Activation of Thrombin-activable Fibrinolysis Inhibitor Requires Epidermal Growth Factor-like Domain 3 of Thrombomodulin and Is Inhibited Competitively by Protein C*

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Hip-R, hippuryl-arginine; TMnc, thrombomodulin.

Thrombomodulin is a cofactor protein on vascular endothelial cells that inhibits the procoagulant functions of thrombin and enhances thrombin-catalyzed activation of anticoagulant protein C. Thrombomodulin also accelerates the proteolytic activation of a plasma procarboxypeptidase referred to as thrombin-activable fibrinolysis inhibitor (TAFI). In this study, we describe structures on recombinant membrane-bound thrombomodulin that are required for human TAFI activation. Deletion of the N-terminal lectin-like domain and epidermal growth factor (EGF)-like domains 1 and 2 had no effect on TAFI or protein C activation, whereas deletions including EGF-like domain 3 selectively abolished thrombomodulin cofactor activity for TAFI activation. Provided that thrombomodulin EGF-like domain 3 was present, TAFI competitively inhibited protein C activation catalyzed by the thrombin-thrombomodulin complex. A thrombomodulin construct lacking EGF-like domain 3 functioned normally as a cofactor for protein C activation but was insensitive to inhibition by TAFI. Thus, the anticoagulant and antifibrinolytic cofactor activities of thrombomodulin have distinct structural requirements: protein C binding to the thrombin-thrombomodulin complex requires EGF-like domain 4, whereas TAFI binding also requires EGF-like domain 3.

Thrombomodulin is an integral membrane glycoprotein on vascular endothelial cells. By binding to thrombin in a 1:1 stoichiometric complex, thrombomodulin inhibits the procoagulant functions of thrombin and acts as a cofactor for thrombin-catalyzed activation of protein C. Activated protein C degrades clotting factors Va and VIIIa and thereby inhibits blood clotting. This cofactor activity of thrombomodulin initiates an essential physiological anticoagulant mechanism (reviewed in Refs. 1–3).

Human thrombomodulin consists of 10 structural elements: an N-terminal domain homologous to the family of C-type lectins (residues 1–226), six tandemly repeated epidermal growth factor (EGF)-like domains (residues 227–462), a Ser/Thr-rich region (residues 463–497), a transmembrane domain (residues 498–521), and a short cytoplasmic tail (residues 522–557) (4–7). Previous studies demonstrated that the anticoagulant function of thrombomodulin is mediated by EGF-like domains 4, 5, and 6 (8). Thrombin binds to EGF-like domains 5 and 6 (9). EGF-like domain 4 is not required for thrombin binding but is essential for accelerating protein C activation (10, 11). Membrane association is not necessary for cofactor activity (12, 13), and the smallest fully active soluble thrombomodulin fragment consists of EGF-like domains 4–6 (8, 14). Although membrane insertion is known to impose additional structural requirements for normal activity (10), the functions of the lectin-like domain and the EGF-like domains 1–3 remain unknown.

Recently, a new activity of thrombomodulin was discovered; it accelerates the proteolytic activation by thrombin of a plasma procarboxypeptidase named thrombin-activable fibrinolysis inhibitor (TAFI) (15). TAFI is identical to the protein first named carboxypeptidase U (16, 17) and later studied as plasma procarboxypeptidase B (18). Activated TAFI (TAFIa) cleaves C-terminal lysine and arginine residues from partially degraded fibrin. The removal of these positively charged residues suppresses the ability of fibrin to catalyze plasminogen activation and thereby delays clot lysis (19). A fragment consisting of the six EGF-like domains of thrombomodulin can promote TAFI activation (3), but the structural elements required for cofactor activity have not been localized more precisely. In the present study, we demonstrate that TAFI activation requires EGF-like domain 3 of thrombomodulin and is inhibited competitively by protein C.

EXPERIMENTAL PROCEDURES

Materials—Pure human thrombin was provided by Dr. M. Wardell (Washington University). Recombinant human protein C was obtained from Dr. S. B. Yang (Eli Lilly, Co.). Human TAFI and rabbit lung thrombomodulin were from Hematologic Technologies (Burlington, VT). Human antithrombin, porcine intestinal heparin, hippuryl-arginine (Hip-R), and D-Phe-Pro-Arg-chloromethane were from Sigma.

