Novel Proteoglycan Linkage Tetrasacccharides of Human Urinary Soluble Thrombomodulin, SO$_4$-3GlcAβ1-3Galβ1-3(±Siaα2-6)Galβ1-4Xyl*

(Received for publication, August 10, 1997, and in revised form, October 30, 1998)

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O-linked sugar chains with xylose as a reducing end linked to human urinary soluble thrombomodulin were studied. Sugar chains were liberated by hydrazinolysis followed by N-acetylation and tagged with 2-amino pyridylidine. Two fractions containing pyridylaminated Xyl as a reducing end were collected. Their structures were determined by partial acid hydrolysis, two-dimensional sugar mapping combined with exoglycosiase digestions, methylation analysis, mass spectrometry, and NMR as SO$_4$-3GlcAβ1-3Galβ1-3(±Siaα2-6)Galβ1-4Xyl. These sugar chains could bind to an HNK-1 monoclonal antibody. This is believed to be the first example of a proteoglycan linkage tetrasaccharide with glucuronic acid 3-sulfate and sialic acid.

Thrombomodulin (TM)$^7$ is a physiologically important anticoagulant (1) that is present not only on the endothelial cell surface but also in soluble form in plasma and urine (2–4). Takahashi et al. (5) have purified the major active forms of human urinary soluble thrombomodulin (uTM) and demonstrated that they possess strong cofactor activity for thrombin-catalyzed protein C activation as well as exhibiting potent anticoagulant activity in vivo. They have also shown that uTM improves disseminated intravascular coagulation without excess prolongation of the activated partial thromboplastin time (6). The protein possesses five potential N-linked glycosylation sites (7) as deduced from its amino acid sequence, whereas the detection of GalNAc suggests the presence of O-linked sugar chains (8). Although uTM does not contain a glycosaminoglycan, recombinant TM and some TMs obtained from cultured human endothelial cells are expressed in both a surface but also in soluble form in plasma and urine (2–4). They have also shown that uTM improves disseminated intravascular coagulation without excess prolongation of the activated partial thromboplastin time (6).

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* The abbreviations used are: TM, thrombomodulin; uTM, urinary soluble TM; PA, pyridylaminated; HPLC, high performance liquid chromatography.
Elution Condition 2 for methylation analysis: column, μBondasphere 5C18–300Å; flow rate, 1.0 ml/min at 25 °C; eluent, 0.1 M ammonium acetate buffer, pH 6.0. After injecting a sample, the acetonitrile concentration was increased linearly from 0 to 70% in 45 min.

Size fractionation HPLC: column, Shodex NH₂-P-50; flow rate, 0.6 ml/min at 25 °C. Two eluents, A and B, were used. Eluent A was acetonitrile:water:acetic acid (95:5:3, v/v/v) titrated to pH 7.3 with triethylamine, and Eluent B was water:acetic acid (100:3, v/v) titrated to pH 7.3 with triethylamine. The column was equilibrated with Eluent A. Three min after injecting a sample, linear gradient elution was performed to Eluent A:Eluent B (90:10, v/v) in 2 min and then to Eluent A:Eluent B (50:50 v/v) in 38 min.

Anion-exchange HPLC: column, TSKgel Sugar AXI; flow rate, 0.3 ml/min at 73 °C. The eluent used was a mixture of 9 parts 0.8 M boric acid adjusted to pH 9.0 with potassium hydroxide and 1 part acetonitrile.

Mono Q HPLC: column, Mono Q HR 5/5; flow rate 1.0 ml/min at 25 °C. The eluent used was water titrated to pH 9.0 with aqueous ammonia. Adsorbed samples were eluted with a linear gradient of ammonium sulfate from 0 to 1.0 M concentration.

Exoglycosidase Digestion—A PA sugar chain (100 pmol) was digested with 100 milliunits of Aspergillus β-galactosidase in 20 μl of 50 mM ammonium acetate buffer, pH 5.0; or with 100 units of β-glucuronidase in 100 μl of 200 mM ammonium acetate buffer, pH 5.0. Enzymatic reactions were carried out at 37 °C for 16 h and then terminated by heating at 100 °C for 3 min.

