, 7.3 ± 0.2, and 7.3 ± 0.2, respectively.

The bindings of the antibodies to thrombomodulin were studies over a wide range of calcium concentrations. ELISA was employed. As shown in Fig. 2, the bindings of KA-1 and -4 were partly dependent on the calcium ion concentration, and reached the maximum at 5 mM Ca^{2+}. Hence the binding of the antibodies to thrombomodulin was carried out in the presence of 5 mM Ca^{2+} in all the other experiments. Dependence of the binding on calcium ions was not apparent when a major active fragment of elastase or trypsin digests was examined (Table I).

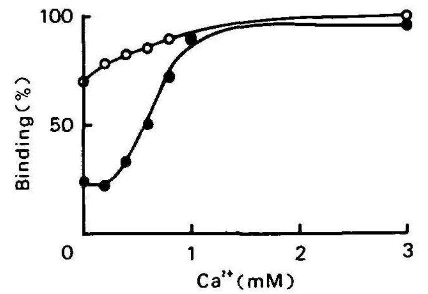

Fig. 2. **Effects of calcium ions on the binding of the antibodies to thrombomodulin.** The binding of the antibodies KA-1 (●) and -4 (○) to thrombomodulin immobilized on the plates was studied in the presence of various concentrations of Ca^{2+} by ELISA. The amount of each antibody bound to the plate in the presence of 5 mM calcium ions was taken as the maximum for the calculation of percent binding.

TABLE I. **Effects of calcium ions on the binding of the antibodies KA-1 and -4 to elastase- or trypsin-digested thrombomodulin as compared with native thrombomodulin.**

Antibodies	Ca^{2+}	Absorbance values[a]		
		Native	Elastase digest	Trypsin digest
KA-1	+	0.80	0.83	0.85
	−	0.42	0.79	0.80
KA-4	+	1.16	1.20	1.17
	−	0.74	1.06	1.03

[a]Binding was assayed by ELISA in the presence (+) or absence (−) of 5 mM calcium ions and the results are expressed as the absorbance at 492 nm.

TABLE II. **Effects of monoclonal antibodies on the binding of ^{125}I-thrombin with thrombomodulin in the presence of 5 mM Ca^{2+}.**

Antibodies	Binding (%)[a]
KA-1	0
KA-2	53
KA-3	95
KA-4	0

[a]Binding is expressed as percent of the binding in the presence of nonimmunized mouse IgG replacing the antibodies.

The effect of the antibodies on the antithrombin activity of thrombomodulin was studied. Thrombomodulin was preincubated with each of the antibodies in the presence of Ca^{2+}, and then antithrombin activity was assayed by the clotting method as described in "MATERIALS AND METHODS." Prolongation of the clotting time by thrombomodulin (≈120 s) was not seen when thrombomodulin was preincubated with antibody KA-1 or KA-4, and the clotting time was nearly the same as the control (≈70 s) which was run without thrombomodulin but with non-immunized IgG. Antibodies KA-2 and -3 had no effect.

The effect of the antibodies on the binding of thrombin to thrombomodulin was studied in the presence of Ca^{2+}. ^{125}I-DIP-thrombin (2.5 μg/ml) and each of the antibodies (500 μg/ml) were separately mixed, and the mixtures were tested for the binding of thrombin to thrombomodulin by IRMA as described in "MATERIALS AND METHODS." Antibodies KA-1 and -4 completely inhibited the binding, whereas the inhibition was partial with KA-2 and not apparent with KA-3 (Table II).

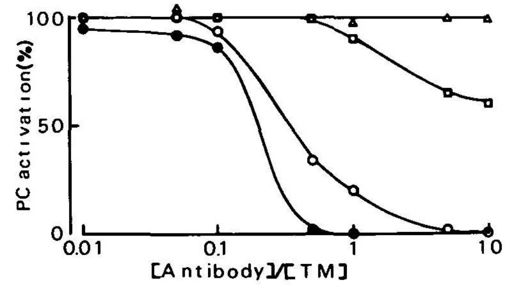

Fig. 3. **Effects of various concentrations of the antibodies on the cofactor activity of thrombomodulin (TM) for protein C (PC) activation.** The cofactor activity of thrombomodulin (0.25 μg/ml) preincubated with various concentrations of the antibody KA-1 (●), -2 (□), -3 (△), or -4 (○) was assessed as described in "MATERIALS AND METHODS." The activities of TM, expressed as percent of the protein C (PC) activation as compared with the activation without antibodies, were plotted against the molar ratios of the antibodies to TM plotted on a logarithmic scale.

