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Amino acid sequence surrounding the chondroitin sulfate attachment site of thrombomodulin regulates chondroitin polymerization

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ABSTRACT

Thrombomodulin (TM) is a cell-surface glycoprotein and a critical mediator of endothelial anticoagulant function. TM exists as both a chondroitin sulfate (CS) proteoglycan (PG) form and a non-PG form lacking a CS chain (α-TM); therefore, TM can be described as a part-time PG. Previously, we reported that α-TM bears an immature, truncated linkage tetrasaccharide structure (GlcAβ1-3Galβ1-3Galβ1-4Xyl). However, the biosynthetic mechanism to generate part-time PGs remains unclear. In this study, we used several mutants to demonstrate that the amino acid sequence surrounding the CS attachment site influences the efficiency of chondroitin polymerization. In particular, the presence of acidic residues surrounding the CS attachment site was indispensable for the elongation of CS. In addition, mutants defective in CS elongation did not exhibit anti-coagulant activity, as in the case with α-TM. Together, these data support a model for CS chain assembly in which specific core protein determinants are recognized by a key biosynthetic enzyme involved in chondroitin polymerization.

1. Introduction

Thrombomodulin (TM) is an integral membrane glycoprotein expressed on the surface of vascular endothelial cells and is present in two forms, a chondroitin sulfate (CS) proteoglycan (PG) (β-TM) and a non-PG form that lacks a CS chain (α-TM). Due to its dual nature, TM is considered to be a part-time PG [1]. TM binds to thrombin with high affinity and acts as a mediator of endothelial anticoagulant function. Comparative studies of the anticoagulant effects of β-TM and α-TM revealed that the CS chain of β-TM plays an indispensable role in regulating anticoagulation [2]. β-TM exhibits a higher affinity for thrombin than α-TM and more effectively inhibits thrombin-mediated clotting activity than β-TM [3]. In addition, platelet factor 4, which prevents the formation of a complex between heparin and antithrombin III, leads to blood coagulation and binds to β-TM but not α-TM [3]. The platelet factor 4/β-TM complex exhibits anticoagulant activity by inactivating the clotting factors Va and VIIIa through limited proteolysis. In addition, a recombinant soluble form of TM has been approved to treat patients suffering from disseminated intravascular coagulation and has greater therapeutic potential than heparin [4–7]. Therefore, the CS moiety of TM is essential for its biological functions. However, the control mechanism for the glycanation step has not been clarified.

CS and heparan sulfate (HS) are attached to specific Ser residues of core proteins through a glycosaminoglycan (GAG)–protein linkage region GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-*O*-Ser. After a linkage-region tetrasaccharide is formed, the repetitive disaccharide unit [(-4GlcAβ1-3GalNAcβ1-)n] characteristic of CS is synthesized by alternate addition of GalNAc and GlcA residues, respectively. It should be noted that the tetrasaccharide linkage region is shared with another sulfated GAG, HS, which contains a repetitive disaccharide unit [(-4GlcAβ1-4GlcNAcα1-)n]. Therefore, the addition of GalNAc instead of the GlcNAc residue triggers the synthesis of CS on the linkage region, indicating that the first *N*-acetylhexosamine transfer is a critical step for the selective assembly of CS and HS chains. Due to its common use, perturbation of the biosynthetic machinery for the tetrasaccharide linkage region affects the function of CS and HS chains [8].

Previously, we reported that an immature truncated GAG structure GlcAβ1-3Galβ1-3Galβ1-4Xyl is attached to recombinant human TM [9]. This finding clearly indicates that GAG biosynthesis

Abbreviations: 2AB, 2-aminobenzamide; CS, chondroitin sulfate; GAG, glycosaminoglycan; ChSy, chondroitin synthase; ChPF, chondroitin polymerizing factor; GalNAc, *N*-acetyl-D-galactosamine; GlcA, D-glucuronic acid; TM, thrombomodulin; HS, heparan sulfate.

