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Thrombin cleaves recombinant soluble thrombomodulin into a lectin-like domain fragment and a fragment with protein C-activating cofactor activity

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SUMMARY Thrombomodulin (TM) is a transmembrane protein that plays an important role in regulating the coagulation system by acting as a cofactor for thrombin in protein C activation. Additionally, TM is involved in inflammation. Previous studies have shown that soluble fragments of TM of varying sizes, which are derived from membrane-bound TM, are present in plasma and urine. Soluble fragments of TM are speculated to exhibit biological activity. Among these, a lectin-like domain fragment (TMD1) is of particular importance. Recombinant TMD1 has previously been shown to attenuate lipopolysaccharide-induced inflammation. Here, we report that thrombin cleaves recombinant soluble TM, which is used for the treatment of disseminated intravascular coagulation associated with sepsis, into TMD1 and a fragment comprising the C-terminal portion of TM (TMD23), the latter of which retains the cofactor activity for activating protein C. Our findings suggest that thrombin not only activates protein C on membrane-bound TM but may also cleave TM to generate TMD1.

Keywords Thrombomodulin, thrombin, cleavage product, protein C-activating cofactor activity

Thrombomodulin (TM) is a transmembrane protein, expressed mainly on vascular endothelial cells, that has an anticoagulant role in the coagulation system (*1*). It is composed of a lectin-like domain (TMD1), a hydrophobic region, six tandem repeats of epidermal growth factor-like domains (TMD2), a Ser/Thr-rich domain (TMD3), a transmembrane segment (TMD4), and a cytoplasmic tail (TMD5). TM serves as a thrombin receptor and cofactor for thrombin. Thrombin and protein C interact with TMD2, resulting in activation of protein C by thrombin. Activated protein C in turn inactivates FVa and FVIIIa in the coagulation system. Studies with recombinant TMD1 have shown that TMD1 has several biological functions, including attenuation of lipopolysaccharide (LPS)-induced inflammation (*2*), suppression of high-mobility group box 1-mediated inflammation (*3,4*), suppression of vascular inflammation (*5*), and inhibition of angiogenesis (*6*). TM also has activating and inactivating functions in alternative pathways of the complement system (*7,8*) and can interfere with complement activation *via* the classical and lectin pathways (*9*). In addition, soluble fragments of TM of varying sizes are present in the plasma and urine (*10*). It has been speculated that proteases such as rhomboids and metalloproteases are involved in the release of TM from the cell membrane (*11,12*).

Soluble TM fragments containing TMD2 exhibit protein C-activating cofactor activity (*13*). Recombinant human soluble TM consisting of TMD1, TMD2, and TMD3 (rTM) has recently been used in the clinic to treat disseminated intravascular coagulation (DIC) associated with sepsis (Figure 1A) (*13-16*). Clinical trials have also assessed rTM in DIC with hematologic malignancy and hemolytic uremic syndrome. Here, we report that thrombin cleaves rTM into two fragments, TMD1 and TMD23, the latter of which retains the cofactor activity for protein C activation by thrombin.

Incubation of rTM with varying concentrations of human thrombin (2-20 μg/mL, *i.e.*, 50-500 nM) resulted in the appearance of a 59-kDa band below rTM (Figure 1B, left panel, arrow 1) and a faint 31 kDa band above the B chain of thrombin (Figure 1B, left panel, arrow 2) on SDS-PAGE in a concentration-dependent manner. An anti-TM antibody bound by immunoblotting to both the 59-kDa and 31 kDa proteins (Figure 1B, right panel, arrows 1 and 2). The sum of the molecular weights of the 59-kDa and 31-kDa fragments nearly match the molecular weight of rTM (89 kDa). These results indicate that thrombin cleaves rTM into these two fragments. The N-terminal amino acid sequence of the 59-kDa fragment was determined to be Gly-Ala-Asp-Phe-Gln-Ala-Leu-