Methanolysis—A PA sugar chain (500 pmol) was methanolyzed with 0.1 M HCl in methanol for 3 h (19). After evaporation to dryness, the desulfated PA sugar chain was N-acetylated with 120 μl of water:pyridine:acetic anhydride (5:25:1) on ice for 1 h. After evaporation of the solution, a small amount of O-acetyl groups was removed by heating the solution with 20 ml of 1.0 M aqueous ammonia at 100 °C for 5 min. The solution was then freeze-dried.

Methylation Analysis—Freeze-dried X2 (2 nmol) was permethylated with 0.1 ml of methylsulfinyl carbanion reagent (prepared from 25 mg of NaH and 0.8 ml of dimethyl sulfoxide) and 0.1 ml of methyl iodide as reported (20). The reaction mixture was placed on a Sephadex LH 20 column (0.9 x 24 cm), and the permethylated X2 was eluted with chloroform. One-ml fractions were collected, and each fraction was

FIG. 1. Gel filtration profiles of PA sugar chains from uTM. PA sugar chains were applied onto a TSKgel HW-40F column (2 x 80 cm) using 0.2 M ammonium acetate buffer, pH 6.0, and 5-ml fractions were collected. An aliquot of each fraction was subjected to reducing-end PA sugar analysis. PA GlcNAc; PA GalNAc; PA Xyl. Fractions indicated by a bar were pooled as Fraction X.

FIG. 2. Subfractionation of Fraction X by reversed-phase HPLC. Elution Condition 1 was used (see text).

FIG. 3. Characterization of structures of X1 and X2 using a two-dimen- sional sugar map. The elution positions relative to that of Galβ1–3GalNAc–PA on reversed-phase HPLC are shown on the abscissa. Elution Condition 1 was used for the reversed-phase HPLC. The molecular sizes of the PA sugar chains measured by size fractionation HPLC are plotted on the ordinate in terms of glucose units using PA isomaltooligosaccharides. Arrows with solid lines indicate changes in coordinate positions caused by chemical desulfation, and other arrows indicate changes in coordinate positions caused by digestion with the following exoglycosidases: dashed lines, β-glucuronidase; dot and dash line; β-galactosidase; dotted line, sialidase. AGGX, GlcAβ1–3Galβ1–3Galβ1–4Xyl-PA; GX, Galβ1–4Xyl-PA.
concentrated to dryness by blowing the solvent with nitrogen. The residue was dissolved in a small amount of chloroform, and the sample was purified by TLC using a silica gel plate and methanol:ethyl acetate (2:8, v/v) with one drop of acetic acid as a solvent. The fluorescent spots revealed under a UV lamp were scraped and combined, and the permethylated X2 was extracted with 2 ml of methanol. The extract was evaporated to dryness, and the permethylated X2 thus purified was then hydrolyzed with 50 μl of 4 N trifluoroacetic acid at 100 °C for 3 h. The solution was freeze-dried, and the residue was pyridylaminated with 10 μl of 2-aminopyridine reagent and 35 μl of borane–dimethylamine complex reagent as reported (21). Excess reagents were evaporated three times with 50 μl of toluene:methanol (1:1 v/v) under a stream of nitrogen at 60 °C for 10 min (18). The residue was dissolved in 5 μl of the electrophoresis buffer, and small amounts of contaminating materials were removed by paper electrophoresis. Paper electrophoresis was performed at 30 V/cm at 4 °C using a filter paper (30 cm) and water:acetic acid:pyridine (60:2:3, v/v). The area that migrated like PA GlcNAc was cut off, and PA derivatives of partially O-methylated PA monosaccharides were extracted from the paper 4 times each with 100 μl of water. A part of the solution (10 μl) was analyzed by reversed-phase HPLC using Elution Condition 2.

**Mass Spectrometric Analysis**—Mass spectra were recorded using a Voyager-DE STR Biospectrometry Workstation, a matrix-assisted laser desorption ionization time of flight mass spectrometer (PerSeptive Biosystems, Framingham, MA). For mass spectrometry, PA sugar chains were dissolved in distilled water (10 pmol/μl). Aliquots of 0.5 μl were applied onto a sample plate. Subsequently, 0.5 μl of a matrix solution (10 mg/ml 2, 5-dihydroxybenzoic acid in 50% (v/v) acetonitrile) was mixed with the aliquot and allowed to dry. The analyzer was used in the linear mode.

