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Equilibrium Binding of Thrombin to Recombinant Human Thrombomodulin: Effect of Hirudin, Fibrinogen, Factor Va, and Peptide Analogues†

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ABSTRACT: Thrombomodulin is an endothelial cell surface receptor for thrombin that acts as a physiological anticoagulant. The properties of recombinant human thrombomodulin were studied in COS-7, CHO, CV-1, and K562 cell lines. Thrombomodulin was expressed on the cell surface as shown by the acquisition of thrombin-dependent protein C activation. Like native thrombomodulin, recombinant thrombomodulin contained N-linked oligosaccharides, had M_r ~100 000, and was inhibited or immunoprecipitated by anti-thrombomodulin antibodies. Binding studies demonstrated that nonrecombinant thrombomodulin expressed by A549 carcinoma cells and recombinant thrombomodulin expressed by CV-1 and K562 cells had similar K_d's for thrombin of 1.3 nM, 3.3 nM, and 4.7 nM, respectively. The K_d for DIP-thrombin binding to recombinant thrombomodulin on CV-1(18A) cells was identical with that of thrombin. Increasing concentrations of hirudin or fibrinogen progressively inhibited the binding of ^{125}I-DIP-thrombin, while factor Va did not inhibit binding. Three synthetic peptides were tested for ability to inhibit DIP-thrombin binding. Both the hirudin peptide Hir$^{53-64}$ and the thrombomodulin fifth-EGF-domain peptide Tm$^{426-444}$ displaced DIP-thrombin from thrombomodulin, but the factor V peptide FacV$^{30-43}$ which is similar in composition and charge to Hir$^{53-64}$ showed no binding inhibition. The data exclude the significant formation of a ternary complex consisting of thrombin, thrombomodulin, and hirudin. These studies are consistent with a model in which thrombomodulin, hirudin, and fibrinogen compete for binding to DIP-thrombin at the same site.

Thrombomodulin is an endothelial cell surface receptor for the blood clotting serine protease thrombin (Esmon & Owen, 1981). The thrombin–thrombomodulin complex activates protein C at least 1000-fold more rapidly than thrombin alone (Esmon et al., 1982). Activated protein C (APC)[1] degrades clotting factors Va and VIIIa (Walker et al., 1979; Vehar & Davie, 1980) and thereby inhibits the further production of thrombin. This and other activities of thrombomodulin make it an important natural anticoagulant (Esmon, C. T., 1987; Esmon, N. L., 1987).

Thrombomodulin was first isolated from rabbit lung (Esmon et al., 1982), and human thrombomodulin was subsequently purified from lung (Maruyama et al., 1985) and placenta (Salem et al., 1984a). Human thrombomodulin has M_r ~ 100 000 and consists of a single polypeptide (Salem et al., 1984a). The nucleotide sequence of human thrombomodulin

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[1] Abbreviations: APC, activated protein C; DIP, diisopropyl fluorophosphate; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HUVE, human umbilical vein endothelial cells; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

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cDNA and genomic DNA has recently been determined (Wen et al., 1987; Suzuki et al., 1987; Jackman et al., 1987; Shirai et al., 1988). The deduced amino acid sequence suggests a structure similar to that of the low-density lipoprotein receptor (Südhof et al., 1985), with six disulfide-linked EGF-like domains.

Thrombomodulin appears to bind two protein ligands, thrombin and protein C, and may also interact with factor Va (Salem et al., 1984a; Maruyama et al., 1984) and factor Xa (Haley et al., 1989). The thrombin binding site on thrombomodulin has recently been shown to be within the fifth and sixth EGF-like domains (Stearns et al., 1989; Suzuki et al., 1989; Zushi et al., 1989). Kinetic analyses have suggested that thrombomodulin inhibits both thrombin inactivation by hirudin, a thrombin inhibitor of leech saliva, and also fibrinogen cleavage by thrombin. Fibrinogen is not only a substrate for thrombin but can also inhibit the interaction of thrombin with other substrates or cofactors. For example, fibrinogen inhibits protein C activation by the thrombin–thrombomodulin complex (Hofsteenge et al., 1986; Jakubowski et al., 1986; Hofsteenge & Stone, 1987; Jakubowski & Owen, 1989). These data suggest that a site on thrombin distinct from the active site but required for binding of hirudin and fibrinogen may overlap that of thrombomodulin binding. However, this model has not been confirmed by direct binding studies, and these data do not exclude the formation of a ternary complex between thrombomodulin, thrombin, and either hirudin or fibrinogen. Analysis of thrombin binding to thrombomodulin on endothelial cells is complicated by nonspecific binding due to other thrombin receptors (Awbrey et al., 1979; Maruyama & Majerus, 1985).

We have expressed active recombinant human thrombomodulin in several heterologous cell lines and have shown that it appears to be structurally and functionally similar to natural thrombomodulin. With this system, we have shown that hirudin, fibrinogen, and synthetic peptides corresponding to the putative thrombin binding domain on thrombomodulin (fifth EGF domain) and hirudin (amino acid residues 53–64) inhibit the binding of DIP-thrombin to thrombomodulin.

Experimental Procedures

Plasmids. Plasmid pSVTM was obtained by cloning the *Sal*I fragment containing the full-length thrombomodulin cDNA from λHTm15 (Wen et al., 1987) into the *Xho*I site of pJC119 (Sprague et al., 1983). Plasmid pUC9RSVgpt was provided by Dr. Timothy Ley (Washington University, Saint Louis, MO). Plasmid pRSVTM was derived from pUC9RSVgpt by replacement of the xanthine–guanine phosphoribosyltransferase gene insert with the thrombomodulin cDNA insert. Briefly, pUC9RSVgpt was digested with *Hind*III. The vector fragment was methylated with *Eco*RI methylase. The *Hind*III termini were filled in by using Klenow fragment and ligated to *Eco*RI linkers (pGGAATTCC, New England Biolabs, Beverly, MA). After *Eco*RI digestion and purification by gel electrophoresis, the vector fragment was ligated to the *Eco*RI fragment from λHTm15 containing the full-length thrombomodulin cDNA. Plasmid pSP65TM was prepared by cloning the thrombomodulin cDNA insert of λHTm15 into the *Sal*I site of pSP65 (Promega, Madison, WI). This plasmid was linearized with *Hind*III, and recombinant thrombomodulin mRNA was prepared by transcription with SP6 DNA-dependent RNA polymerase for translation in a rabbit reticulocyte lysate system (BRL, Gaithersburg, MD) in the presence of [^{35}S]methionine.

Cell Culture and Cell Lines. COS-7 and CV-1 cells were provided by Dr. David Schlessinger (Washington University, Saint Louis, MO). CHO cells were obtained from the Tissue Culture Support Center (Washington University, Saint Louis). K562 erythroleukemia cells (Lozzio & Lozzio, 1975) and mouse hemangioma cells (Fry et al., 1980) were obtained from Drs. Timothy Ley (Washington University, Saint Louis) and John C. Hoak (University of Iowa, Iowa City), respectively. These cells were grown in DMEM containing 4.5 mg/mL glucose, 3.7 mg/mL $NaHCO_3$, 10% FCS, 2 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. For selection of transformants, antibiotic G418 (Gibco, Grand Island, NY) was added to the medium to a final concentration of 1 mg/mL. HUVE cells (CRL 1730) and A549 lung carcinoma cells (CCL 185) were purchased from American Type Culture Collection. HUVE cells were cultured in flasks coated with human fibronectin at 1 μg/cm^2 in 45% 199 medium, 45% F-12 medium 10% FCS, 90 μg/mL heparin, 1 μg/mL hydrocortisone, 10 ng/mL epidermal growth factor, 30 μg/mL endothelial cell growth supplement (Collaborative Research, Lexington, MA), and 100 μg/mL gentamicin. A549 cells were cultured in 199 medium, containing 20% fetal calf serum, 2 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin.

Antibodies. Monoclonal, polyclonal, and affinity-purified polyclonal antibodies to human thrombomodulin were prepared as described previously (Maruyama & Majerus, 1985; Wen et al., 1987). The protein content of the three stocks of antibodies listed above was 230 μg/mL, 1.93 mg/mL, and 84 μg/mL, respectively. Rabbit antiserum to VSV G glycoprotein was a gift of Dr. Milton Schlesinger (Washington University, Saint Louis, MO).

Transfections. For transient expression, 10^6 COS-7 cells were transfected with 10–20 μg of supercoiled plasmid DNA in 35-mm wells by using a DEAE-dextran method (Adams & Rose, 1985). Transfection of CHO and CV-1 cells for the establishment of stably transformed cell lines was done by using the calcium phosphate method (Graham & Van der Eb, 1973; Parker & Stark, 1979). Confluent monolayers of cells in 35-mm wells (~10^6 cells) were cotransfected with 5 μg of *Sca*I-linearized pRSVTM and 0.5–2.5 μg of *Eco*RI-linearized pSV2neo (Southern & Berg, 1982).