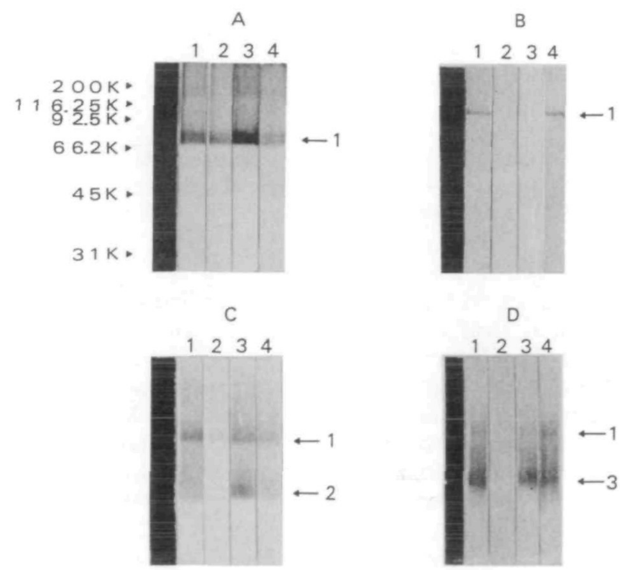

Fig. 4. **Immunoblotting of thrombomodulin, elastase- or trypsin-digested thrombomodulin.** Thrombomodulin (5 μg) was incubated with elastase (0.1 μg) or trypsin (10 ng) at 37°C for 30 min. Native thrombomodulin (Panels A and B), elastase-digested thrombomodulin (Panel C), and trypsin-digested thrombomodulin (Panel D) were subjected to SDS-PAGE without reduction (Panels A, C, and D) or with reduction (Panel B), followed by immunoblotting with antibody KA-1 (lane 1), KA-2 (lane 2), KA-3 (lane 3), or KA-4 (lane 4). Arrows 1, 2, and 3 indicate native thrombomodulin, a major fraction of elastase-digested thrombomodulin, and a major fraction of trypsin-digested thrombomodulin, respectively. On the left of each panel is a molecular weight calibration scale constructed using molecular weight standards stained with Coomassie Brilliant Blue.

The effect of the antibodies on protein C activation by thrombin and thrombomodulin was studied. A fixed amount of thrombomodulin was preincubated with various concentrations of the antibodies, and the mixtures were tested for their cofactor activities in thrombin-catalyzed protein C activation as described in "MATERIALS AND METHODS." Antibodies KA-1, -4, and -2 inhibited the cofactor activity, whereas KA-3 did not inhibit the cofactor activity even at a molar ratio (antibody : thrombomodulin) of 10 (Fig. 3).

TABLE III. **Dissociation constants for anti-thrombomodulin monoclonal antibodies to native thrombomodulin, elastase-digested, and trypsin-digested thrombomodulins.**

IgG	K_d[a]		
	Native thrombomodulin	Elastase-digested[b] thrombomodulin	Trypsin-digested[b] thrombomodulin
KA-1	3.0×10^{-9}	4.0×10^{-9}	3.0×10^{-9}
KA-2	2.4×10^{-9}	>1,000	>1,000
KA-3	2.6×10^{-9}	6.4×10^{-9}	3.0×10^{-9}
KA-4	1.2×10^{-9}	1.4×10^{-9}	1.2×10^{-9}

[a]For determination of the dissociation constants (K_d), a solid-phase IRMA using ^{125}I-labeled monoclonal antibodies was performed in the presence of calcium ions as described under "MATERIALS AND METHODS." [b]A major active fragment of enzyme-digested thrombomodulin obtained by HPLC.

TABLE IV. **Competition between monoclonal antibodies for binding to thrombomodulin.**

Conjugated IgG	Binding of HRP-conjugated antibodies[a]				
	Competitor				
	KA-1	KA-2	KA-3	KA-4	Nonimmunized mouse IgG
KA-1	8	118	125	27	100
KA-2	91	7	102	102	100
KA-3	86	80	6	93	100
KA-4	13	96	96	11	100

[a]HRP-conjugated antibody bound to the plates coated with thrombomodulin in the presence of nonconjugated antibodies (competitor) is shown as a percentage of that bound in the presence of nonimmunized mouse IgG replacing competing antibodies.

At equimolar concentrations of the antibody and thrombomodulin, the inhibition was 100, 78, and 8% with KA-1, -4, and -2, respectively (Fig. 3).