**Nuclear Magnetic Resonance (NMR) Spectroscopy**—PA sugar chains were exchanged twice with 2H2O at room temperature with intermediate lyophilization and finally dissolved in 2H2O (99.999 atom % 2H; Isotec, Miamisburg, OH). Samples were analyzed at 30 or 50 °C on a Varian Unity Inova 750 MHz NMR spectrometer. Chemical shifts (δ) are expressed by reference to internal acetone (δ 2.213 ppm at 50 °C, δ 2.218 ppm at 30 °C).

**Equilibrium Dialysis**—A commercial HNK-1 monoclonal antibody was purified by gel filtration on a YMC-Pack Dia-200G column, and the IgM fraction was concentrated by ultrafiltration with an Amicon YM-30 membrane. Equilibrium dialysis was performed by heating with sodium hydroxide at 250 °C for 30 min. Neu5Ac was quantified by the method of Hara et al. (26). The value of PA Xyl was taken as 1.00.

**Other Analytical Methods**—The structures of the PA sugar chains were assessed by two-dimensional sugar mapping. A PA sugar chain was chromatographed by reversed-phase (Elution Condition 1) and size fractionation HPLC, and its elution position was compared with those of standard PA sugar chains on a two-dimensional sugar map. The PA sugar chain was then digested sequentially with exoglycosidases, and the structure of the product was analyzed on the two-dimensional sugar map as reported (23).

| Fraction | PA Xyl | Sulfate | Neu5Ac |
|----------|--------|---------|--------|
| X1       | 1.00   | 0.97    | 0.00   |
| X2       | 1.00   | 1.11    | 0.88   |

![Matrix-assisted laser desorption ionization mass spectra of Fraction X1 and X2.](image-url)
The reducing ends of PA sugar chains were analyzed according to the reported method (24). PA sugar chains were hydrolyzed with 100 μl of 4 M trifluoroacetic acid at 100 °C for 3 h in evacuated sealed tubes. The solution was evaporated to dryness by a centrifugal concentrator, and the residue was N-acetylated with saturated sodium bicarbonate solution and acetic anhydride. The PA monosaccharides obtained were separated and quantified by anion-exchange HPLC.

Component sugar analysis was done as reported (24). Glycopeptides were hydrolyzed with 100 μl of 4 M trifluoroacetic acid at 100 °C for 3 h. The solution was freeze-dried, and monosaccharides liberated were N-acetylated with saturated sodium bicarbonate solution and acetic anhydride. After desalting with Dowex 50 (H⁺), the solution was freeze-dried, and the monosaccharides were pyridylaminated with 2-aminopyridine and borane–dimethylamine complex. The excess reagents were removed under a stream of nitrogen gas with 40 ml of toluene at 40 °C. The residue was dissolved in a small amount of water, and a part of the solution was analyzed by anion-exchange HPLC.

Sulfate ion was measured by ion chromatography. A PA sugar chain (2 nmol) was mixed with 20 μl of 20 mM sodium hydroxide. After heating at 250 °C for 30 min, the resulting sulfate ion was measured with a Dionex 2021i ion chromatography system using an IonPac AS4A column and 2.8 mM Na₂CO₃, 2.25 mM NaHCO₃ at a flow rate of 1 ml/min (25). Sialic acid was measured by the reported method (26).

TABLE II

| Chemical shift | X1 50 °C | X2 50 °C | R1 30 °C | R2 30 °C | R3 ppm |
|---------------|---------|---------|---------|---------|--------|
| Xyl-1 H2      | 4.002   | 4.011   | 4.016   | 4.016   | 4.016  |
| Gal-2 H1      | 4.605   | 4.590   | 4.595   | 4.595   | 4.617  |
|               | 4.170   | 4.196   | 4.214   | 4.214   | 4.200  |
| Gal-3 H1      | 4.668   | 4.654   | 4.653   | 4.653   | 4.668  |
|               | 4.170   | 4.164   | 4.170   | 4.170   | 4.160  |
| GlcNAc H1     | 4.757   | 4.755   | 4.756   | 4.756   | 4.668  |
|               | 4.317   | 4.318   | 4.320   | 4.320   | 4.328  |
| Neu5Ac H3e    | 2.688   | 2.688   | 2.688   | 2.688   | 2.688  |
|               | 1.634   | 1.641   | 1.641   | 1.641   | 1.641  |
| NAc           | 2.012   | 2.012   | 2.012   | 2.012   | 2.012  |