Transfection of K562 cells by electroporation was carried out with a procedure modified from that previously described (Chu et al., 1987). Briefly, 10^7 K562 cells from late log phase of growth were resuspended in 1 × HEBS buffer (20 mM Hepes, pH 7.05, 137 mM NaCl, 5 mM KCl, 6 mM dextrose, and 0.7 mM Na_2HPO_4). Sixty-five microliters of DNA in 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA (5 μg of *Sca*I-linearized pSV2neo DNA and 5–50 μg of *Eco*RI-linearized pRSVTM DNA), 25 μL of 5 μg/μL salmon sperm DNA, and 10 μL of 10 × HEBS buffer were mixed and added to the cell suspension. This cell–DNA mixture was electroporated in a 600-μL semimicrocuvette at 600 μF with a single pulse of 225 V delivered from the BTX Transfector 300 (Biotechnologies and Experimental Research Inc., San Diego, CA) through a 1.9-mm gap stainless steel electrode. After electroporation, the whole cell–DNA mixture was transferred to 9.5 mL of DMEM containing 5% FCS and 5% controlled process serum replacement 4 (Sigma, Saint Louis, MO) and incubated at 37 °C for 24 h before G418 selection.

Northern Blotting. Total RNA was prepared from cultured cells by the procedure of Chomczynski and Sacchi (1987). Thrombomodulin cDNA probes were random primer labeled with [α-^{32}P]dCTP to a specific activity of ~4 × 10^9 dpm/μg of DNA (Feinberg & Vogelstein, 1983). Electrophoresis on agarose gels, blotting, and hybridization were performed ac-

cording to standard protocols (Lehrach et al., 1977). After 12 h of hybridization, the RNA blots were washed in 1.5 mM sodium citrate, pH 7.0, 15 mM NaCl, and 0.1% SDS at 65 °C for 30 min.

Biosynthetic Labeling and Immunoprecipitation. In a typical experiment, cells grown in 35-mm wells (~1 × 10^6 cells/well) were labeled for 3 h at 37 °C in 0.5 mL of serum-free and cysteine-free medium to which 100 μCi of [^{35}S]cysteine was added. After labeling, the medium was removed and saved. The cells were washed once with ice-cold PBS (137 mM NaCl, 3 mM KCl, 15 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2) and lysed in 0.5 mL of 0.6% (v/v) Triton X-100, 0.1 M Tris-HCl, pH 8.1, 135 KIU/mL aprotinin, and 1 mM *Nα-p*-tosyl-L-lysine chloromethyl ketone. Both culture medium and cell lysate were centrifuged at 4 °C, 12000*g* for 10 min, and the supernatants were saved for immunoprecipitation. Immunoprecipitation from the lysate was performed by incubating 0.5 mL of lysate supernatant with 50 μL of 1% (w/v) SDS, 15 μL of protein A–Sepharose beads (1:1 slurry in PBS), and 15 μL of either 1.93 mg/mL rabbit anti-thrombomodulin polyclonal antibodies or 84 μg/mL affinity-purified anti-thrombomodulin polyclonal antibodies at 4 °C for 12 h with gentle shaking. Immunoprecipitation from the culture medium was done as from the lysate except that 30 μL of 10% (v/v) Triton X-100 was first added to 0.5 mL of culture medium. The beads were washed three times by centrifugation and resuspension in 1 mL of ice-cold washing solution [0.1% (v/v) Triton X-100, 0.02% (w/v) SDS, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 135 KIU/mL aprotinin] and then resuspended in either sample buffer for SDS–PAGE or 0.15% (w/v) SDS for further analysis by glycosidase digestion.

For inhibition of N-linked glycosylation, cells were incubated in medium containing 1 μg/mL tunicamycin for 1.5 h before labeling and in medium containing 0.75 μg/mL tunicamycin for 3 h during labeling. Digestion of immunoprecipitated proteins with endoglycosidase F, neuraminidase, and *O*-glycanase was done as previously described (Ye et al., 1988), except that *O*-glycanase was used at a final concentration of 0.15 unit/mL.

The amount of thrombomodulin synthesized as a percentage of total protein synthesis was estimated by biosynthetic labeling as above and comparison of the recovery of radioactivity in immunoprecipitates to total radioactivity precipitated with trichloroacetic acid. Briefly, 30 μL of 1 mg/mL bovine serum albumin was mixed with 5 μL of cell lysate, precipitated in 1 mL of 10% trichloroacetic acid for 10 min, and filtered through Whatman glass microfiber GF/A filters.

Gel Electrophoresis and Autoradiography. Immunoprecipitates from cell lysates or culture medium were reduced in sample buffer containing 5% (v/v) β-mercaptoethanol at 100 °C for 4 min and electrophoresed by using an SDS–PAGE with 5% polyacrylamide in stacking gel and 10% polyacrylamide in separating gel (Laemmli, 1970). Autoradiography was done as described (Bonner & Laskey, 1974).

Proteins and Peptides. Purified human protein C, APC, and prothrombin were gifts of Dr. Joseph Miletich (Washington University, Saint Louis), and human antithrombin III was provided by Dr. Douglas Tollefsen (Washington University, Saint Louis). Human thrombin was prepared by activation of prothrombin with *Echis carinatus* venom (Sigma) (Kawabata et al., 1985) and purification on Amberlite CG-50 (Fenton et al., 1977). Thrombin concentration was determined by absorbance at 280 nm, using the extinction coefficient $E^{1\%}$ = 18.3. Diisopropyl fluorophosphate was purchased from Sigma (St. Louis). Human fibrinogen was purchased from Kabi-Vitrum (Sweden). Factor V was prepared as previously described (Kane & Majerus, 1981). The peptides were synthesized with an ABI Model 430 peptide synthesizer using Boc chemistry and standard cycle conditions and purified by reverse-phase HPLC (Crimmins et al., 1988). The purity of Hir^{53-64}, $FacV^{30-43}$, and $Tm^{426-444}$ as determined by reverse-phase HPLC analysis was 95%, 96%, and 83% respectively. DIP-thrombin was prepared by incubating 1 mg of diisopropyl fluorophosphate with 0.7 mg of thrombin in 1 mL of 20 mM HEPES, pH 8.0 and 0.75 M NaCl at 4 °C for 12 h with continuous mixing followed by dialysis against 2 L of 0.15 M NaCl and 0.05 M sodium citrate, pH 6.5, for 48 h. After modification, thrombin clotting activity decreased by over 99%.

Three preparations of hirudin were employed. Most of the data were obtained with partially purified natural hirudin (Sigma, 1050 antithrombin units/mg), hereafter referred to as "hirudin" or "partially pure hirudin". This preparation exhibited a single major band of ~8000 daltons and several minor larger species on SDS–PAGE. Highly purified natural hirudin (Calbiochem, 10900 units/mg) and pure recombinant hirudin ([Lys^{47}] variant, Sigma, 8750 units/mg) were used for some experiments. Highly purified natural hirudin exhibited a single major band of ~8000 daltons on SDS–PAGE accounting for ≥80% of protein detected either by silver staining or by radioiodination and autoradiography. Recombinant hirudin exhibited a single species of ~8000 daltons on SDS–PAGE by either silver staining or radioiodination and autoradiography.

Thrombomodulin Cofactor Activity Assay. Cell surface thrombomodulin cofactor activity was assayed as described (Suzuki et al., 1987). Approximately 10^6 cells were washed with ice-cold PBS and suspended in 174 μL of ice-cold assay buffer (50 mM Tris-HCl, pH 8.0, 2 mM $CaCl_2$, 100 mM NaCl, 0.1% bovine serum albumin). Thrombin and protein C were added to a concentration of 14.9 nM and 0.89 μM, respectively, in a final volume of 100 μL. The number of CV-1 cells/35-mm well was determined by counting the cells trypsinized from an identically plated well with a Coulter Counter ZM (Luton Beds, England). The amount of APC present in the assay was calculated from a standard curve generated with purified APC [pmol of APC = (13.4)(ΔA_{405}/min)]. Control assays in the absence of cells gave ΔA_{405}/min values of 0.005–0.01 min^{-1}. Values are expressed in pmol of APC generated/(30 min·10^6 cells).

For monoclonal antibody inhibition, CV-1(18A) or HUVE were washed twice with cold PBS and once with cold thrombomodulin assay buffer. One to two million cells were then lysed in 1 mL of assay buffer containing 0.6% Triton X-100. The nuclei were removed by centrifugation at 12000*g*, 4 °C for 10 min. Then 63 μL of the supernatants was mixed with increasing amounts of monoclonal anti-thrombomodulin IgG in a final volume of 87 μL of assay buffer containing 0.6% Triton X-100. After 15-min incubation at 37 °C, the cell lysate–IgG mixtures were assayed for thrombomodulin cofactor activity.