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is initiated on α-TM, but remains truncated, thus leaving polymerization incomplete. Recently, we demonstrated that chondroitin is polymerized with alternating GalNAc and GlcA residues on the linkage-region tetrasaccharide of α-TM when any two of four proteins—chondroitin synthase-1 (ChSy-1), ChSy-2, ChSy-3, and chondroitin polymerizing factor (ChPF)—were co-expressed [10–13]. In addition, ChSy family members co-expressed in various combinations exhibited distinct but overlapping acceptor substrate specificities toward the two synthetic acceptor substrates [12]. Therefore, it is suggested that enzyme complexes interact with the core proteins, and that the affinity for the core proteins differed among the enzyme complexes.

To investigate whether the amino acid sequence around the CS attachment site of TM might be important in determining CS synthesis, we altered the amino acids surrounding the CS attachment site of TM. In this study, we show that the amino acid sequence surrounding the CS attachment site influences the efficiency of chondroitin polymerization. In addition, mutants defective in CS elongation did not exhibit anti-coagulant activity, as in the case with α-TM. Together, these data suggest a model for CS chain assembly in which specific core protein determinants regulate CS chain polymerization.

2. Methods

2.1. Establishment of a TM expression vector

The cDNA fragment encoding TM was amplified from a human leukocyte cDNA library (Clontech) with primers containing a *Bam*HI or *Hind*III restriction site (5′-forward: 5′-CGGGATCCGGTAACATGCTTGGGGTCCTG-3′, and 3′-reverse: 5′-CCAAGCTTCGAATGCACGAGCCCCACGG-3′, respectively). The PCR fragments were subcloned into pcDNA3.1(−) myc/His (Invitrogen) (sTM-myc), as described previously [14].

2.2. Site-directed mutagenesis

A two-stage PCR mutagenesis method was used to construct TM mutants. Two separate PCR reactions were performed to generate two overlapping gene fragments using the full-length form of TM cDNA as a template. In the first PCR, the sense 5′-forward primer (described above) was used with each of the following antisense internal mutagenic primers: S472E 5′-GTGGACGGTGGCGAC-**GAA**GGCTCTGGCGAG-3′, D471A 5′-AAGGTGGACGGTGGCG**C**CAGCG GCTCTGGC-3′, D471N 5′-AAGGTGGACGGTGGC**A**ACAGCGGCTCT GGC-3′, E476A 5′-GACAGCGGCTCTGGCG**C**GCCCCCGCCCAGC-3′, G470S D471G 5′- AAGGTGGACGGT**A**GCG**G**CAGCGGCTCTGGC-3′, (the mutated nucleotides are underlined and in bold). In the second round of PCR, the corresponding sense internal mutagenic primers (complementary to the antisense internal mutagenic primers) and the antisense 3′-reverse primer (described above) were used. The two PCR products were gel-purified and used as a template for a third PCR reaction containing the sense 5′-forward and antisense 3′-reverse primers described above. The final PCR fragment was subcloned into the pcDNA3.1(−) myc/His (sTM-myc), as described above. The fidelity of the plasmid constructs was confirmed by DNA sequencing.

2.3. Expression and purification of recombinant sTM-myc

CHO–K1 cells were maintained in Ham's F12 medium (Wako) with 10% fetal bovine serum in 5% CO_2 at 37 °C. For cDNA transfection, cells were grown overnight and transfected using FuGENE 6 (Roche Applied Science) according to the manufacturer's instructions. Two days after transfection, 7 ml of the culture medium was collected and incubated with 20 μl of Ni-NTA agarose (Qiagen) overnight at 4 °C. The beads recovered by centrifugation and washed with 50 mM sodium phosphate with 0.3 M sodium chloride and 5 mM imidazole, pH 8.0. To quantify the protein absorbed onto the Ni-NTA agarose beads, the bound protein was eluted with 50 mM sodium phosphate with 0.3 M sodium chloride and 250 mM imidazole, pH 8.0, and subsequently quantified using the BCA Protein Assay Reagent (enhanced protocol, Pierce).

2.4. Western blotting analysis

The beads recovered by centrifugation were washed three times with TBS buffer containing Tween 20. The samples were subjected to SDS-PAGE (7% gel) and proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked in PBS containing 2% skim milk and 0.1% Tween 20, incubated with TM antibody (D3), and then treated with anti-rabbit IgG conjugated with horseradish peroxidase (Amersham Bioscience). Proteins bound to the antibody were visualized using an enhanced chemiluminescence (ECL) advance kit (Amersham Bioscience).

