Demonstration of the Immature Glycosaminoglycan Tetrasaccharide Sequence GlcAβ1-3Galβ1-3Galβ1-4Xyl on Recombinant Soluble Human α-Thrombomodulin

AN OLIGOSACCHARIDE STRUCTURE ON A “PART-TIME” PROTEOGLYCAN*

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Thrombomodulin (TM), a cell surface glycoprotein, is a critical mediator of endothelial anticoagulant defenses occurring both as a chondroitin sulfate proteoglycan (β-TM) and a protein (α-TM) unsubstained by chondroitin sulfate (CS), hence its description as a “part-time” proteoglycan (PG) (Fransson, L. A. (1987) Trends Biochem. Sci. 12, 406–411). Sugar analysis was performed on α-TM to investigate a possible biosynthetic mechanism for part-time PGs. Recombinant human α-TM, which was expressed in CHO-K1 cells, separated by anion-exchange chromatography from β-TM, and purified by immunoaffinity chromatography (Nawa, K., Sakano, K., Fujiwara, H., Sato, Y., Sugiyama, N., Teruuchi, T., Iwamoto, M., and Marumoto, Y. (1990) Biochem. Biophys. Res. Commun. 171, 729–737), was used for analysis. Preliminary sugar composition analysis after acid hydrolysis showed Xyl in addition to Gal, GalNAc, GlcNAc, Man, Fuc, and Glc. O-Glycosidically-linked oligosaccharides were liberated by mild alkaline treatment and purified. The isolated oligosaccharide fraction was derivatized with a fluorophore 2-aminobenzamide (2AB), resulting in two fluorescent components, a 2AB-Glc. Based on structural analysis by a combination of sequential exoglycosidase digestion and 500-MHz 1H NMR spectroscopy of the 2AB-oligosaccharide, the structure of the oligosaccharide was elucidated as GlcAβ1-3Galβ1-3Galβ1-4Xyl, which turned out to represent a glycosaminoglycan (GAG)-protein linkage region tetrasaccharide common to various PGs and was considered to be a biosynthetic intermediate of an immature GAG chain. The results may indicate that at least one class of the so-called part-time PGs bear the linkage tetrasaccharide at the GAG attachment sites and that the critical determining step or the rate-limiting step for PG biosynthesis is the transfer of the fifth sugar residue, the first hexosamine, rather than xylose.

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dues substituted by GAGs (9, 10). Mann et al. (11) proposed that the amino acids surrounding the serine residue generate a conformation whereby the GAG attachment site is bound poorly by xylosyltransferase, and consequently some molecules are substituted and others unsubstituted with GAG, resulting in a "part-time" PG. In addition, Lin et al. (4) indicated that the conformation well beyond the immediate site of GAG addition plays an important role in GAG attachment. Alternatively, another hypothesis for the formation of part-time PG involves competition for a serine residue between xylosyltransferase and N-acetylgalactosaminyltransferase (12). However, neither model satisfactorily explains the biosynthetic mechanism of part-time PG.

We recently identified a novel α-N-acetylgalactosaminyltransferase in fetal bovine serum, and also in mouse mastocytoma cells, which catalyzes the transfer of an α-GalNAc residue to the linkage tetrasaccharide-serine, GlcAβ1→3Galβ1→4Xyl1-O-Ser, derived from PGs, although its role in GAG biosynthesis remains unclear (13, 14). In addition, Manzi et al. (15) reported that the α-GalNAc-capped pentasaccharide sequence bound to an artificial primer was secreted by human melanoma cells, Chinese hamster ovary cells, and several other human cancer cell lines when carbohydrate synthesis was stimulated using a primer 4-methylumbelliferyl-β-D-xyloside. More recently, we found that the α-GalNAc-capped pentasaccharide serine GalNAcc1→4GlcAβ1→3Galβ1→4Xyl1-O-Ser, a reaction product of the α-GalNAc transferase, was not utilized as an acceptor for a glucuronyltransferase involved in further chain elongation, creating a part-time PG, and hence the critical determining step for PG biosynthesis is the transfer of the fifth sugar residue, the first hexosamine, rather than xylose.