Competition Equilibrium Binding. Thrombin, DIP-thrombin, fibrinogen, and hirudin were labeled with $Na^{125}I$ (~13 mCi/μg ^{125}I) for 10 s with the chloramine T method (Tollefsen et al., 1974) to ~3 × 10^6 cpm/μg (~0.06 μCi/pmol of protein, ~0.03 mol of ^{125}I/mol of protein). Thrombin, thrombin derivatives, and fibrinogen were recovered essentially quantitatively after iodination as determined by SDS–PAGE, staining with Coomassie Blue, and densitometry. Clotting activity of thrombin was decreased ~50%. Hirudin activity

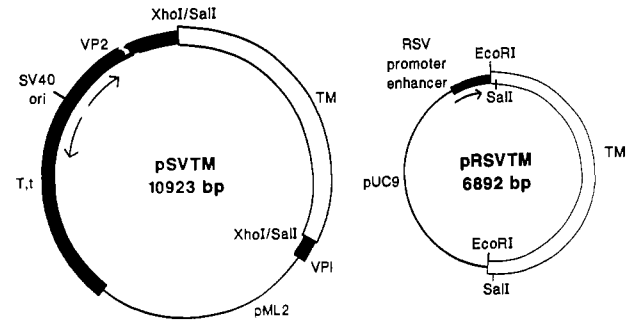


FIGURE 1: Structure of human thrombomodulin expression vectors pSVTM and pRSVTM. Open box, thrombomodulin cDNA; filled box, viral sequence; line, prokaryotic plasmid sequence (pML2 or pUC9). The arrows indicate the direction of transcription. VP1, major structural protein 1; VP2, minor structural protein 2; T, large T antigen; t, small T antigen, SV40 ori, simian virus 40 replication origin. pSVTM was employed mainly for transient expression in COS-7 cells; it contains a polyadenylation signal from the SV40 late genes that is 138 nucleotides downstream from the 3'-terminus of the cDNA insert. pRSVTM was used for stable transfection of CHO, CV-1, and K562 cells. It contains no additional 3' polyadenylation or processing signal.

was recovered essentially quantitatively after iodination as determined by inhibition of fibrinogen clotting by thrombin.

Equilibrium binding was performed by using a modification of a previously described method (Owensby et al., 1988). Competition binding studies with increasing concentrations of unlabeled ligand or receptor (thrombin, DIP-thrombin, hirudin, fibrinogen, factor Va, or synthetic peptides Hir^{53-64}, $FacV^{30-43}$, and $Tm^{426-444}$) were performed with 0.3 nM ^{125}I-thrombin or ^{125}I-DIP-thrombin. For adherent CV-1(18A) and A549 cells, binding was done at 4 °C for 2 h in 22-mm wells ($\sim 4 \times 10^5$ cells/well) in 1 mL of binding medium (100% DMEM, 20 mM Hepes, pH 7.3, 0.1 mg/mL cytochrome *c*) containing various concentrations of unlabeled ligand. Binding for 1 h was shown to be sufficient to achieve equilibrium (data not shown).

After binding, the monolayers were washed four times by immersion in cold PBS for 3–5 s. The total time of washing was ≤15 s. Each monolayer was then solubilized in 1 mL of 1 M NaOH for γ counting. For K562(5/50K) cells, which grow in suspension, binding was done in Eppendorf tubes using 1 mL of the same binding medium containing a suspension of $\sim 1 \times 10^6$ cells. After binding, the cell suspension was overlayed on a cushion of 500 μL of heat-inactivated FCS and centrifuged. The supernatant fraction was removed and the cell pellet solubilized as described above. With the exception of the experiment in Figure 7A, specific binding was calculated by substracting from total binding the number of counts bound to an equal number of untransfected cells. In Figure 7A, specific binding was obtained by subtracting the number of counts bound in the presence of 600 nM unlabeled thrombin. Under these conditions, the binding of ^{125}I-thrombin to CV-1(18A) cells is indistinguishable from binding to untransfected CV-1 cells in the absence of unlabeled thrombin (data not shown).

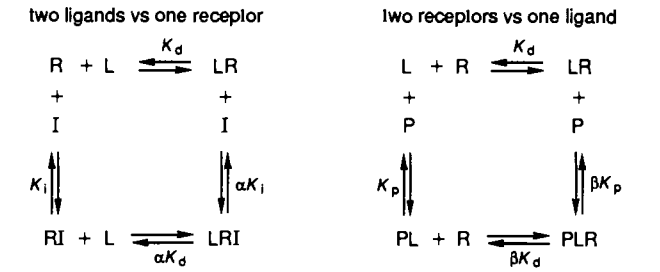
Direct Equilibrium Binding. Increasing concentrations (0.5–200 nM) of ^{125}I-DIP-thrombin in 1 mL of binding medium were incubated with CV-1 or CV-1(18A) cells in 22-mm wells (5×10^5 cells/well). Binding of ^{125}I-DIP-thrombin to untransfected CV-1 cells and binding of ^{125}I-DIP-thrombin (0.5–8 nM) to CV-1(18A) cells in the presence of 800 nM unlabeled thrombin gave similar values for nonspecific thrombin binding. Total receptor concentration R_m and dissociation constant K_d* of ^{125}I-DIP-thrombin ligand *L were determined by fitting the following equations, where N is the affinity constant for nonspecific binding of ligand L:

$$\text{nonspecific binding} = N[{}^*\text{L}] \quad (1)$$

$$\text{total binding} = \frac{R_m[{}^*\text{L}]}{K_d{}^* + [{}^*\text{L}]} + N[{}^*\text{L}] \quad (2)$$

$$\text{specific binding} = \frac{R_m[{}^*\text{L}]}{K_d{}^* + [{}^*\text{L}]} \quad (3)$$

Analysis of Equilibrium Binding of Ligand to Receptor in the Presence of a Third Component. For equilibrium binding of ligand L to receptor R, the third component can be either a second ligand I that binds to receptor R or a second receptor P that binds to ligand L:



$R_m = [R] + [RI] + [LR] + [LRI]$
$L_m = [L] + [LR] + [LRI]$
$I_m = [I] + [RI] + [LRI]$

$L_m = [L] + [LR] + [PL] + [PLR]$
$R_m = [R] + [LR] + [PLR]$
$P_m = [P] + [PL] + [PLR]$

if (α or β) = $+\infty$	competitive binding
if (α or β) > 1	partially competitive binding
if (α or β) = 1	noncompetitive binding
if 0 < (α or β) < 1	"promotional" binding

Depending on the values of α or β, the binding of an increasing amount of the third component can be either competitive, partially competitive, noncompetitive, or "promotional" with respect to the binding of ligand L to receptor R. In our experiments this represents the cell-bound state of labeled thrombin, (LR + LRI) or (LR + PLR), which can be measured.

Competitive Equilibrium Binding (α *or* $\beta = +\infty$). For competitive binding no ternary complex can form and LR is the sole R-bound state of ligand L. The expressions of [LR] can be derived for each of the two cases: (1) two ligands and one receptor or (2) two receptors and one ligand.

(1) The Case of Two Ligands and One Receptor. When K_d and K_i are much larger than R_m, $L_m \simeq [L]$ and $I_m \simeq [I]$, and [LR] is given by the expression:

$$[LR] = \frac{R_m L_m}{K_d(1 + I_m/K_i) + L_m} \quad (4)$$

By simple substitution, [LR] can be expressed as a quantity, Y, that is a function of X, where $X = I_m$. A fractional displacement function $D(X)$ can be defined that is the ratio of $Y = [LR]$ at any concentration of $X = I_m$ over $Y = [LR]$ when $X = 0$:

$$D(X) = \frac{Y(X = I_m)}{Y(X = 0)} = \frac{K_i(1 + L_m/K_d)}{K_i(1 + L_m/K_d) + X} = \frac{EC_{50}}{EC_{50} + X} \quad (5)$$

By definition, $D(EC_{50}) = 0.5$, and

$$EC_{50} = K_i(1 + L_m/K_d) \quad (6)$$

(2) The Case of Two Receptors and One Ligand. When K_d is much larger than R_m, $L_m \simeq [L] + [PL]$, and [LR] is given by the expression:

$$[LR] = \frac{R_m(L_m - P_m - K_p + \sqrt{(P_m + K_p - L_m)^2 + 4K_pL_m})}{2K_d + (L_m - P_m - K_p + \sqrt{(P_m + K_p - L_m)^2 + 4K_pL_m})} \quad (7)$$

Again, [LR] can be expressed as a function, Y, of $X = P_m$, and the fractional displacement function $D(X)$ of ligand L by receptor P is defined as

$$D(X) = \frac{Y(X = P_m)}{Y(X = 0)} = \frac{(1 + K_d/L_m)(L_m - X - K_p + \sqrt{(X + K_p - L_m)^2 + 4K_pL_m})}{2K_d + (L_m - X - K_p + \sqrt{(X + K_p - L_m)^2 + 4K_pL_m})} \quad (8)$$

and also

$$EC_{50} = L_m\left(1 + \frac{K_p}{K_d} - \frac{K_d}{L_m + 2K_d}\right) + K_p \quad (9)$$

In this case the displacement function $D(X)$ is sigmoidal rather than hyperbolic in shape on a linear scale.