Thrombomodulin was digested with elastase or trypsin, electrophoresed, and subjected to immunoblotting using the monoclonal antibodies. Monoclonal antibodies KA-1, -3, and -4 bound to enzyme-digested thrombomodulins, but KA-2 did not appear to bind to enzyme-digested thrombomodulins (Fig. 4). The fragments of the enzyme digests, that react with the antibodies, correspond to the major active fragments. KA-2 and -3 did not appear to bind to thrombomodulin when thrombomodulin had been treated with reduction (Fig. 4B).

Dissociation constants (K_d) for the antibodies to native thrombomodulin and the major active fragment of elastase- or trypsin-digested thrombomodulin were assessed to estimate the avidity of the antibodies (Table III). The K_d values for thrombomodulin binding to all four antibodies were similar: 1.2 to 3.0 nmol/liter. Elastase or trypsin digestion of thrombomodulin did not much alter the K_d for the antibodies except in the case of KA-2. The K_d values for antibody KA-2 binding to the elastase or trypsin-digested thrombomodulin were more than 1 μmol/liter, indicating virtually no avidity of KA-2 to the enzyme-digested thrombomodulins. It should be noted that elastase digestion of thrombomodulin doubled the K_d for KA-3. These data are consistent with the results of the immunoblotting studies.

Competition between antibodies for binding to thrombomodulin was examined by ELISA as described in "MATERIALS AND METHODS." Antibodies KA-1 and -4 competed with each other for binding to thrombomodulin (Table IV), but KA-2 and -3 did not compete with the other antibodies in binding to thrombomodulin.

TABLE V. **Functional properties of monoclonal antibodies to thrombomodulin (TM).** Presence or absence of a property of each antibody is indicated by + or −, respectively. Double or triple + sign indicates a higher degree of expression of a property of an antibody as compared with the other antibodies.

Antibodies	Binding to		Inhibition of		Ca^{2+} Dependency
	Elastase-digested TM	Trypsin-digested TM	Thrombin binding	Protein C activation	
KA-1	+	+	++	+++	+
KA-2	−	−	+	+	−
KA-3	+	+	−	−	−
KA-4	+	+	++	++	+

The properties of the monoclonal antibodies described above are summarized in Table V.

DISCUSSION

In the present investigation, we have shown that binding of the monoclonal antibodies (KA-1 and -4) to human thrombomodulin is influenced by calcium concentration; higher concentrations of calcium ions result in more binding of the antibodies to thrombomodulin (Fig. 2). This may suggest that thrombomodulin undergoes a calcium-dependent conformational change which increases the binding of these antibodies to the molecule. Dependence of the binding on calcium ions was not apparent when the major active fragments of the enzyme digests of thrombomodulin were tested for their binding to the antibodies, and the K_ds to the fragments were not appreciably different from those to the native thrombomodulin in the presence of calcium ions (Table III). These facts may suggest that the conformational change brought about by calcium binding or a proteolytic cleavage of the molecule may have removed steric hindrance to the access of the antibodies, and the epitopes may have become more exposed. Since the binding still occurs in the absence of calcium ions, it is unlikely that a calcium-induced conformational change has created new epitopes for the antibodies.

It is known that protein C undergoes a conformational change when it is bound to calcium. The conformational change allows protein C to be sterically fit for its binding to thrombin-thrombomodulin complex and to be effectively activated (*14*). Calcium ions also act as a bridge between thrombomodulin and Gla residues of protein C (*14*). The calcium-induced conformational change of thrombomodulin suggested in the present study may be an additional mechanism of calcium ion involvement in protein C activation catalyzed by thrombin-thrombomodulin complex.

Since the calcium-dependent antibodies, KA-1 and -4, strongly inhibit the binding of thrombin to thrombomodulin (Table II), the epitopes recognized by these antibodies may be located in proximity to a thrombin-binding site. Hence the thrombin-binding domain may undergo a conformational change when calcium binds to the molecule. These calcium-dependent antibodies inhibited the cofactor activity of thrombomodulin in thrombin-catalyzed protein C activation (Fig. 3), most likely by interfering with the binding of thrombin to thrombomodulin. The thrombin-binding domain of thrombomodulin is located in the fifth and sixth epidermal growth factor (EGF)-like structural repeats in the EGF-homology domain which follows the amino-terminal domain (*15*). This suggests that the calcium-dependent antibodies, KA-1 and -4, bind to the EGF-homology domain, particularly to the fifth and sixth EGF-like structural repeats in that domain, and these regions undergo a calcium-induced conformational change. The calcium-dependent antibodies also bind to the major fragment of elastase or trypsin digests of thrombomodulin (Table III and Fig. 4), indicating that the thrombin-binding domain is present in those major fragments. This is in conformity to the facts that these fragments are active as a cofactor in thrombin-catalyzed protein C activation (*16*) and contain mainly of EGF-homology domain (*15*).