Plasmid pTM456 that encodes a fragment of human thrombomodulin corresponding to EGF-like domains 4–6 (residues Asp349–Cys462) was described previously (20). To reduce sensitivity to oxidative damage, the mutation Met388 Thr was engineered into pTM456 by a polymerase chain reaction-based method to generate plasmid pTM456T. Large scale production of recombinant protein TM456 was performed into baculovirus expression vector pAcGP67A (PharMingen, San Diego, CA) between the BamHI and NotI sites to yield expression plasmid pAcGP67TM456, which was transfected into insect Sf9 cells (Life Technologies, Inc.) with BaculoGold® DNA to produce recombinant virus. Large scale production of recombinant protein TM456 was performed...
by infection of High Five cells (PharMingen). TM456 was purified from serum-free culture medium by a combination of affinity chromatography on α-thrombin-Aff-Gel 10 (Bio-Rad) and Mono-Q ion exchange chromatography (Amersham Pharmacia Biotech). Upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining, purified TM456 migrated as a single band with an apparent mass of 23 kDa. By automated Edman degradation the N-terminal sequence was Ala-Asp-Pro-Cys-Arg-Ala-Asn-Cys-Glu-. Residue Ala1 is derived from the expression vector, and the sequence beginning at Asp2 corresponds to that expected for thrombomodulin. TM456 concentrations were determined spectrophotometrically using the calculated (22) extinction coefficient $\varepsilon_{280\text{ nm}}^\text{cm}^{-1}\text{mM}^{-1} = 5595$. Five stably transfected CV-1 cell lines expressing human thrombomodulin mutants TMnc, NdL, NdE1, NdE2, and NdE3 were cultured as described previously (10, 23). Subsequent procedures were performed at 4°C or on ice.

Confluent cells from six T75 flasks were scraped into 20 mM sodium phosphate, pH 7.4, 150 mM NaCl (phosphate-buffered saline; PBS; 5 ml/flask), resuspended in 5 ml of 20 mM Tris-HCl, pH 7.4, and disrupted by 10 strokes in a 7-ml glass Dounce homogenizer. Nuclei were removed by centrifugation at 600 x $g$ for 10 min, and cell membranes were collected by centrifugation of the supernatant at 100,000 x $g$ for 60 min. The membrane pellets were resuspended in 250 μl of 20 mM Tris-HCl, pH 7.4, and stored at −70°C. The thrombomodulin concentration of each preparation was determined by protein C activation assay as described previously (10) with a rabbit lung thrombomodulin standard.

**Results**

**Protein C Activation Assay**—Thrombomodulin cofactor activity for protein C activation by thrombin was measured by a modification of a two-stage assay described previously (23). In the first stage, thrombomodulin was incubated for 30 min at 37°C in 25 μl of 20 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM CaCl₂, with the indicated concentrations of thrombin and protein C. The reaction was stopped by adding 5 μl of 150 μg/ml antithrombin containing 150 units/ml heparin. Amidolytic activity of activated protein C was assayed in the second stage by adding 5 μl of 100 μg/ml S-2366 (Molecular Devices). The kinetics of TAFI activation were investigated for each thrombomodulin variant (Fig. 2). Preliminary studies indicated that the apparent $K_m$ of thrombin binding to full-length thrombomodulin (TMnc) has 557 amino acid residues and consists of a lectin domain (L), six EGF-like domains (1–6), a Ser/Thr-rich domain (O), a transmembrane domain (T), and a cytoplasmic tail (C). The domain composition of other constructs is indicated. Activation of 0.5 μM protein C (B) or 0.5 μM TAFI (C) by 5 nM thrombin was determined with thrombomodulin constructs TMnc (●), NdL (○), NdE1 (×), NdE2 (■), and NdE3 (□). The reaction conditions are described under “Experimental Procedures.” The curves represent the results of nonlinear regression of the data for TMnc to the Michaelis-Menten equation.

**Fig. 1. Thrombomodulin concentration dependence of protein C activation and TAFI activation.** A, thrombomodulin constructs used in these studies. Full-length thrombomodulin (TMnc) has 557 amino acid residues and consists of a lectin domain (L), six EGF-like domains (1–6), a Ser/Thr-rich domain (O), a transmembrane domain (T), and a cytoplasmic tail (C). The domain composition of other constructs is indicated. Activation of 0.5 μM protein C (B) or 0.5 μM TAFI (C) by 5 nM thrombin was determined with thrombomodulin constructs TMnc (●), NdL (○), NdE1 (×), NdE2 (■), and NdE3 (□). The reaction conditions are described under “Experimental Procedures.” The curves represent the results of nonlinear regression of the data for TMnc to the Michaelis-Menten equation.

**Fig. 1.** Thrombomodulin concentration dependence of protein C activation and TAFI activation. A, thrombomodulin constructs used in these studies. Full-length thrombomodulin (TMnc) has 557 amino acid residues and consists of a lectin domain (L), six EGF-like domains (1–6), a Ser/Thr-rich domain (O), a transmembrane domain (T), and a cytoplasmic tail (C). The domain composition of other constructs is indicated. Activation of 0.5 μM protein C (B) or 0.5 μM TAFI (C) by 5 nM thrombin was determined with thrombomodulin constructs TMnc (●), NdL (○), NdE1 (×), NdE2 (■), and NdE3 (□). The reaction conditions are described under “Experimental Procedures.” The curves represent the results of nonlinear regression of the data for TMnc to the Michaelis-Menten equation.
modulin NdE3 (Fig. 2E) was extremely slow and indistinguishable from that observed in the absence of thrombomodulin (Fig. 2F). These data indicate that the N-terminal lectin domain and EGF-like domains 1 and 2 of thrombomodulin are dispensable for the acceleration of TAFI activation, whereas EGF-like domain 3 is required.