Equilibrium Binding Other Than Competitive ($0 < \alpha$ or $\beta < +\infty$). For the remaining cases where the values of α and β are finite, the sum of the ternary complex (LRI or PLR) and LR represents the R-bound state of ligand L. Again, we have the following two cases:

(1) The Case of Two Ligands and One Receptor.

$$[LR] = \frac{[L]R_m - [LRI](\alpha K_d + [L])}{K_d + [L]} \quad (10)$$

$$[LRI] = \left\{\alpha K_i\left(\frac{K_d}{[L]} + 1\right) + (R_m + I_m)\left(\frac{\alpha K_d}{[L]} + 1\right) - \left[\left[\alpha K_i\left(\frac{K_d}{[L]} + 1\right) + (R_m + I_m)\left(\frac{\alpha K_d}{[L]} + 1\right)\right]^2 - 4I_mR_m\left(\frac{\alpha K_d}{[L]} + 1\right)^2\right]^{1/2}\right\} \Big/ 2\left(\frac{\alpha K_d}{[L]} + 1\right)^2 \quad (11)$$

where $[L] = L_m - [LR] - [LRI]$. Notice that the expressions of [LR] and [LRI] do not contain the term L_m. Given K_d, K_i, R_m, and L_m, the curves of [LR] and [LRI] as a function of I_m can be calculated by successive approximation. The method consists of using L_m as a first approximation for [L] in the expressions of [LR] and [LRI]. The approximate values of [LR] and [LRI] thus calculated can then be used to obtain a better value for [L]. This process is reiterated until the values converge to a limit.

(2) The Case of Two Receptors and One Ligand.

$$[LR] = \frac{[L](R_m - [PLR])}{K_d + [L]} \quad (12)$$

$$[PLR] = \left\{\beta K_d + \beta K_p + \beta[L] + \frac{\beta K_dK_p}{[L]} + R_m + P_m - \left[\left(\beta K_d + \beta K_p + \beta[L] + \frac{\beta K_dK_p}{[L]} + R_m + P_m\right)^2 - 4R_mP_m\right]^{1/2}\right\} \Big/ 2 \quad (13)$$

When K_d is much larger than R_m, $L_m \simeq [L] + [PL] + [PLR]$, and [L] is given by the expression:

$$[L] = \frac{L_m - P_m - K_p + \sqrt{(P_m + K_p - L_m)^2 - 4K_p([PLR] - L_m)}}{2} \quad (14)$$

Given K_d, K_p, R_m, and L_m, successive approximation can be used to calculate the curves of [LR] and [PLR] as a function of P_m.

Determination of Dissociation Constants. In a competition binding experiment the fractional specific binding B at a given concentration of unlabeled competitor C can be calculated from

$$B = \frac{(C^*L) - NS}{(^*L) - NS} \quad (15)$$

where (*L) = binding of radiolabeled ligand *L in the absence of unlabeled competitor C, (C*L) = binding of radiolabeled ligand *L in the presence of unlabeled competitor C, and NS = nonspecific binding of radiolabeled ligand *L to transformed cells in the presence of sufficient unlabeled competitor C such that $[C] \gg K_c$ (the dissociation constant of C), or to homologous untransformed cells.

When the radiolabeled ligand is ^{125}I-thrombin or ^{125}I-DIP-thrombin and the unlabeled competitor C is another ligand I (thrombin or DIP-thrombin), specific binding B has the functional form of eq 5:

$$B = \frac{EC_{50}{}^n}{EC_{50}{}^n + [I]^n} \quad (16)$$

where [I] = concentration of unlabeled ligand used as competitor, EC_{50} = concentration of unlabeled ligand that causes a 50% displacement of radiolabeled ligand *L, and n = pseudo Hill coefficient (Limbird, 1986). To obtain the values of n and EC_{50}, eq 16 was fitted with the RS/1 system (BBN Software Products Corporation, Cambridge, MA) to binding data. The range of values given to RS/1 to perform the fit was as follows: n = 0.4–1.2 by 0.1; EC_{50} = 0.5–6 by 0.5. The relationship between the equilibrium dissociation constants K_i of unlabeled ligand I and K_d* of radiolabeled ligand *L and EC_{50} is given by the following equation, which results from the application of eq 6:

$$EC_{50} = K_i(1 + [^*L]/K_d^*) \quad (17)$$

It can be seen from eq 17 that if $[^*L] \ll K_d^*$, then $EC_{50} \simeq K_i$. Moreover, if iodination of thrombin does not change its affinity for thrombomodulin, then $K_i = K_d^*$. Equation 17 can then be solved for K_i after substituting K_i for K_d^*.

When the radiolabeled ligand is ^{125}I-DIP-thrombin and the unlabeled competitor C is a receptor P (hirudin, fibrinogen, Hir^{53-64}, or $Tm^{426-444}$), specific binding B is given by the following equation, which results from the application of eq 8:

$$B = [(1 + K_d^*/[^*L])([^*L] - [P] - K_p + \sqrt{([P] + K_p - [^*L])^2 + 4K_p[^*L]})]/[2K_d^* + ([^*L] - [P] - K_p + \sqrt{([P] + K_p - [^*L])^2 + 4K_p[^*L]})] \quad (18)$$

where K_d^* = dissociation constant of ^{125}I-DIP-thrombin with respect to thrombomodulin (previously determined), K_p = dissociation constant of receptor P with respect to ^{125}I-DIP-thrombin (unknown), [*L] = concentration of ^{125}I-DIP-thrombin (fixed), and [P] = concentration of competing receptor P used (variable). To obtain the value of K_p, eq 18 was fitted with the RS/1 system to binding data.

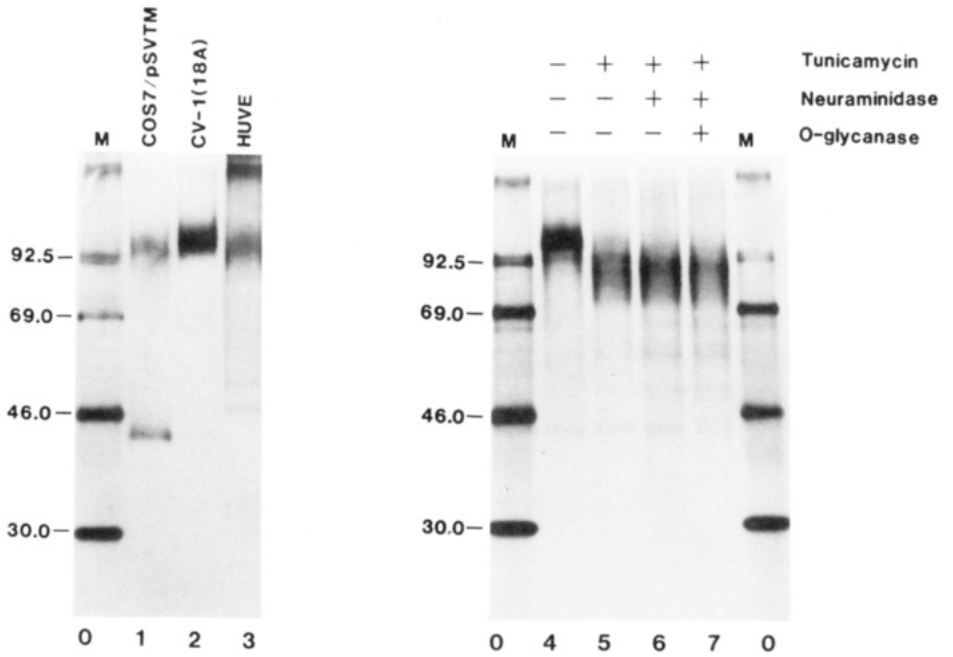


FIGURE 2: Biosynthesis and glycosylation of recombinant human thrombomodulin by CV-1(18A) cells. Biosynthetic labeling, treatment with tunicamycin, immunoprecipitation with polyclonal anti-thromomodulin antibody, glycosidase digestion, and gel electrophoresis were performed as described under Experimental Procedures. Lanes 1–3, comparison of thromomodulin immunoprecipitated from lysates of pSVTM transfected COS-7 cells (COS7/pSVTM), CV-1(18A) cells, or human umbilical vein endothelial cells (HUVE). Lanes 4–7, thrombomodulin immunoprecipitated from lysates of CV-1(18A) cells treated with or without tunicamycin and disgested with or without neuraminidase and *O*-glycanase as indicated.

