Alanine-scanning Mutagenesis of the Epidermal Growth Factor-like Domains of Human Thrombomodulin Identifies Critical Residues for Its Cofactor Activity*

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Thrombomodulin (TM) is an endothelial cell surface-bound cofactor in thrombin-dependent formation of activated protein C, a potent anticoagulant. Cofactor activity has been localized to the carboxyl-terminal half of the six epidermal growth factor-like (EGF) domains of TM (TM₆). To identify residues in TM₆ that are critical for activity, 77 alanine point mutants were made between Cys-333 and Cys-462 by site-directed mutagenesis (all residues except Ala, Cys, Gly, and Pro). Mutants were expressed in Escherichia coli, and cofactor activity measured directly in periplasmic extracts obtained by osmotic shock. Critical residues were defined as those which when mutated had less than 25% cofactor activity of a reference TM₆. Western blots of non-reduced samples confirmed that alanine substitutions did not significantly decrease expression levels or result in the formation of dimers. In EGF₄, which is essential for protein C activation by the thrombin-TM complex, critical residues were: Asp-390, Asp-400, Asp-423, Asp-425, Glu-426, Ile-424, Asp-425, and Glu-426. A potential Ca²⁺-binding site, which is comprised of residues Asp-423, Asp-425, Glu-426, Asn-439, Leu-440, and Phe-444, was also identified and overlaps the thrombin-binding region. Asp-461, in the C-loop of EGF₆ previously shown to be critical for thrombin binding, was also critical. Asp-308, Asp-400, Asn-402, and Asn-429 in EGF₅ were also critical.

Thus, rapid alanine-scanning mutagenesis of TM₆ has identified 22 critical residues in the region comprising EGF₄-₆, which is essential for thrombin binding and protein C activation by the thrombin-TM complex.

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MATERIALS AND METHODS

T₄ DNA ligase, pSELECT-1 vector, and pGEM-3ZI were from Promega Corp., Madison, WI. E. coli strain DH₅₀ was from Gibco/BRL, Grand Island, NY. T₄ DNA polymerase and E. coli strain CJ236 (dut ung) were from Bio-Rad. Restriction enzymes were obtained from either New England Biolabs, Beverly, MA or Boehringer Mannheim. Plasmid pKT279 was from Stratagene, La Jolla, CA. Human α-thrombin (~400 NIH units/mg) and hirudin (300-1000 units/mg) were from Sigma. Recombinant human protein C was from Dr. John McPherson, Genzyme Corp., Framingham, MA and was purified as described (18). Chronogenic substrate D-valyl-L-leucyl-L-arginine-p-nitroanilide (S-2266) was from Kabi Vitrum, Franklin, OH. Rabbit polyclonal antiserum raised against purified reduced and alkylated TM₆ expressed in insect cells was kindly provided by Julia Gray, Berlex Biosciences. Biotinylated goat anti-rabbit IgG and \( ECL \) detection kit were from Amersham Corp. VECTASTAIN ABC kit was from Vector Laboratories, Burlingame, CA.

Construction of E. coli Expression Plasmids—A DNA fragment coding for TM₆ (amino acids 227-462) was obtained by polymerase chain reaction of human genomic DNA using primers 5'CCGGG-

1 The abbreviations used are: TM, thrombomodulin; EGF, epidermal growth factor. TM₆, wild-type TM comprising EGF domains 1-6. TM₆-M, mutant TM containing Leu instead of Met at position 388; Tricine, N-[2-hydroxy-1.1-bis(hydroxymethyl)ethyl]glycine.
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RESULTS AND DISCUSSION

TM Secreted in E. coli Is Functionally Active—TM₆ was expressed in E. coli under the control of the β-lactamase promoter and directed to the periplasmic space with the β-lactamase signal sequence. Secreted TM₆ cofactor activity in thrombin-dependent activation of protein C could be measured directly in the periplasmic extracts without prior protein purification. Using this system, wild-type TM₆ had a cofactor activity of 8.6 ± 2.8 units/ml (n = 18). Assays were performed at 2.5 mM Ca²⁺ to reduce the background rate of protein C activation by thrombin alone. Background rates of protein C activation in extracts of E. coli transfected with control plasmid pSelect-1 were <0.5% of those obtained with wild-type TM₆.