EXPERIMENTAL PROCEDURES

Materials—Two forms of recombinant soluble human TM with or without GAG modification were expressed in CHO-K1 cells using the expression vector pRS7TM-neo, which contained the DNA fragment encoding soluble TM (Ala1–Ala491) (17).

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Oligosaccharides were liberated from the purified α-TM by mild alkaline treatment and purified by passage through a cation-exchange column as described under "Experimental Procedures." The isolated oligosaccharide fraction was derivatized with a fluorophore 2AB and fractionated by gel filtration HPLC, resulting in two fluorescent components, a 2AB-oligosaccharide (fraction 1) and a putative Glc-2AB (fraction 2) (Fig. 2). The latter peak was co-eluted with the authentic Glc-2AB when they were co-injected (data not shown). The 2AB-derivatized oligosaccharide was analyzed below, whereas the putative Glc-2AB seemed to disappear in the 2AB-derivatized authentic unsaturated linkage tetrasaccharide-serine (27) and the corresponding unsaturated linkage hexasaccharide-serine (26) (Table I). These results altogether indicate that the structure of the compound in fraction 1 is most likely GlcA

| Component | Molar ratio |
|-----------|-------------|
| GalNAc    | 2.0         |
| Xyl        | 1.0         |
| GlcNAc    | 5.0         |
| Glc        | 0.75        |
| Man        | 3.0         |
| Fuc        | 1.0         |
| Gal        | 6.0         |

The eluates were monitored by fluorescence at an excitation wavelength of 330 nm and an emission wavelength of 420 nm. Arrows indicate the elution positions of the 2AB-derivatives of authentic trisaccharide and monosaccharides: 1, Galβ1–3Galβ1–4Xyl-2AB; 2, GlcNAc-2AB; 3, Gal-2AB; 4, Glc-2AB; 5, Gal-2AB; 6, Xyl-2AB; 7, Fuc-2AB.

FIG. 2. Size fractionation of the 2AB-derivatized oligosaccharide fraction from α-TM. The 2AB-derivatized oligosaccharide fraction from α-TM was fractionated by gel-filtration HPLC on a TSK-gel G2500PW column using 20 mM CH₃COONH₄, (pH 7.5) as eluent at a flow rate of 1 ml/min as described under "Experimental Procedures." The elutes were monitored by fluorescence at an excitation wavelength of 330 nm and an emission wavelength of 420 nm. Arrows indicate the elution positions of the 2AB-derivatives of authentic trisaccharide and monosaccharides: 1, Galβ1–3Galβ1–4Xyl-2AB; 2, GlcNAc-2AB; 3, Gal-2AB; 4, Glc-2AB; 5, Gal-2AB; 6, Xyl-2AB; 7, Fuc-2AB.

FIG. 1. SDS-PAGE analysis of recombinant soluble α-TM. Recombinant soluble α-TM was analyzed by SDS-PAGE using the buffer system of Laemmli (46) with an 8% gel under non-reducing conditions and proteins were stained with Coomassie Brilliant Blue. Lane 1, molecular weight standards (Amersham Pharmacia Biotech); lane 2, α-TM 4.0 μg.

Isolation of the Oligosaccharides—O-Glycosidically-linked oligosaccharides were liberated from the purified α-TM by mild alkaline treatment and purified by passage through a cation-exchange column as described under "Experimental Procedures." The isolated oligosaccharide fraction was derivatized with a fluorophore 2AB and fractionated by gel filtration HPLC, resulting in two fluorescent components, a 2AB-oligosaccharide (fraction 1) and a putative Glc-2AB (fraction 2) (Fig. 2). The latter peak was co-eluted with the authentic Glc-2AB when they were co-injected (data not shown). The 2AB-derivatized oligosaccharide was analyzed below, whereas the putative Glc-2AB was not analyzed further. Although α-TM seemed to contain O-glycosidase-sensitive oligosaccharides as revealed by the detection of GalNAc by the preliminary sugar composition analysis, O-linked oligosaccharides with a GalNAc residue at the reducing terminus were not detected. However, when 3H-labeled O-linked oligosaccharides released from α-TM by alkaline β-elimination with NaBH₄ were fractionated by HPLC and used for the structural analysis, 3H-labeled galactosaminitol as well as 3H-labeled xyitol were detected indeed by acid hydrolysis of the fractions (data not shown). Therefore, O-linked oligosaccharides with a GalNAc residue at the reducing terminus were actually contained in α-TM but not released by mild alkaline treatment used prior to 2AB-derivatization in the present study.