RESULTS

Expression of Recombinant Human Thrombomodulin. Two expression vectors containing a full-length human thrombomodulin cDNA were constructed (Figure 1). The human thrombomodulin cDNA insert of λHTm15 (Wen et al., 1987) possesses four potential polyadenylation sites in its 3′ noncoding region. CV-1 cells were chosen as one host cell for these studies because thrombomodulin had been expressed successfully in COS cells (Suzuki et al., 1987), which were derived from CV-1 cells by integration of origin-defective SV40 DNA (Gluzman, 1981). K562 human erythroleukemia cells were also used because they grow readily in suspension and because they have been useful for the study of transferrin binding and endocytosis (Stein & Sussman, 1986). In all cases stable cell lines were obtained by cotransfection with pSV2neo and selection with the antibiotic G418. The resultant CHO and K562 cell lines were readily cloned. CV-1 cell lines were difficult to grow from single cells and instead were generally propogated as pools of several clones. For each CV-1 cell line the approximate number of clones that it contains is indicated by the number in parentheses in its name.

Untransfected COS-7, CHO, CV-1, and K562 cells did not express detectable thrombomodulin mRNA by Northern blotting under stringent hybridization conditions with a human thrombomodulin cDNA probe (data not shown). COS-7 cells transiently transfected with pSVTM and CV-1 and CHO cells stably transfected with pRSVTM expressed thrombomodulin RNA species of expected sizes on Northern blot analysis (data not shown).

Biological Activity of Recombinant Human Thrombomodulin. The expression of active recombinant thrombomodulin at the cell surface was demonstrated by functional assays of intact cultured cells (Table I). Untransfected CV-1 and K562 cells had no detectable endogenous thrombomodulin cofactor activity. In contrast, CHO cells had ~50% as much thrombomodulin activity as a mouse hemangioma cell line that is known to express high levels of mouse thrombomodulin. This is presumably due to the expression of hamster thrombomodulin. As a consequence of this endogenous activity, transfected CHO cell lines were not used further for functional assays.

The expression of pSVTM in transiently transfected COS-7 cells gave detectable thrombomodulin activity compared to cells transfected with the pJC119 vector alone. Transfected CV-1 cell lines expressed 5–30 times as much thrombomodulin activity as was obtained by transient expression. Cell lines CV-1(18A), CV-1(18B), CV-1(20), and CV-1(22) expressed thrombomodulin as 0.8%, 0.6%, 0.2%, and 0.2% of total protein synthesized, respectively. The thrombomodulin activity expressed in CV-1(18A) cells was ~1.4 times that of both cultured endothelial cells and mouse hemangioma cells and ~1.6 times that of A549 human lung carcinoma cells. The CV-1(18A) cell line had the highest level of thrombomodulin expression whether assayed by functional assay or by immunoprecipitation, and this line was used for the subsequent characterization of recombinant thrombomodulin.

Biosynthesis and Glycosylation of Recombinant Human Thrombomodulin. Recombinant human thrombomodulin was initially expressed in COS-7 cells by transfection with pSVTM. After biosynthetic labeling for 3 h with [^{35}S]cysteine, a protein similar in size to natural thrombomodulin with M_r ~100 000 was immunoprecipitated with polyclonal anti-thrombomodulin antibodies (Figure 2). This species was not immunoprecipitated by antiserum to the VSV G glycoprotein, and anti-thrombomodulin antibodies did not yield a similar species from cells transfected with the pJC119 vector alone (data not shown). A radiolabeled protein of M_r ~105 000 was immunoprecipitated from lysates of stably transfected CV-1(18A) cells that was absent in lysates of the parent CV-1 cells and similar in size to that expressed in transiently transfected COS-7 cells (Figure 3, lanes 1–3).

The glycosylation of thrombomodulin was investigated by synthesis in the presence of tunicamycin and by digestion with glycosidases. Thrombomodulin contains 5 potential N-linked

Table I: Cell Surface Thrombomodulin Activity of Transfected and Untransfected Cells

cells	thrombomodulin activity [pmol of APC/(30 min·10^6 cells)]
untransfected cells	
mouse hemangioma	38.2[a]
HUVE	39.2[b]
A549	33.8
CHO	18.2[c]
CV-1	−0.2[c]
K562	−0.1[d]
transfected cells	
COS-7 pJC119 (20 μg)	0.45
COS-7 pSVTM (20 μg)	2.0
CV-1(18A)	54.6
CV-1(18B)	13.4
CV-1(20)	20.5
CV-1(22)	24.4
K562(5/50A)	23.9
K562(5/50C)	27.3
K562(5/50K)	28.6

[a] Average of 4 experiments. [b] Averages of 5 experiments. [c] Average of 3 experiments. [d] Average of 2 experiments.

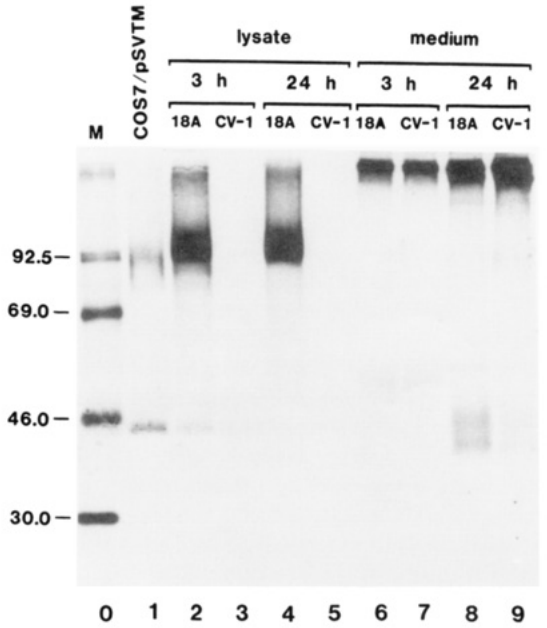


FIGURE 3: Time course of recombinant human thrombomodulin synthesis in CV-1(18A) cells. Biosynthetic labeling, immunoprecipitation with polyclonal anti-thrombomodulin antibody, and gel electrophoresis were performed as described under Experimental Procedures. Lane 0, ^{14}C-labeled protein standards; lane 1, lysates of COS-7 cells transfected with pSVTM and labeled for 3 h; lanes 2–9, lysates or culture medium from cell line CV-1(18A) or the parent CV-1 cells labeled for 3 or 24 h, as indicated.

glycosylation sites and 8 clustered hydroxyamino acids that may be O-glycosylated (Wen et al., 1987). Thrombomodulin synthesized by CV-1(18A) cells in the presence of tunicamycin was detected as a broad band, perhaps a doublet, of M_r ~ 87 000–92 000 (Figure 2, lane 5). Digestion of thrombomodulin expressed by transfected COS-7 cells with endoglycosidase F also reduced its size to M_r ~87 000 (data not shown). This decrease of M_r ~13 000–18 000 is due to the loss of N-linked oligosaccharides. Translation in a rabbit reticulocyte lysate system of synthetic thrombomodulin mRNA yielded a product with M_r ~68 000, which is comparable to the size predicted from the cDNA sequence of M_r 60 300 (data not shown). The remaining difference between M_r ~87 000 and M_r ~68 000 may be due to O-linked glycosylation or other posttranslational modifications. However, digestion of thrombomodulin from tunicamycin-treated CV-1(18A) cells

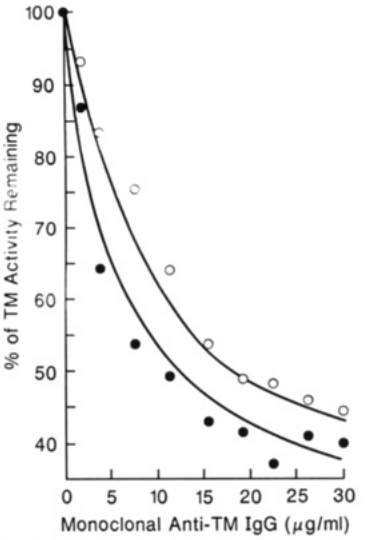


FIGURE 4: Inhibition of thrombomodulin cofactor activity in lysates of CV-1(18A) cells and umbilical vein endothelial cells by monoclonal anti-thrombomodulin IgG. (○) CV-1(18A) lysate; (●) HUVE lysate.

with neuraminidase and *O*-glycanase did not further reduce the size of the protein (Figure 2, lanes 6 and 7), suggesting that the remaining oligosaccharides, if any, are resistant to these enzymes.