Glaser et al. (22) have demonstrated that substitution of Met-388 with Leu (TM₆-M388L) in purified TM₆ results in approximately 2-fold higher specific activity when compared with wild-type TM₆. In periplasmic extracts, TM₆-M388L had a cofactor activity of 20.1 ± 5.2 units/ml (n = 18). The ratio of TM₆-M388L/wild-type TM₆ cofactor activities was 2.35 ± 0.99, with an inter assay coefficient of variation of 16.7% (n = 18). Since total expression levels of wild-type TM₆ and TM₆-M388L in extracts were similar by western blot analysis using polyclonal anti-TME antibodies (see Fig. 2), the ratio of TM₆-M388L to wild-type TM₆ activities in extracts was consistent with those of purified proteins. This result was confirmed when specific activities for TM₆ and TM₆-M388L were determined by measuring antigen levels in E. coli shockates using purified recombinant human thrombomodulin expressed in mammalian cells and a sandwich enzyme-linked immunosorbent assay comprising two monoclonal anti-TME antibodies (43B and 531). The specific activities of TM₆ and TM₆-M388L were 437,000 ± 24,000, and 1,019,000 ± 87,000 units/mg of protein, respectively, giving a TM₆-M388L/TM₆ specific activity ratio of 2.33 (n = 2). Thus, the current system provided a fast and easy method of comparing cofactor activities of different mutants.

Identification of TM₆ Residues That Are Critical for Cofactor Activity—We previously identified the C-loop of EGF3 to EGF6 as the smallest TM₆ fragment with full cofactor activity (18). In order to investigate the contribution of individual residues in this region to cofactor activity, each residue between Cys-333 and Cys-492 (except Ala, Cys, Gly, and Pro) was replaced with alanine by site-directed mutagenesis. A total of 77 mutants were constructed using TM₆-M388L as a template. Three independent clones were isolated for each alanine mutant, and each was assayed at least twice. Results were expressed as mean percentages of TM₆-M388L activity (Fig. 1). Of the 77 mutants, 22 had <25% of TM₆-M388L cofactor activity. Other mutations produced proteins with activity ranging between 25 and 100% of TM₆-M388L. Two mutants, Q365A and L369A, were found to have 10–15% higher activity than TM₆-M388L. These increases were found to be additive in a double mutant (results not shown). None of the 22 critical mutations caused gross changes in the growth rate of DH₅α cells. To eliminate the possibility that loss of activity in the 22 critical mutants could be accounted for by gross changes in disulfide bonding or by reduced expression levels, a Western blot of periplasmic extracts was performed under non-reducing conditions (Fig. 2). No evidence for the formation of multimers was found, indicating that none of the low activity mutants arose from disulfide scrambling. In contrast to TM₆-M388L, and TM₆ which migrated as single bands, some mutants migrated as doublets. The basis for these doublets is not clear, and the possibility that they arise from alternative disulfide pairing cannot be excluded. Most of the mutants were also expressed at similar levels to TM₆ and TM₆-M388L standards, again indicating that loss of activity could not be accounted for by decreased expression. For those

1. E. Campbell and M. McCaman, personal communication.
mutants showing lower expression levels (e.g. Asp-416 and Asp-417), laser densitometric scans were performed to normalize cofactor activity for expression level relative to TM-M388L. In all cases, the normalized cofactor activity was still <25% of TM-M388L (data not shown).

Of the 22 critical residues identified, there were 11 negatively charged residues but no positively charged residues. Furthermore, all but 4 of the 22 residues (Asn-402, Glu-408, Asn-429, and Leu-440) were either identical to or conservative substitutions of the amino acids in the corresponding positions in human, mouse, and bovine TM.

Residues in the Thrombin-binding Region—Six of the negatively charged residues (Glu-408, Asp-416, Asp-417, Asp-423, Asp-425, and Glu-426) were found within a region spanning the C-loop of EGF5 to the interdomain between EGF5 and EGF6. This region is known to play a key role in thrombin binding (16, 17, 23). While overall negative charges were presumably important for the binding to the anion-binding exosite of thrombin, substitutions of larger hydrophobic residues in this region with smaller hydrophobic residue also resulted in the loss of activity (I414A, I415A, I424A). Preliminary experiments indicated that I424A had a much lower thrombin affinity than TM-M388L (see Table I). The bulky side chains of leucine and isoleucine may be required for the correct spatial arrangement of TM to be recognized by thrombin. Decreased cofactor activity of the D461A mutant might also be due, in part, to the decreased affinity for thrombin. In earlier studies using purified deletion mutants, it was shown that the deletion of residues 447-462 from wild-type TM increased $K_d$ for thrombin about 6-fold without affecting $k_{cat}/K_m$ (18).