2.5. Measurement of the anti-coagulant activity of TM

Direct anti-coagulant activity of TM was determined by its ability to inhibit thrombin clotting activity with fibrinogen (Sigma), as described [14]. Fibrinogen clotting was monitored visually. Thrombin (18 nM, Sigma) was incubated for 1 min at 37 °C with TM or mutant TM at various molar ratios. Fibrinogen (2.0 mg/ml, final concentration) was then added and the clotting time was determined. The assays were performed in 0.02 M Tris–HCl buffer (pH 7.5) containing 0.1 M NaCl and 1 mg/ml bovine serum albumin, as described previously [14].

2.6. Preparation of 2-aminobenzamide (2AB)-derivatives of GAG chains from TM

Purified TM (200 μg core protein) was treated with 0.5 m LiOH at 4 °C for 13 h to liberate *O*-linked saccharides from the core protein [9,15]. After neutralization, the sample was applied to an AG 50W-X2 (H^+ form, Bio-Rad) column (2.5 ml bed volume). The flow-through fraction containing the *O*-linked oligosaccharide components was pooled and neutralized with 1 mM NH_4HCO_3. Derivatization of the oligosaccharide component of α-TM with 2AB was performed as described [9,16]. The 2AB-derivatized oligosaccharide fraction was analyzed by anion-exchange HPLC on an amine-bound silica PA-03 column (4.6 × 250 mm, YMC Co., Kyoto, Japan) as described previously [9,16].

3. Results and discussion

3.1. CS attachment site amino acid sequence of TM is recognized by CS polymerases

In our previous study, we demonstrated that α-TM contained a truncated linkage tetrasaccharide, GlcAβ1-3Galβ1-3Galβ1-4Xyl [9], and suggested that the critical determining step for CS proteoglycan biosynthesis might be the transfer of the fifth sugar residue (the first GalNAc) by CS synthesizing enzyme complexes [10–13,17,18]. To investigate if the amino acids close to the attachment site are important in determining CS chain polymerization, site-directed mutagenesis was performed to generate a collection of substitution mutants, as shown in Table 1. Although TM is an integral membrane protein, sTM-myc is designed to be secreted into the culture medium through the truncation of its transmembrane domain [2]. The soluble form of myc/His-tagged TM (sTM-myc) or

Table 1
Sequences of CS-attachment regions in wild-type and mutant TMs.

Construct	CS attachment site[a]
TM	GGDSGSGEPPPSP
D471A	GG**A**SGSGEPPPSP [b]
D471N	GG**N**SGSGEPPPSP
E476A	GGDSGSG**A**PPPSP
G470S D471G	G**SG**SGSGEPPPSP
S472E	GGD**E**GSGEPPPSP

[a] Amino acid sequence surrounding the Ser474 (underlined) CS attachment site is shown in single-letter code, beginning with G469 and ending with P481.
[b] The mutated nucleotide is in bold.