The time course of thrombomodulin synthesis was studied by immunoprecipitation of biosynthetically labeled thrombomodulin from CV-1(18A) cells. During a 24-h labeling of these cells, no detectable thrombomodulin was secreted into the medium and there was no change in the size of the species immunoprecipitated from cell lysates (Figure 3, lanes 3–9). The unidentified radiolabeled material detected in culture medium near the origin of the gel (Figure 3, lanes 6–9) was observed in both transfected and untransfected cells in these experiments but not in other experiments (data not shown), and therefore it is not thrombomodulin.

Inhibition of Recombinant Human Thrombomodulin by Monoclonal Antibody. A monoclonal antibody that was known to inhibit human thrombomodulin cofactor activity (Maruyama & Majerus, 1985) was employed to compare natural and recombinant thrombomodulin (Figure 4). Lysates prepared from CV-1(18A) cells (2×10^6/mL) and from human umbilical vein endothelial cells (1×10^6/mL) were incubated with increasing concentrations of anti-thrombomodulin antibody and assayed for residual cofactor activity. Prior to the addition of antibody, the CV-1(18A) and endothelial cell lysates generated 300 and 54 pmol of APC/(30 min·10^6 cells), respectively, in the standard thrombomodulin cofactor assay. The average specific activity for CV-1(18A) cell lysate among three experiments was 108 pmol of APC/(30 min·10^6 cells). The average value for endothelial cell lysate among six experiments was 85 pmol of APC/(30 min·10^6 cells). Both natural and recombinant thrombomodulin activities were inhibited by monoclonal anti-thrombomodulin IgG with a similar dose response (Figure 4). The antibody concentration required for 50% inhibition was 12.5 μg/mL for endothelial cell thrombomodulin. This value was 16.6 μg/mL for CV-1(18A) thrombomodulin in the experiment of Figure 4, and the average among three experiments was 13.3 μg/L. This result suggests that recombinant thrombomodulin is immunologically very similar to natural thrombomodulin.

Binding Affinity of Thrombin for Recombinant Thrombomodulin. Control experiments demonstrated that ^{125}I-DIP-thrombin binding to recombinant thrombomodulin expressed by CV-1(18A) cells reached equilibrium by approximately 60

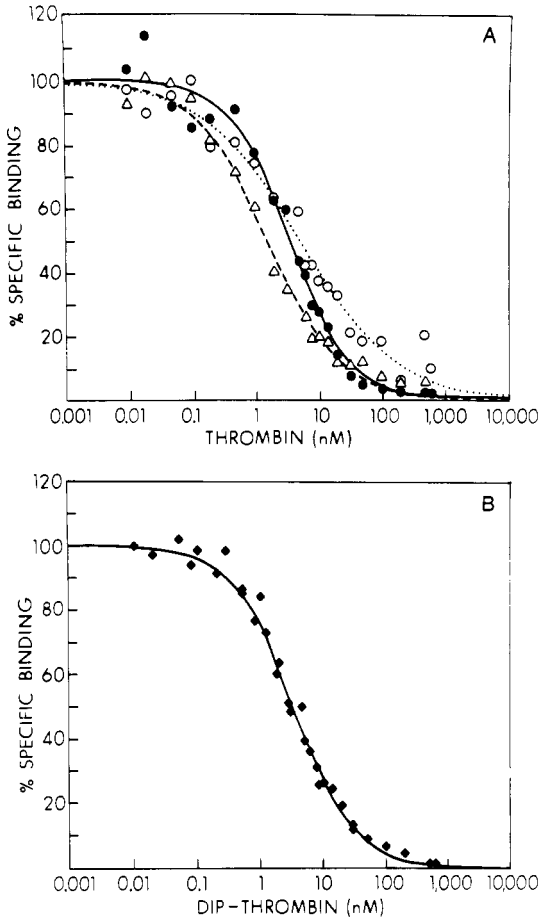


FIGURE 5: Binding of thrombin and DIP-thrombin to thrombomodulin. Panel A: ^{125}I-Thrombin at 0.3 nM was incubated with cells at 4 °C for 2 h in the presence of increasing concentrations of unlabeled thrombin up to 600 nM. Cells were washed and bound thrombin was quantitated by γ counting. Filled circles, CV-1(18A) cells; open circles, K562(5/50K) cells; open triangles, A549 cells; continuous line, binding curve fitted to data for CV-1(18A) cells (n = 0.98, K_d = 3.26 nM); dotted line, binding curve fitted to data for K562(5/50K) cells (n = 0.59, K_d = 4.67 nM); dashed line, binding curve fitted to data for A549 cells (n = 0.78, K_d = 1.26 nM). The signal (100% binding) to background (0% binding) ratio was 7.5 for CV-1(18A) cells, 3.3 for K562(5/50K) cells, and 9 for A549 cells. Binding data were fitted to eqs 16 and 17. Panel B: Same experimental protocol as in panel A, except that thrombin and ^{125}I-thrombin were replaced by DIP-thrombin and ^{125}I-DIP-thrombin. The binding was performed on CV-1(18A) cells, and the signal (100%) to background (0%) ratio was 15.

min (data not shown). The dissociation of ^{125}I-thrombin from thrombomodulin on CV-1(18A) cells at 4 °C was studied in the presence of 400 nM unlabeled thrombin. Binding was ≥92% reversible, and dissociation appeared to be a first-order process with a half-time of 4.6 min (data not shown). To determine the equilibrium dissociation constant of thrombin from thrombomodulin, we performed competition binding of 0.3 nM ^{125}I-thrombin with increasing concentrations of unlabeled thrombin up to 600 nM using CV-1(18A), K562(5/50K), and A549 human lung carcinoma cells. Binding data were analyzed by using eq 16 and 17. For the individual experiments shown in Figure 5A, the values of the pseudo Hill coefficient, n, and the dissociation constant, K_d, were 0.98 and 3.26 ± 0.29 nM for CV-1(18A) cells, 0.59 and 4.67 ± 0.63 nM for K562(5/50K) cells, and 0.78 and 1.26 ± 0.12 nM for A549 cells. Competition of ^{125}I-DIP-thrombin binding to CV-1(18A) cells with increasing concentrations of unlabeled DIP-thrombin produced similar values for n and K_d of 0.94 and 3.26 ± 0.15 nM (Figure 5B). To estimate the number of recombinant thrombomodulin molecules on the surface of

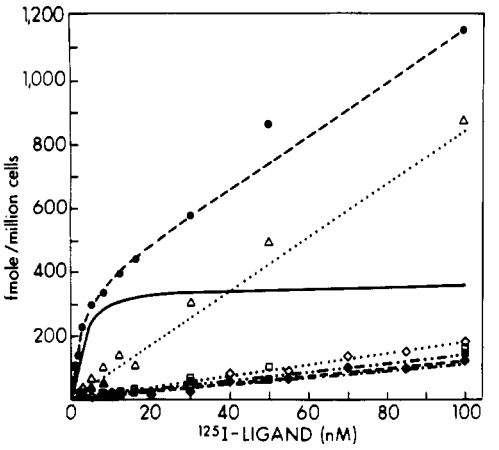


FIGURE 6: Binding of DIP-thrombin and hirudin to CV-1(18A) cells. Increasing concentrations of ^{125}I-DIP-thrombin (0–200 nM) or ^{125}I-hirudin (0–100 nM) were incubated with either CV-1(18A) or CV-1 cells at 4 °C for 2 h. Cells were washed and bound ligand was quantitated by γ counting. Binding data were fitted to eq 2. Filled circles, total binding of ^{125}I-DIP-thrombin to CV-1(18A) cells; filled triangles, nonspecific binding of ^{125}I-DIP-thrombin (0–8 nM) to CV-1(18A) cells in the presence of 800 nM thrombin; open triangles, nonspecific binding of ^{125}I-DIP-thrombin to CV-1 cells; solid line, calculated specific binding curve; filled diamonds, binding of ^{125}I-hirudin to CV-1(18A) cells; open diamonds, binding of ^{125}I-hirudin to CV-1 cells; filled squares, binding of ^{125}I-fibrinogen to CV-1(18A) cells; open squares, binding of ^{125}I-fibrinogen to CV-1 cells.