Enzymatic Characterization of the Isolated 2AB-derivatized Oligosaccharide—Fraction 1 was subjected to HPLC on an amine-bound silica PA03 column. Only one peak was detected at the elution position of the authentic unsaturated linkage tetrasaccharide ΔHexAα1–3Galβ1–3Galβ1–4Xyl-2AB (Fig. 3A), and the molar ratio of the oligosaccharide to the core protein was calculated to be in the range of 0.77–1.33 based on the peak area on HPLC. The peak was shifted to a position corresponding to Galβ1–3Galβ1–4Xyl-2AB by β-glucuronidase digestion (Fig. 3B). In contrast, the peak was not shifted by digestion with chondroitinases ABC or AC-II (data not shown), demonstrating no repeating disaccharide unit. It was not sensitive to neuraminidase or α-N-acetylgalactosaminidase either, indicating that it is not covered with neuraminic acid or α-GalNAc. Fraction 1 was further analyzed by sequential enzymatic digestion using gel-filtration HPLC on a column of GS-320. One major peak was again observed at the elution position slightly ahead of the 2AB-derivatized authentic unsaturated linkage tetrasaccharide ΔHexAα1–3Galβ1–3Galβ1–4Xyl-2AB upon exhaustive glucuronidase digestion (Fig. 4B) and then to the position of Xyl-2AB upon subsequent exhaustive β-galactosidase digestion (Fig. 4C). These results altogether indicate that the structure of the compound in fraction 1 is most likely GlcA

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Data are expressed as molar ratios relative to α-thrombomodulin core protein (65 kDa).
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Fig. 3. HPLC analysis of fraction 1 isolated from α-TM. Fraction 1 isolated from α-TM (see Fig. 2) was analyzed by HPLC on an amine-bound silica column using a linear gradient of 16–530 mM NaH₂PO₄ over a 60-min period before (panel A) and after β-glucuronidase digestion (panel B) as described under “Experimental Procedures.” The eluates were monitored by fluorescence at an excitation wavelength of 330 nm and an emission wavelength of 420 nm. The arrows denote the elution positions of the following authentic compounds: 1, Galβ₁–3Galβ₁–4Xyl-2AB; 2, αHexAα₁–3Galβ₁–3Galβ₁–4Xyl-2AB. Note that a given unsaturated oligosaccharide and the corresponding saturated oligosaccharide, which differ only in the non-reducing terminal structure, were hardly separated under these conditions.

Fig. 4. Sequential glycosidase digestions of fraction 1 isolated from α-TM. Fraction 1 isolated from α-TM (see Fig. 2) was analyzed by gel-filtration HPLC on a column of GS-320 (7.6 × 500 mm), which was eluted with 50 mM CH₃COONH₄ at a flow rate of 1 ml/min, as described under “Experimental Procedures.” The eluates were monitored by fluorescence at an excitation wavelength of 330 nm and an emission wavelength of 420 nm. Panel A, before enzymatic digestion; panel B, after β-glucuronidase digestion; panel C, after β-glucuronidase digestion followed by β-galactosidase digestion. The peaks marked by asterisks were attributable to the buffer salts. The arrows denote the elution positions of the following authentic compounds: 1, αHexAα₁–3GalNAcβ₁–4GlcAβ₁–3Galβ₁–3Galβ₁–4Xyl-2AB; 2, αHexAα₁–3Galβ₁–3Galβ₁–4Xyl-2AB; 3, Galβ₁–3Galβ₁–4Xyl-2AB; 4, Xyl-2AB.