Requirement for EGF-like Domain 4 to Promote TAFI Activation—In addition to EGF-like domain 3, EGF-like domains 5 and 6 of thrombomodulin are necessary for TAFI activation, because they are required for thrombin binding (9). EGF-like domain 4 is known to be essential for protein C activation (8). To investigate the role of EGF-like domain 4 in TAFI activation, two soluble thrombomodulin mutants were compared. Construct TM456 contains amino acid residues Asp349–Cys462 and corresponds to EGF-like domains 4–6. Construct TM356 is similar, but EGF-like domain 4 (residues Cys351–Cys386) is replaced by EGF-like domain 3 (Cys311–Cys344) (20). Both TM456 and TM356 bind thrombin with similar affinity (20). As reported previously (20), protein C was activated by thrombin in the presence of TM456, and replacement of EGF-like domain 4 with EGF-like domain 3 in TM356 resulted in complete loss of cofactor activity (Fig. 3A). TM456 was inactive when tested for the ability to promote TAFI activation (Fig. 3B), and this result is consistent with the observation that membrane-associated thrombomodulin NdE3 has no cofactor activity for TAFI activation (Fig. 1C). TM356 also did not support TAFI activation (Fig. 3B). Thus, EGF-like domain 4 may be required for thrombomodulin-dependent activation of both protein C and TAFI.

Inhibitory Effect of TAFI on Protein C Activation—EGF-like domain 3 may contribute to TAFI activation by promoting either substrate binding or catalysis. These possibilities were addressed by determining whether TAFI could inhibit protein C activation in the presence of thrombomodulin TMnc, NdL, NdE1, NdE2, and NdE3 (Fig. 4). In the absence of TAFI, all three thrombomodulins had similar cofactor activity for protein C activation. In the presence of TAFI, protein C activation catalyzed by TMnc and NdE2 was inhibited ~40%. Separate control experiments showed that neither TAFI nor TAFIa inhibits the amidolytic activity of activated protein C (data not shown). Furthermore, protein C activation in the presence of thrombomodulin lacking EGF-like domain 3 (NdE3) was resistant to inhibition by TAFI (Fig. 4). These results suggest that TAFI does not interact directly with protein C and thereby prevent protein C binding to thrombin-thrombomodulin. In addition, EGF-like domain 3 appears to be required for TAFI binding to thrombin-thrombomodulin.

Competition between Protein C and TAFI Activation on Thrombin-Thrombomodulin—Activation of TAFI or protein C...
by the thrombin-thrombomodulin complex depends on distinct EGF-like domains, suggesting that these substrates might bind independently even though they are cleaved by the same protease active site. To characterize the relationship between protein C and TAFI binding, the kinetic interactions between them were investigated. As shown in Fig. 5A, TAFI inhibits protein C activation in a concentration-dependent manner. Lineweaver-Burk analysis (Fig. 5A, inset) indicates that TAFI is a competitive inhibitor of protein C with a $K_i$ (TAFI) of 1.89 $\mu M$. This value is similar to the $K_m$ (TAFI) of 1.79 $\mu M$ determined with TMnc (Fig. 2A). As expected, the reciprocal experiment showed that protein C is a competitive inhibitor of TAFI activation with a $K_i$ of 2.98 $\mu M$ (Fig. 5B, inset). This value for $K_i$ is similar to the value for $K_m$ (protein C) of 2.35 $\mu M$ determined independently with the same thrombomodulin preparation (data not shown). For comparison, $K_m$ values for protein C have been reported with recombinant soluble human thrombomodulin variants of 1.4 $\mu M$ (with glycosaminoglycan modification) to 2.3 $\mu M$ (without glycosaminoglycan modification) (24). Control experiments demonstrated that TAFIa activity decays with a half-life of $\sim 10$ min as reported previously (25), that neither protein C nor activated protein C affect the stability of TAFIa, and that the rate of TAFI activation by thrombin-TMnc was linear during the 10-min assay (data not shown). Thus, TAFI and protein C cannot bind simultaneously to the thrombin-thrombomodulin complex.