Table II: Primary Structure of Synthetic Peptides and Their Affinity for Thrombin

Peptides	Sequence[a]	K_p[b]
$Tm^{426-444}$	E C P E G Y I L D D G F I C T D I D E	85 ± 7 μM
Hir^{53-64}	N G D F - - E E I P E E Y L	0.8 ± 0.1 μM
$Facv^{30-43}$	I E D S - - D D I P E D T T Y K	≥1000 μM

[a] Sequences are aligned to maximize the apparent similarity among the peptides. [b] The dissociation contant K_p is determined by fitting eq 18 to the competition binding data.

CV-1(18A) cells, direct equilibrium binding of ^{125}I-DIP-thrombin was performed with 5 × 10^5 cells/mL (Figure 6). By fitting eq 2 to the binding data, calculated values of K_d (2.82 ± 0.81 nM) and R_m (220000 ± 15000 molecules/cell) were determined. The K_d of ^{125}I-DIP-thrombin determined by direct binding was essentially the same as that of unlabeled DIP-thrombin determined by competition binding, showing that the K_d was unaffected by iodination.

Effect of Hirudin and Hir^{53-64} on the Equilibrium Binding of Thrombin to Thrombomodulin. Previous kinetic studies have suggested that hirudin binds to thrombin at both the active site and a lower affinity site which may also interact with thrombomodulin (Stone et al., 1987). To study directly the binding interactions of hirudin with thrombomodulin, we conducted competition binding with active site blocked DIP-thrombin. CV-1(18A) cells were incubated with 0.3 nM ^{125}I-DIP-thrombin and increasing concentrations of hirudin or the synthetic hirudin peptide Hir^{53-64} (Table II). Both hirudin and Hir^{53-64} displaced ≥90% of bound DIP-thrombin at 500 nM and 50 μM, respectively (Figure 7A). No binding of hirudin to thrombomodulin was observed when ^{125}I-hirudin was incubated with CV-1 or CV-1(18A) cells (Figure 6), justifying the choice of the two receptor–one ligand model for hirudin and the use of eq 18 to analyze the binding data. These data suggest that the displacement of ^{125}I-DIP-thrombin by hirudin is due to competition with thrombomodulin for a site on thrombin and are consistent with purely competitive binding of hirudin and thrombomodulin to thrombin (β = +∞), al-

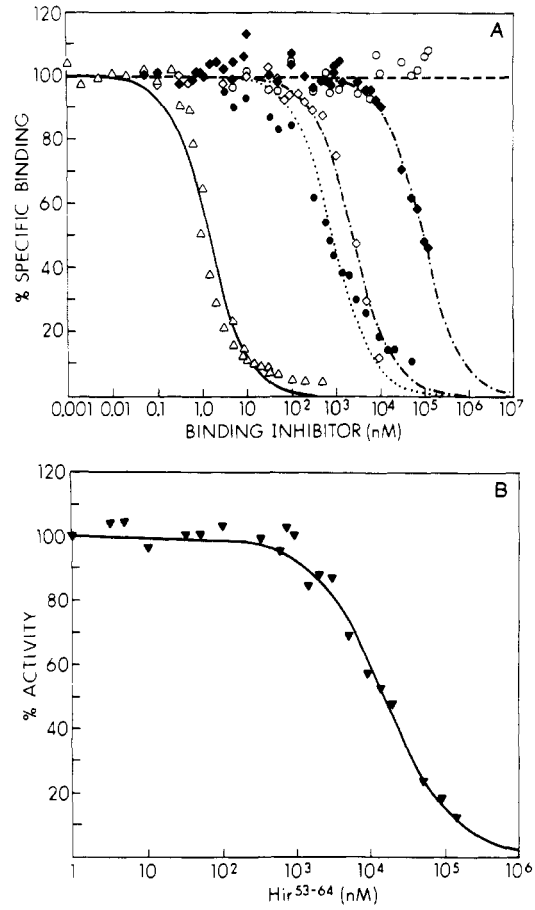


FIGURE 7: Inhibition of DIP-thrombin binding to thrombomodulin. Panel A: Displacement of ^{125}I-DIP-thrombin from thrombomodulin. ^{125}I-DIP-thrombin at 0.3 nM was incubated with CV-1(18A) cells at 4 °C for 2 h in the presence of increasing concentrations of hirudin (open triangles), human fibrinogen (open diamonds), Hir$^{53-64}$ (filled circles), FacV$^{30-43}$ (open circles) and Tm$^{426-444}$ (filled diamonds). Curve fitting was performed by using eq 18 with L_m = 0.3 nM and K_d = 3.26 nM. The signal (100%) to background (0%) ratio was 15–23. Panel B: Inhibition of thrombomodulin cofactor activity by Hir$^{53-64}$. The thrombomodulin cofactor activity assay was performed as described under Experimental Procedures except that 3.2×10^5 CV-1(18A) cells in assay buffer were preincubated with 0.34 nM thrombin and increasing concentrations of Hir$^{53-64}$ at 4 °C for 30 min before the addition of protein C to 0.89 μM. The final concentrations of thrombin and cells were 0.3 nM and 3200 cells/μL, respectively.

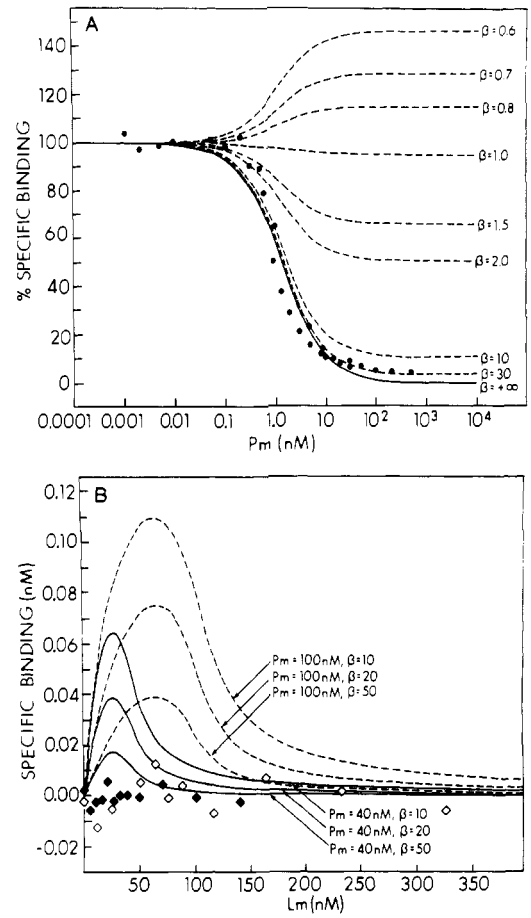


FIGURE 8: Comparison of binding data to the theoretical curves of equilibrium binding when the third component is a second receptor. Panel A: The theoretical curves were generated by plotting the sum [LR] + [PLR] given by eqs 12 and 13 with P_m as variable and the constant parameters R_m = 0.2 nM, L_m = 0.3 nM, K_d = 3.26 nM, K_p = 1.13 nM, and β = 0.6 to $+\infty$ (as indicated). Dots, the hirudin data from Figure 7. Panel B: ^{125}I-Hirudin at 40 nM (filled diamonds) and 100 nM (open diamonds) was incubated with CV-1(18A) cells at 4 °C for 2 h with increasing concentrations of unlabeled DIP-thrombin. The theoretical curves were generated by plotting eq 13 with L_m as the variable parameter and the constant parameters R_m = 0.2 nM, P_m = 40 or 100 nM, K_d = 3.26 nM, K_p = 1.13 nM, and β = 10–50 as indicated.

though partially competitive binding with β having a very large value cannot be ruled out.

To estimate the magnitude of the coefficient β if the binding were partially competitive, the sum [LR] + [PLR] given by eqs 12 and 13 was plotted as a function of the hirudin concentration P_m with L_m = 0.3 nM, R_m = 0.2 nM, K_d = 3.26 nM, and different values of β. Figure 8A shows that the limit of displacement when β = 30 is $\simeq$95%, which is comparable to the limit reached by the competition binding data. Another approach to estimate β is to look at the formation of the ternary complex PLR independently of LR by incubating various concentrations of unlabeled DIP-thrombin at a fixed concentration of ^{125}I-hirudin. Since ^{125}I-hirudin does not bind specifically to CV-1(18A) cells, any specific binding should be due to the formation of the ternary complex when unlabeled DIP-thrombin was added. Figure 8B shows that the binding data are consistent with $\beta \geq 50$ when compared with the theoretical curves. This excludes the significant formation of a ternary complex containing thrombomodulin, thrombin, and hirudin.