RESULTS

Preparation and Analysis of a PA Sugar Chain Fraction from uTM—Glycopeptides were prepared from 374 mg of uTM by digestion with actinase E, and the digest was purified by gel filtration. After component analysis of each fraction, the fractions that contained sugars were combined (data not shown). Sugar chains were liberated from the combined fraction by hydrazinolysis followed by N-acetylation, and the reducing ends of the sugar chains were pyridylaminated. To obtain an overall view of the sugar structures, the reducing ends of the PA sugar chain fraction were first analyzed. The acid hydrolysates of the PA sugar chain fraction were found to contain 0.39 mol of PA Xyl, 3.0 mol of PA GlcNAc, and 0.45 mol of PA GalNAc, as major components. Small fractions of the PA sugar chain fraction were found to contain 0.39 mol pf PA Xyl, 3.0 mol of PA GlcNAc, and 0.45 mol of PA Gal. Other fractions and the fraction eluted between 25 and 60 ml did not contain appreciable amounts of PA Xyl. Other major peaks contained PA GalNAc, PA Gal, or PA GlcNAc as major components. Small amounts of contaminating materials were removed by size fractionation HPLC, and the final fractions were desalted on a Sephadex G-25 column (1 x 10 cm) using 10 mM ammonium acetate, pH 6.0, as an eluent. Starting from Fraction X, X1, and X2 were recovered at 17 and 26% (including losses during purification), respectively. X1 and X2 thus purified showed a single peak when analyzed by reversed-phase HPLC (data not shown) using Elution Condition 1 and size fractionation HPLC, indicating that X1 and X2 were pure.

Structure Analysis of X1—The fluorescent fragments obtained by partial acid hydrolysis (100 °C in 1 M trifluoroacetic acid for 15 min) of X1 appeared at the elution positions of GlcAβ1→3Galβ1→3Galβ1→4Xyl-PA, GlcAβ1→4Xyl-PA, and PA Xyl on a two-dimensional sugar map (Fig. 3), indicating that X1 contained the linkage tetrasaccharide structure of proteoglycans. X1 had 1 mol of sulfate but no sialic acid (Table 1). The elution position of X1 on the map was not changed by digestion with β-glucuronidase, chondro-4-sulfatase, or chondro-6-sulfatase (data not shown). When X1 was desulfated by methanolytic digestion, the product appeared at the position of GlcAβ1→3Galβ1→4Xyl-PA (Fig. 3). X1 was not digested with β-glucuronidase, but desulfated X1 was then susceptible to β-glucuronidase digestion. These results indicated that the sulfate group was linked to the GlcA-Gal structure. On further digestion of X1 with chondroitinase ABC (data not shown), the product appeared at the elution position of GlcAβ1→3Galβ1→4Xyl-PA (27). R1, SO₃-3GlcNAcβ1→4GlcAβ1→3Galβ1→4Xyl (27). R2, SO₃-3GlcNAcβ1→4GlcAβ1→3Galβ1→4Xyl (27).

The above findings were confirmed by measuring the molecular weight of X1 by mass spectrometry (Fig. 4). In the positive ion mass spectrum, the peak of the molecule-related ion, [M + Na]+ (m/z 831.2, calculated 831.2), was observed. Loss of sodium sulfite with hydrogen transfer from the cation also oc-
curred, resulting in an intense peak at m/z 729.2 (calculated 729.3). In the negative-ion mass spectrum also, the results verified the proposed structures of X1. The linkage position of the sulfate group was analyzed by 1H NMR spectroscopy. Reported data on the unsaturated linkage hexasaccharide of proteoglycans (ΔGlcAβ1→3GalNAcβ1→4GlcAβ1→3Galβ1→3Galβ1→4Xylol) (27), the synthetic trisaccharide, YM677 (SO₄-3GlcAβ1→3Galβ1→4GlcNAc) (28), and an oviducal mucin oligosaccharide (SO₄-3GlcAβ1→3Galβ1→4Galβ1→3GalNAcol) (29) provided the necessary reference data for assignment of the proton signals of X1 (Table II). The proton signal at 4.605 ppm was assigned to H1 of Gal-2 and that at 4.668 ppm, to H1 of Gal-3 as compared with those of the unsaturated linkage hexasaccharide (R1). Analysis of the two-dimensional NMR spectra of the SO₄-GlcA residues in X1 (Fig. 5) revealed identical spin patterns to the SO₄-3GlcA residues in X1. Therefore, the proton signals at 4.757 and 4.317 ppm were, respectively, assigned to H1 and H3 of SO₄-3GlcA, confirming that the HNK-1 disaccharide element was present in X1. Taking these results together with those given above, the structure of X1 was determined to be SO₄-GlcAβ1→3Galβ1→3Galβ1→4Xyl-PA.