Identification of a Putative Ca$^{2+}$-binding Site—As shown in Fig. 3, the region of TM spanning residues 423-444 contains a consensus sequence for a Ca$^{2+}$-binding site, Asp-Ile-Asp-Glu-Cys-X-X-X-X-Cys-X-X-X-X-Cys-X-Asn-X-X-X-X-Phe-X-Cys-X-Cys, which is highly homologous to known Ca$^{2+}$-binding EGF domains in protein S and factor IX (24, 25). In the present study we found that alanine substitution of Asp-423, Ile-424, Asp-425, Glu-426, Asn-429, Asn-439, Leu-440, and Phe-444 led to large decreases in TM cofactor activity. Identification of every single non-cysteine residue
within the consensus sequence for the Ca$^{2+}$-binding site as being critical for TM activity is a significant finding. Although indirect, these are the first experimental results supporting the presence of a Ca$^{2+}$-binding site in this region of TM and suggest that this may be an important structural feature of this thrombin-binding region of TM (see below).

**Residues That May Alter the Substrate Specificity of Thrombin**—Binding of TM to thrombin causes multiple conformational changes around the catalytic site of thrombin, leading to alteration in its substrate specificity. Using fluorescent labeled thrombin, Ye et al. (26) have shown that a fragment consisting of EGF5 and EGF6 can induce only partial conformational changes, which do not lead to the activation of protein C. In the current studies, we found 4 residues outside EGF5 and -6 that were critical for cofactor activity (Asp-349, Glu-357, Tyr-358, and Phe-376). While it remains possible that these alanine mutations introduced major structural perturbations, these residues could be interacting with thrombin to bring about additional conformational changes within thrombin required for protein C activation. Preliminary kinetic data (see Table I) suggest that Phe-376 may be involved in thrombin binding.

Le Bonnec et al. (27, 28) have reported that Glu-192 and Glu-39 near the active site of thrombin were responsible for its inability to accept Asp residues in the P3 and P3’ positions of the protein C cleavage sequence. They also postulated that TM could function, in part, by alleviating these inhibitory effects. It would be interesting to investigate if any of these 4 residues in EGF4 were responsible for these effects. In our deletion studies, the mutant consisting of residues 351-462 had the same $K_a$ for thrombin, but 90% reduction in $k_{cat}/K_m$ compared with wild-type TM (18). Deletion or mutation of Asp-349 might be responsible for this loss of catalytic activity (29).

**Decreased Thrombin Binding Affinity of Alanine Mutants**—In the present study, critical residues were defined as those which when mutated to alanine had 25% or less of M388L cofactor activity. This loss of activity could arise from direct effects on $K_a$ for thrombin, $k_{cat}/K_m$ of the thrombin-TM complex for protein C, or from effects on both $K_a$ and $k_{cat}/K_m$. To address this issue we have performed preliminary kinetic experiments in which the apparent $K_a$ of the low activity mutants for thrombin was determined directly in extracts from the dependence of cofactor activity on thrombin concentration. The data are summarized in Table I. The data show that many of the alanine mutations in the thrombin-binding region and the putative Ca$^{2+}$-binding site cause increases in the apparent $K_a$ for thrombin. These data suggest that the effects of these mutations are on the function of the correctly folded protein and not on protein folding per se. This conclusion is supported by the Western blot analysis. Definitive kinetic analyses to differentiate between functional loss of activity and loss of structural integrity in all 22 critical mutants will require larger amounts of purified proteins expressed in mammalian cells. Our results do suggest, however, that both EGF5 and EGF6 are important in thrombin binding. The presence of bound Ca$^{2+}$ in EGF6 may serve to coordinate the carboxylate groups of Asp-423, Asp-425, and Glu-426 at the protein surface. Such a structure, together with the anionic residues in the C-loop of EGF5, could form an extended negatively charged surface for interaction with the anion-binding exosite of thrombin.

In conclusion, we have utilized alanine-scanning mutagenesis in an E. coli expression system as a rapid screen to evaluate the contribution of individual residues in TM to its cofactor activity. While we were unable to perform detailed kinetic studies using this system, it allowed us to identify 22 critical residues in EGF4, EGF5, and EGF6, including those reported earlier by others (16, 17, 23, 29). Some of these residues are likely to be surface contact residues that interact directly with thrombin and regulate $K_a$ for thrombin and $k_{cat}/K_m$ for protein C of the thrombin-TM complex.

**Acknowledgments**—We would like to thank Raymond P. Muffly for synthesis and purification of mutagenic oligonucleotides, John McClary for DNA sequencing, and Sam Lipson for help with statistical analysis. We also thank Elisabeth Campbell and Michael McCaman for the enzyme-linked immunosorbent assay results with E. coli shockates using monoclonal antibodies 43B and 531.
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