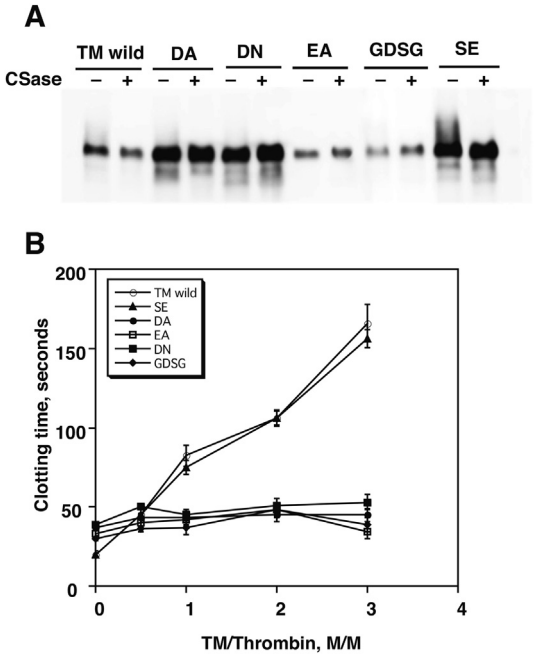

Fig. 1. Effects of site-directed mutagenesis of TM near the CS attachment site. (A) sTM-myc, sTM-D471A, -D471N, -E476A, -G470S D471G, and -S472E were transiently expressed in CHO–K1 cells. Secreted sTM-myc was pulled down from the culture medium and subjected to western blotting using anti-TM mAb. sTM-myc was treated with chondroitinase ABC (CSase) to remove the CS moiety, and western blotting was performed with anti-TM mAb. (B) sTM-myc (TM wild, open circle), -S472E (SE, filled triangle), -D471A (DA, filled circle), -E476A (EA, open square), -D471N (DN, closed square), and -G470S D471G (GDSG, filled diamond) were mixed with thrombin at different molar ratios, as indicated, and clotting time was measured.

each mutant was transiently expressed in CHO–K1 cells. The secreted sTM-myc was pulled down with nickel-nitrilotriacetic acid (Ni-NTA)-conjugated agarose beads from the culture medium, followed by western blotting analyses. As a result, when sTM-myc was expressed alone, both of the glycoforms, chain-bearing CS (β-TM) and non-chain-bearing CS (α-TM), were detected with the anti-TM antibody (Fig. 1A). Upon chondroitinase ABC (CSase) digestion, the upper smear band (β-TM) disappeared and converged into a 100 kDa band (α-TM) (Fig. 1A), indicating that sTM-myc CS chain was modified when produced by the CHO–K1 cells.

To date, negatively charged and hydrophobic residues located proximal to a Ser–Gly (SG) site have been thought to play a role in GAG assembly at the SG site [19–21]. In addition, a primary CS attachment site at Ser474 and a secondary site at Ser472 have also been identified [22]. To investigate whether the amino acid sequence around the CS attachment site of TM might be important in determining CS synthesis, we altered amino acids adjacent to Ser474 (Table 1). Interestingly, when Ala (D471A) or Asn (D471N) was substituted for Asp471, no β-TM production was detected (Fig. 1A and also see Table 2). In addition, when Ala was substitution for Glu476 (E476A), β-TM production was diminished. As previously reported, the substitution of Glu for Ser472 (S472E) increased the relative amounts of β-TM compared with native wild-type sTM (Fig. 1A and also see Table 2) [22]. These results suggest that the presence of acidic residues flanked by hydrophobic residues upstream and/or downstream of the SG motif is necessary for the addition of CS.

In addition, a repetitive SG site has been shown to enhance GAG assembly [21]. It was therefore expected that Gly–Asn substituted for Ser470–Gly471 would result in an increase in the degree of β-TM. However, this substitution markedly decreased β-TM compared with wild-type sTM (Fig. 1A and also see Table 2). These results suggest that triplication of the SG motif inhibits CS modification of the TM.

3.2. Immature truncated GAG structure GlcAβ1–3Galβ1–3Galβ1–4Xyl is attached to sTM and sTM-mutants

Next, we determined if the sTM-mutants contained the truncated linkage tetrasaccharide. The linkage-oligosaccharides were isolated after mild alkaline treatment with LiOH as described previously [9,16]. The isolated oligosaccharides were derived with the fluorophore 2AB, then digested with CSase and analyzed using HPLC. Only one peak was detected at the elution position of the authentic linkage tetrasaccharide GlcAβ1–3Galβ1–3Galβ1–4Xyl-2AB in the sTM-D471A, D471N, E476A, and G470S D471G mutants (Table 2). The peak was shifted to a position that corresponded to Galβ1–3Galβ1–4Xyl-2AB after β-glucuronidase digestion (data not shown), thus demonstrating no repeating disaccharide units in these four mutants. As expected, two peaks were detected at the elution position of the authentic linkage tetrasaccharide GlcAβ1–3Galβ1–3Galβ1–4Xyl-2AB and the authentic 2AB-labeled nonsulfated hexasaccharide ΔHexAα1–3GalNAcβ1–4GlcAβ1–3Galβ1–3Galβ1–4Xyl–2AB (ΔHexA represents 4-deoxy-Δ-L-

Table 2
Proportion of α-type and β-type TM linkage regions from purified sTM-myc.