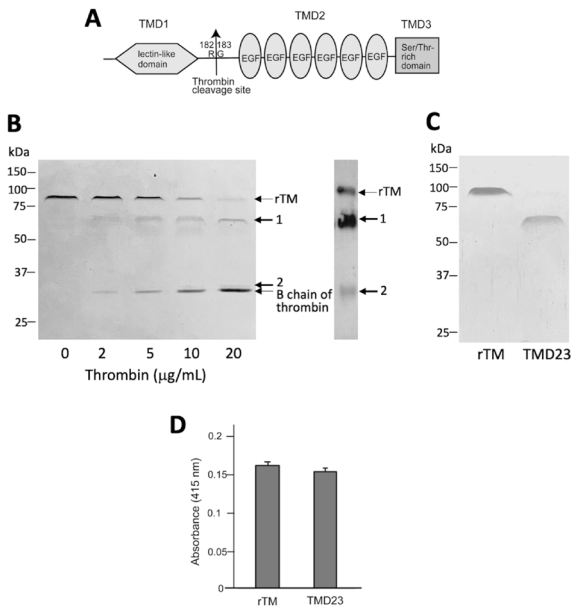

Figure 1. Cleavage of rTM into TMD1 and TMD23 by thrombin, and thrombin-cofactor activity of TMD23. (A) The domain structure of rTM and the identified thrombin cleavage site (arrow). **(B)** SDS-PAGE and immunoblot of thrombin-treated rTM. rTM (50 μg/mL) and thrombin (0-20 μg/mL) were incubated in 20 mM Tris, 150 mM NaCl, pH 8.0 at 37°C for 3 hrs, followed by SDS-PAGE under reducing condition (10% gel). The gel was stained with Coomassie Brilliant Blue R-250 (left panel). In the right panel, after incubation of rTM (50 μg/mL) and human thrombin (20 μg/mL), SDS-PAGE, and transfer to a PVDF membrane, the blots were probed with a rabbit polyclonal antibody against human TM. Horseradish peroxidase-conjugated anti-rabbit IgG was used as the secondary antibody and visualized using 3,3',5,5'-tetramethylbenzidine. In the both panels, arrow 1 and arrow 2 indicate a 59-kDa protein (TMD23) and a 31-kDa protein (TMD1), respectively. Positions of molecular weight markers are indicated on the left. **(C)** SDS-PAGE of purified TMD23. After rTM and thrombin were incubated, the reaction mixtures were subjected to anion-exchange chromatography on a Mono Q column, and TMD23 was purified. **(D)** Thrombin-cofactor activity of TMD23. TMD23 or rTM at concentrations of 1μM were incubated with thrombin and protein C at 37°C for 10 min. Then, hirudin was added, and the reaction mixture was incubated at 37°C for 10 min. After incubation, S-2366, a substrate for protein C, was added, followed by additional incubation at 37°C for 20 min. After adding acetic acid to terminate the reaction, absorbance was measured at 415 nm (n = 3).

Pro-Val-Gly, which is identical to the sequence spanning amino acids 183-192 of rTM. This region is located in the hydrophobic domain between TMD1 and TMD2, indicating that thrombin cleaves rTM between Arg^{182} and Gly^{183} to generate TMD1 and TMD23, respectively (Figure 1A).

Among the three domains of rTM, TMD2 is responsible for the thrombin cofactor activity. Therefore, we next examined whether TMD23 generated from rTM by thrombin retained its protein C-activating cofactor activity. We purified TMD23 from the reaction mixture of rTM and thrombin by anion-exchange chromatography using Mono Q. Purified TMD23 migrated as a single band on SDS-PAGE (Figure 1C). Using purified TMD23, we examined its protein C-activating cofactor activity and found that it exhibited activity similar to that of rTM (Figure 1D).

Thrombin acts on a variety of biological substrates. In the coagulation system, thrombin cleaves fibrinogen, FXIII, FVIII, FXI, protein C, and other coagulation factors. In addition, proteins such as C3 and C5 of the complement system are thrombin substrates, which shows its relatively broad specificity. Based on phage-display analysis, Gallwitz *et al*. proposed the following consensus recognition sequences of optimal substrates for thrombin: P2-Pro, P1-Arg, P1′-Ser/Ala/Gly/Thr, P2′-not acidic, and P3′-Arg (*17*). However, no natural substrates of thrombin display this consensus sequence, suggesting that exosite cooperativity is important for determining specificity and cleavage rate. The observed cleavage site of rTM has the sequences P2-Ala, P1-Arg, P1′-Gly, P2′-Ala, and P3′-Asp, which align to three positions in the consensus recognition sequence.

The physiological concentrations of free thrombin during coagulation reactions are estimated to range from 1 nM to approximately 500 nM (*18,19*). Thrombin at a relatively higher concentration (50 nM-500 nM) was used to examine rTM cleavage in this study. High plasma thrombin levels can be observed in certain pathophysiological conditions such as DIC (*20*).

The physiological relevance of thrombin cleavage of rTM into TMD1 and TMD23 is that thrombin may be involved in the generation of soluble TMD1 in plasma. It has been reported that an approximately 30-kDa protein that is regarded as possibly TMD1 is present in the plasma (*21*). Thrombin may cleave membrane-bound and/or soluble TM shed from the cells to generate TMD1. The involvement of metalloproteases in TMD1 shedding has also been previously reported (*22*). As described above, TMD1 has been shown to have several biological functions, including the attenuation of inflammation mediated by LPS. Soluble TMD1, but not membrane-bound TMD1, may play a crucial role in the attenuation of circulating LPS-induced inflammation.

We demonstrated that TMD23 generated from rTM by thrombin digestion has cofactor activity for protein C activation. It was previously reported that recombinant TMD23 has cofactor activity and exerts a 4.6-fold higher activity than rTM on a molar basis (*13*). However, TMD23 did not show a higher activity than rTM in this study (Figure 1D). A possible explanation for this observation is that TMD23 partially loses its cofactor activity during purification. The present results imply that TMD23 may retain its cofactor activity under conditions in which rTM is cleaved by thrombin in the blood.

In summary, thrombin cleaves rTM to generate TMD1 and TMD23, the latter of which retains its cofactor activity for protein C activation by thrombin. This finding suggests that thrombin not only activates protein C on membrane-bound TM but may also cleave TM to generate TMD1, which exerts several biological functions in the blood.

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