Protein species in which only some of the molecules are substituted with GAG have been termed “part-time” PGs (5). In addition to TM, collagen type IX (32), the invariant chain of the class II major histocompatibility complex (33), the high molecular weight receptor for transforming growth factor-β (34), and a lymphocyte homing receptor (35) are well known as part-time PGs. The addition of GAG chains on these proteins has generated profound effects on their various properties since it depresses the biological activity by blocking the active site on the protein or adds a new binding site to the molecule. Hence, it is of particular importance to investigate the biosynthetic mechanism generating part-time PGs and to understand how the addition of GAG chains is regulated. So far, the addition of GAG chains has been considered to occur in an all-or-none manner. Namely, no obvious intermediate oligosaccharide chains for the GAG biosynthesis have been found on core proteins of part-time PGs including α-TM. In this study, based on the structural analysis by a combination of enzymatic digestion and ¹H NMR spectroscopy, we demonstrated the following O-linked oligosaccharide containing a Xyl residue on recombinant human α-TM: GlcAβ₁–3Galβ₁–3Galβ₁–4Xyl. This finding clearly indicates that the biosynthesis of GAG has actually
been initiated on α-TM but is truncated leaving the polymerization incomplete. Therefore, we may have to revise the previous concept regarding the biosynthetic mechanism for generating a part-time PG.

Previous studies suggested that the transfer of a Xyl residue was the critical step determining GAG biosynthesis since xylosylation is the first step in the carbohydrate modification of the core protein (36). Mann et al. (11) proposed that the amino acids surrounding the serine residue generate a conformation whereby the GAG attachment site is bound poorly by xylosyltransferase and consequently only some of the molecules are substituted with GAG. They also showed using site-directed mutagenesis that the xylosyltransferase responsible for the initiation of the GAG chain can use a threonine residue for the substitution instead of a serine residue, but that such substitution is only partial, creating a part-time PG. Thus, it seems that the conformation of the GAG substitution site may be important for recognition by xylosyltransferase (11). On the other hand, Gerlitz et al. (12) presented the “acceptor consensus overlap” model involving glycosyltransferase competition for the serine residue to account for the expression of TM in its two distinct glycoforms, α- and β-TMs, since there is an apparent overlap at Ser 474 in human TM in terms of the acceptor consensus sequences of xylosyltransferase and GalNAc transferase. In this context, several studies suggested that these two glycosyltransferases reside in the same subcellular compartment (cis-Golgi) (37–39), and it is therefore possible that direct

\[ \text{GlcAβ1-3Galβ1-3Galβ1-4Xyl-2AB} \]

\[ \text{Before derivatization with 2AB} \]

\[ \text{ANOMERIC PROTONS} \]

\[ \text{GlcA-4} \]

\[ \text{Gal-3} \]

\[ \text{H-1} \]

\[ \text{H-2} \]

\[ \text{H-3} \]

\[ \text{H-4} \]

\[ \text{H-5} \]

\[ \text{Gal-2} \]

\[ \text{H-1} \]

\[ \text{H-2} \]

\[ \text{H-3} \]

\[ \text{H-4} \]

\[ \text{H-5} \]

\[ \text{Xyl-1} \]

\[ \text{H-1} \]

\[ \text{H-2} \]

\[ \text{H-3} \]

\[ \text{H-4} \]

\[ \text{H-5} \]

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In conclusion, our studies demonstrated the immature truncated GAG structure Glcαβ1–3Galβ1–3Galβ1–4Xyl on the recombiant human α-TM. The findings indicate that the anti-coagulation functions of TM are regulated by the transfer of a GalNAc residue to the linkage tetrasaccharide rather than the transfer of a Xyl residue to the core protein and that β-GalNAc transferase I, which catalyzes the transfer of a β-GalNAc residue to the linkage tetrasaccharide, may play a critical role as the rate-limiting enzyme in the biosynthesis of part-time PGs. Further study of the carbohydrate structure on other part-time PGs is necessary to confirm the generality of the mechanism.

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