Assuming that the binding is purely competitive, the dissociation constant K_p of hirudin from DIP-thrombin was determined by fitting eq 18 to the binding data of Figure 7A. This yielded a K_p value of 1.13 ± 0.12 nM for partially purified hirudin and a K_p value of 0.79 ± 0.10 μM for Hir$^{53-64}$ (Figure 7A). Similar studies with purified hirudins gave values for K_p of 0.64 nM and 0.16 nM for purified natural and recombinant hirudin, respectively (data not shown). Thrombomodulin cofactor activity was also inhibited by Hir$^{53-64}$ with an EC_{50} of 15.40 μM (Figure 7B). For comparison, a K_p of 26 pM for hirudin was determined kinetically by assaying the ability of DIP-thrombin to relieve the inhibition of thrombin by hirudin (Stone et al., 1987). Similarly, an EC_{50} value of ~6.2 μM Hir$^{53-64}$ was reported to cause half-maximal prolongation of a modified prothrombin time (Maraganore et al., 1989). The differences among these values may be explained by differences in reaction conditions. In particular, assays of cofactor activity contain protein C which may alter the affinity of thrombin derivatives for thrombomodulin. Kinetic assays of thrombin require the presence of thrombin substrates, which may alter the affinity of hirudin for thrombin. Finally, the interaction of thrombin with thrombomodulin may alter the K_d of hirudin binding to thrombin.

Effect of Factor Va, Fibrinogen, and Peptide Analogues on the Equilibrium Binding of Thrombin to Thrombomodulin.

Factor Va and fibrinogen have also been reported to interact with the thrombomodulin–thrombin complex, and the effect of these factors was evaluated on ^{125}I-DIP-thrombin binding (Figure 7A). Binding data were fitted to eq 18. Neither EDTA-dissociated factor Va (data not shown) nor FacV$^{30-43}$, a synthetic peptide from factor V with a charge composition similar to Hir$^{53-64}$ (Table II), at concentrations up to 400 nM and 120 μM, respectively, were able to displace ^{125}I-DIP-thrombin. In contrast, fibrinogen achieved a displacement of 88% at 9 μM with a K_p of 2.25 ± 0.26 μM. Radiolabeled fibrinogen (0.5–200 nM) did not bind significantly more to CV-1(18A) cells compared to the parent CV-1 cell line, indicating that fibrinogen does not bind to thrombomodulin (Figure 6), justifying the choice of the two receptor–one ligand model for fibrinogen. Peptide Tm$^{426-444}$ (Table II), corresponding to the most acidic loop of the proposed thrombin binding domain of human thrombomodulin (Stearns et al., 1989; Suzuki et al., 1989; Zushi et al., 1989), also displaced ^{125}I-DIP-thrombin but with a K_p of 85.4 ± 7.2 μM (Figure 7A).

Discussion

In this report we describe the biosynthesis, glycosylation, and thrombin binding characteristics of recombinant human thrombomodulin. Untransfected host cells did not express detectable endogenous thrombomodulin activity except for CHO cells. Both transfected CV-1 and K562 cell lines expressed thrombomodulin activity that was comparable to or greater than that of either human endothelial cells or mouse hemangioma cells. In particular, CV-1(18A) cells express ~220000 molecules of thrombomodulin per cell as determined by direct equilibrium binding.

The recombinant thrombomodulin specifically immunoprecipitated from transfected cells has an apparent size similar to that of natural thrombomodulin from cultured endothelial cells (Figures 2 and 3). The slight variation in the size of thrombomodulin expressed by different host cells might be due to differences in glycosylation. Recombinant thrombomodulin was not recovered from the medium of transfected COS-7 cells (data not shown) or CV-1(18A) cells (Figure 3), nor was natural thrombomodulin recovered from the medium of HUVE cells (data not shown).

Digestion with endoglycosidase F or synthesis in the presence of tunicamycin showed that recombinant thrombomodulin contains N-linked oligosaccharides (Figure 2). The resistance of recombinant thrombomodulin to further digestion with a combination of neuraminidase and *O*-glycanase may indicate the presence of O-linked oligosaccharide structures that do not contain the disaccharide Galβ1–3GalNAcα1–Ser/Thr or are not accessible to the enzyme. Alternatively, other post-translational modifications may be present.

We used a competition binding assay to determine the equilibrium dissociation constant K_d of thrombin from recombinant thrombomodulin. Similar dissociation constants were obtained for recombinant thrombomodulin expressed on CV-1(18A) and K562(5/50K) cells. The K_d for natural thrombomodulin expressed on A549 human lung carcinoma cells was found to be comparable to a previously reported value of 1.4 nM for the same cells (Maruyama & Majerus, 1985). This value is also comparable to values for K_i determined kinetically for purified bovine thrombomodulin (0.5 nM) (Jakubowski et al., 1986) and rabbit thrombomodulin (0.8–1.8 nM) (Hofsteenge et al., 1986; Hofsteenge & Stone, 1987) binding to human or bovine thrombin. The slight difference between the affinity of thrombin for natural and recombinant thrombomodulin might reflect differences among the environments in which the protein is expressed, or differences in details of biosynthesis and posttranslational modification.

Diisopropyl fluorophosphate inactivation of thrombin did not change its binding affinity for recombinant thrombomodulin expressed on CV-1(18A) cells as shown by both competition and direct binding studies. This allowed us to use DIP-thrombin to study the binding interactions of hirudin, fibrinogen, factor Va, and thrombomodulin independently of the catalytic site on thrombin. Our data showed that hirudin does not bind to thrombomodulin but is able to displace DIP-thrombin bound to thrombomodulin without the significant formation of the ternary complex, hirudin–DIP-thrombin–thrombomodulin. This is consistent with an essentially competitive model in which thrombomodulin and hirudin bind at the same site or overlapping sites on DIP-thrombin. However, an alternative model in which hirudin and thrombomodulin bind to distinct, allosterically competitive sites cannot be ruled out by this experimental approach. The competition binding assay also showed that Hir$^{53-64}$, a small hirudin peptide previously reported to efficiently inhibit thrombin-catalyzed fibrinogen cleavage (Maraganore et al., 1989), competes with thrombomodulin for DIP-thrombin binding with a K_d of 0.79 μM. Because Hir$^{53-64}$ did not inhibit thrombin-catalyzed hydrolysis of a small tripeptidyl substrate, it was suggested that Hir$^{53-64}$ interacted with thrombin at a site required for specificity toward fibrinogen (Maraganore et al., 1989). Our observation of the ability of fibrinogen to displace ^{125}I-DIP-thrombin from thrombomodulin suggests that the direct anticoagulant effect of thrombomodulin may result from the inaccessibility of a fibrinogen-specific binding site on thrombin when bound to thromomodulin. Our direct binding results are consistent with previous indirect kinetic data suggesting that bovine or rabbit thrombomodulin competes with hirudin and fibrinogen for binding to thrombin (Hofsteenge et al., 1986; Jakubowski et al., 1986; Jakubowski & Owen, 1989).

The thrombin binding site of thrombomodulin appears to reside in the fifth or sixth EGF-like domain (Stearns et al., 1989; Suzuki et al., 1989; Zushi et al., 1989). This segment of thrombomodulin contains one particularly acidic peptide, Tm$^{426-444}$, that resembles the thrombin-binding hirudin peptide, Hir$^{53-64}$, in charge, composition, and sequence. This peptide was synthesized and shown to displace ^{125}I-DIP-thrombin from thrombomodulin, suggesting that it may form part of the thrombin binding site of thrombomodulin. Previous reports suggested that factor Va and factor Va light chain can serve as cofactors for protein C activation (Salem et al., 1983) and that factor Va light chain at concentrations greater than 30 nM inhibits thrombin–thrombomodulin-catalyzed protein C activation (Salem et al., 1984b; Maruyama et al., 1984). In the present studies, EDTA-dissociated factor Va (400 nM) was unable to displace ^{125}I-DIP-thrombin (data not shown), suggesting that factor Va light chain does not compete with thrombomodulin for thrombin binding. Peptide FacV$^{30-43}$, which corresponds to a region of factor Va light chain similar in sequence and composition to Hir$^{53-64}$, also did not displace ^{125}I-DIP-thrombin. This demonstrates that the inhibitory activity of peptides Hir$^{53-64}$ and Tm$^{426-444}$ depends on specific sequences and not only upon charge or composition.

This system offers a simple method to study, by equilibrium binding, the interaction of various factors with the thrombin–thrombomodulin complex without interference from other thrombin binding sites present on endothelial cells. In comparison with studies employing solubilized thrombomodulin, studies with cell surface expressed recombinant thrombomo-

dulin have the potential advantage that the structural and functional properties of the phospholipid bilayer are preserved. Site-directed mutagenesis should provide further insights into the structure–function relationships of thrombomodulin in this system.

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Registry No. EGF, 62229-50-9; $Tm^{426-444}$, 129047-87-6; Hir^{53-64}, 121822-19-3; $FacV^{30-43}$, 129708-33-4; factor Va, 65522-14-7; thrombin, 9002-04-4; hirudin, 8001-27-2.

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