**DISCUSSION**

The discovery that thrombomodulin promoted TAFI activation (15) immediately raised the question of whether this reaction might depend on structural features of thrombomodulin distinct from those required for protein C activation. Fragments of thrombomodulin that contain only EGF-like domains 4–6 have essentially normal cofactor activity for the promotion of protein C activation. The other domains of thrombomodulin are conserved among mammals, however, suggesting they may have important biological functions, possibly unrelated to the protein C anticoagulant pathway. As previously noted for protein C activation, TAFI activation did not require the thrombomodulin lectin domain (3) or membrane association (15). In a preliminary experiment, we found that a recombinant thrombomodulin fragment consisting of EGF-like domains 4–6 was unable to promote TAFI activation, even though it was fully functional in protein C activation; this observation focused attention on EGF-like domains 1–3.

Further studies of deletion mutants have identified a domain of thrombomodulin that appears to be specifically required for TAFI activation (Figs. 1 and 2). Thrombomodulin NdE2 promoted the activation of both protein C and TAFI, whereas thrombomodulin NdE3 only supported the activation of protein C. The difference between these constructs encompasses thrombomodulin residues Asp309–Pro347. This segment includes EGF-like domain 3 (residues Cys311–Cys344) as well as two N-terminal (Asp-Asp) and three C-terminal (Val-Glu-Pro) flanking amino acids. Therefore, a 39-amino acid residue segment consisting mostly of EGF-like domain 3 is necessary for thrombomodulin-dependent TAFI activation. The properties of thrombomodulin fragments TM456 and TM356 (Fig. 3) suggest that EGF-like domain 3 cannot promote TAFI activation in the absence of EGF-like domain 4. Because we have no independent evidence that EGF-like domain 3 is correctly folded in TM356, this conclusion will require confirmation by other approaches. Because TAFI activation does not depend on sequences C-terminal to EGF-like domain 6 (3), the smallest thrombomodulin fragment with full anticoagulant and antifibrinolytic cofactor activity may consist of EGF-like domains 3–6. The required features of EGF-like domain 3 and its flanking residues remain to be defined by additional mutagenesis and biochemical analysis.

Additional features of the thrombomodulin cofactor mechanism were identified by the analysis of enzyme kinetics. TAFI and protein C bind competitively to thrombin-thrombomodulin (Fig. 5), even though activation of these substrates depends on different structural features of thrombomodulin. The binding sites for TAFI and protein C may therefore overlap or be linked conformationally. Interestingly, deletion of EGF-like domain 3 prevented TAFI from interfering with the activation of protein C (Fig. 4). Thus, EGF-like domain 4 interacts with protein C but may not interact with TAFI, and EGF-like domain 3 is required for TAFI binding but is dispensable for protein C binding.

The inhibition data (Fig. 4) suggest that TAFI cannot even bind unproductively to a complex of thrombin-thrombomodulin that lacks EGF-like domain 3. In contrast to this result, TAFI clearly binds to free thrombin because thrombin cleaves TAFI with a $K_i$ of 2.1 $\mu M$, and TAFI inhibits thrombin cleavage of a chromogenic substrate with a $K_i$ of $\sim 1.2$ $\mu M$ (15). These findings are consistent with a model in which thrombin and thrombin-thrombomodulin have different modes of binding to TAFI;
in the absence of EGF-like domain 3, thrombin binding prevents TAFI binding to thrombomodulin. If EGF-like domain 3 is present, a TAFI binding site is created or restored in the thrombin-thrombomodulin complex.

Although TAFI and protein C bind competitively to the thrombin-thrombomodulin complex (Fig. 5), this competition probably cannot directly regulate the anticoagulant and anti-fibrinolytic activities of thrombomodulin in vivo. The plasma concentration of protein C is approximately 71 nm (26), and this is at least 20-fold lower than the $K_m$ values of 1.4–2.4 $\mu$m for cleavage of protein C by human thrombin-thrombomodulin (data not shown and Ref. 24). Similarly, the plasma concentration of TAFI is approximately 73 nm (15) compared with its $K_m$ values of 0.5–2.1 $\mu$m (Fig. 2 and Refs. 15 and 25). Consequently, changes in the concentration of one substrate would not significantly affect the activation of the other. However, decreases in the plasma concentration of protein C cause thrombosis (27); changes in the level of TAFI or thrombomodulin defects that selectively impair the activation of either protein C or TAFI might also disrupt the balance between anticoagulant and anti-fibrinolytic pathways and thereby cause a predisposition to bleeding or thrombosis.

Until recently, the only known biological function of thrombomodulin was to bind thrombin and promote the activation of protein C, thus initiating an essential anticoagulant reaction pathway. The discovery of thrombomodulin-dependent TAFI activation showed that thrombomodulin also can initiate an anti-fibrinolytic reaction pathway. The present study characterizes a previously unknown function for EGF-like domain 3 in promoting TAFI activation, demonstrating that the anticoagulant and anti-fibrinolytic activities of thrombomodulin depend on distinct EGF-like domains. Further genetic and biochemical analysis will be required to determine whether additional structural domains of thrombomodulin have analogous independent functions.

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