The calcium-dependent antibodies, KA-1 and -4, compete with each other for the binding to thrombomodulin (Table IV), indicating that epitopes recognized by these antibodies must be in very close proximity to each other. However, the extents of inhibition of thrombin binding and protein C activation attained by KA-4 were remarkably less than those attained by KA-1 (Table II and Fig. 3), suggesting that the epitope for KA-4 may be more distant from the thrombin-binding site than that for KA-1. KA-4 was also less dependent on calcium ions for its binding to thrombomodulin, supporting the proposal that calcium-induced conformational change occurs in a region located close to the thrombin binding site. Calcium ions, however, do not influence the binding of thrombin to thrombomodulin (*14*), indicating that the calcium-induced conformational change has no relation with thrombin binding but may be related to other functions such as binding to the substrate protein C.

Antibody KA-3, which is not dependent on calcium ions for its binding to thrombomodulin, bound to the major active fragments of the enzyme digests of thrombomodulin (Table III and Fig. 4) but did not inhibit the binding of thrombin to thrombomodulin (Table II) or the activation of protein C (Fig. 3), suggesting that KA-3 binds to somewhere other than the fifth and sixth EGF-like repeats in the EGF-homology domain. Another calcium-independent monoclonal antibody, KA-2, inhibited to some extent the binding of thrombin to thrombomodulin (Table II) and also inhibited the protein C activation to a small extent at a molar excess of antibody over antigen (Fig. 3). However, KA-2 did not bind to the major fragments of the enzyme digests of thrombomodulin (Table III and Fig. 4), suggesting that the epitope recognized by KA-2 may be in the O-glycosylation site-rich domain or the transmembrane domain since these domains are close to the thrombin-binding site (the fifth and sixth EGF-like repeats) but are cleaved off from the major fragments by the proteases (*15*).

In summary, the monoclonal antibodies described in this paper and summarized in Table V recognize various regions in the thrombomodulin molecule and suggest that a conformational change of the molecule is induced by calcium ions in the vicinity of the thrombin binding site.

REFERENCES

1. Esmon, C.T. (1987) *Science* **235**, 1348-1352
2. Esmon, N.L., Owen, W.G., & Esmon, C.T. (1982) *J. Biol. Chem.* **257**, 859-864
3. Maruyama, I., Bell, C.E., & Majerus, P.W. (1985) *J. Cell Biol.* **101**, 363-371
4. Johnson, A.E., Esmon, N.L., Laue, T.M., & Esmon, C.T. (1983) *J. Biol. Chem.* **258**, 5554-5560
5. Kurosawa, S. & Aoki, N. (1985) *Thromb. Res.* **37**, 353-364
6. Suzuki, K., Kusumoto, H., & Hashimoto, S. (1986) *Biochim. Biophys. Acta* **882**, 343-352
7. Suzuki, K., Stenflo, J., Dahlback, B., & Teodorsson, B. (1983) *J. Biol. Chem.* **258**, 1914-1920
8. Lundblad, R.L., Uhteg, L.C., Vogel, C.N., Kingdon, H.S., & Mann, K.G. (1975) *Biochem. Biophys. Res. Commun.* **66**, 482-489
9. Köhler, G. & Milstein, C. (1975) *Nature* **256**, 495-497
10. Nakane, P.K. & Kawaoi, A. (1974) *J. Histochem. Cytochem.* **22**, 1084-1091
11. Laemmli, U.K. (1970) *Nature* **227**, 680-685
12. Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.* **76**, 4350-4354
13. Frankel, M.E. & Gerhard, W. (1979) *Mol. Immunol.* **16**, 101-106
14. Esmon, C.T. (1987) *Science* **235**, 1348-1352
15. Kurosawa, S., Stearns, D.J., Jackson, K.W., & Esmon, C.T. (1988) *J. Biol. Chem.* **263**, 5993-5996
16. Kurosawa, S., Galvin, J.B., Esmon, N.L., & Esmon, C.T. (1987) *J. Biol. Chem.* **262**, 2206-2212