Protein	pmol/μg[a]	
	α-type GlcAβ1-3Galβ1-3Galβ1-4Xyl-2AB	β-type (after CSase treatment) ΔHexAα1-3GalNAcβ1-4GlcAβ1-3Galβ1-3Galβ1-4Xyl-2AB[b]
Wild-type	0.56 ± 0.03 (77)	0.17 ± 0.04 (23)
D471A	0.41 ± 0.04 (100)	ND (0)[c]
D471N	0.44 ± 0.02 (100)	ND (0)
E476A	0.61 ± 0.02 (100)	ND (0)
G470S D471G	0.78 ± 0.04 (100)	ND (0)
S472E	0.87 ± 0.03 (63)	0.52 ± 0.04 (37)

[a] The values are the mean ± S.D. of three measurements, expressed in pmol/μg protein, with mol% in parentheses.
[b] ΔHexA represents 4-deoxy-Δ-L-*threo*- hex-4-enepyranosyluronic acid.
[c] ND, not detected (<0.01).

threo- hex-4-enepyranosyluronic acid) in the wild-type TM and S472E mutant (Table 2). The relative amounts of ΔHexAα1–3GalNAcβ1–4GlcAβ1–3Galβ1–3Galβ1–4Xyl–2AB (β-TM) to GlcAβ1–3Galβ1–3Galβ1–4Xyl-2AB (α-TM) in S472E mutant was increased compared with that of the wild-type TM. These results were consistent with previous results [22]. Taken together, these results indicate that the sTM-D471A, D471N, E476A, and G470S D471G mutants only contain the truncated tetrasaccharide linkage, GlcAβ1–3Galβ1–3Galβ1–4Xyl-2AB.

3.3. Regulation of anti-coagulant activity of TM through modulation of CS attachment

TM is expressed on the endothelial cell surface and plays a pivotal role in the anti-coagulation system, including the inhibition of the procoagulant activity of thrombin [2]. Previously, we demonstrated that sTM-myc caused a dose-dependent delay in clotting time, while removal of the CS chain by pre-treatment with CSase greatly abrogated the anti-coagulant activity of sTM-myc [14]. Since the anti-coagulant activity of TM is highly dependent on its CS chain, we examined whether sTM-mutant-myc is able to regulate the function of TM through modulation of its CS attachment. To measure anti-coagulant activity, we purified recombinant sTM-myc or sTM-mutant-myc from the culture medium of CHO–K1 cells. Thrombin-dependent clotting assays were then performed to evaluate the anti-coagulant activity of purified sTM-myc. As a result, sTM-myc (TM in Fig. 1B) and sTM-S472E (SE in Fig. 1B) produced a dose-dependent delay in clotting time. In comparison, sTM-D471A, D471N, E476A, and G470S D471G showed almost no inhibition of thrombin-induced clotting (Fig. 1B). These data are consistent with the present findings that the sTM-D471A, D471N, E476A, and G470S D471G mutant CS chains are largely unmodified, as shown in Table 2.

Previously, we demonstrated that the initiation and elongation of CS chains are performed by an enzyme complex that contains chondroitin synthases (ChSy-1, -2, -3) and chondroitin polymerizing factor (ChPF) [10–13]. In addition, we found that when ChSy family members are co-expressed in various combinations they exhibit distinct but overlapping acceptor substrate specificities toward the two synthetic acceptor substrates, GlcAβ1-3Galβ1-*O*-naphthalenemethanol and GlcAβ1-3Galβ1-*O*-C_2H_4NH-benzyloxycarbonyl, both of which share the disaccharide sequence with the GAG-protein tetrasaccharide linkage region [12]. These results suggest that the amino acids close to the CS attachment site of core proteins are recognized by the enzyme complexes, and that the affinity toward the core proteins differs among the enzyme complexes. Thus, much of the information that regulates this step might be encoded in the PG core protein.

Previous studies have shown that repetitive Ser–Gly dipeptides with a flanking cluster of acidic residues represent a common motif in a variety of HSPGs [21], although most CSPG do not contain nearby clusters of acidic residues or a common motif. Therefore, it was expected that CS priming most likely occurs by default. However, the present study shows that CS biosynthetic enzymes also recognize the amino acid sequence surrounding the CS attachment site. Therefore, we propose here a model for CS chain assembly in which specific core protein determinants are recognized by a key biosynthetic enzyme involved in chondroitin polymerization.

Conflict of interest

None.

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