Structure Analysis of X2—Partial acid hydrolysis of X2 gave the same fluorescent fragments as found for X1, indicating that X2 also contained the linkage tetrasaccharide structure. X2 had 1 mol each of a sulfate and Neu5Ac (Table I). The sialidase digest of X2 was eluted at the position of X1 on the two-dimensional sugar map (Fig. 3), indicating that X2 was a sia-

sialylated form of X1. The elution position of X2 on the map was also not changed by digestion with β-glucuronidase, chondro-4-sulfatase, or chondro-6-sulfatase (data not shown). When X2 was desulfated by methanolysis, the product appeared at position A (Fig. 3). Desulfated X2 was then susceptible to β-glucuronidase digestion, and the digest was eluted at position B. These results indicated that the sulfate group was linked to the GlcA-Gal structure. On further digestion of the β-glucuronidase digest with Aspergillus β-galactosidase, a new peak with a molecular size 0.9 glucose units smaller appeared at position C. The sialidase digest of the β-galactosidase digest was eluted at the position of Galβ1→4Xyl-PA, indicating that Neu5Ac was linked to the Gal-Xyl structure (Fig. 3). Methylation analysis of X2 indicated 3,6-disubstituted and 3-substituted Gal structures (Fig. 6). These results suggested that X2 was SO₄-GlcAβ1→3Galβ1→3(Neu5Acα2→6)Galβ1→4Xyl-PA. The structure was confirmed by measuring the molecular weight of X2 by mass spectrometry (Fig. 4). In the positive-ion mass spectrum, peaks of the molecule-related ions, [M + Na]⁺ (m/z 1122.2, calculated 1122.3) and [M + K⁺]⁺ (m/z 1138.5, calculated 1138.3), were observed. Loss of sodium sulfate with hydrogen transfer from these cations also occurred, resulting in an intense peak at m/z 1020.2 (calculated 1020.4). In the negative-ion mass spectrum also, the results verified the proposed structure of X2. The linkage position of the sulfate group was analyzed by 1H NMR in the same manner as for X1. Analysis of the two-dimensional NMR spectra of the SO₄-GlcA residue in X2 (Fig. 4) revealed identical spin patterns to the SO₄-GlcA residues in X1. Therefore, the proton signal at 4.756 ppm and that at 4.320 ppm were, respectively, assigned to H1 and H3 of SO₄-3GlcA, confirming that the HNK-1 disaccharide element was also present in X2. Three typical signals (H3e 2.688, H3a 1.634, and NAc 2.012 ppm) of the Neu5Ac residue were observed. The NMR spectra of X2 were almost the same as those of X1 besides Neu5Ac and H4 of Gal-2. The H4 signal of Gal-2

Fig. 5. Analysis of sugar structures of Fraction X1 and X2 by total correlated spectroscopy. Parts of the total correlated spectroscopy spectra of X1 (A) and X2 (B) are shown. Specific spin patterns observed for GlcA are shown by dashed lines.
the elution positions of the derivatives of PA galactose: 1, 3,4-di-O-methyl; 2, 2,3-di-O-methyl and 2,6-di-O-methyl; 3, 3,6-di-O-methyl; 4, 4,6-di-O-methyl; 5, 2,4-di-O-methyl; 6, 3,4,6-tri-O-methyl; 7, 2,3,6-tri-O-methyl; 8, 2,3,4-tri-O-methyl; 9, 2,4,6-tri-O-methyl; 10, 2,3,4,5-tetra-O-methyl. Arrow A indicates the elution position of the derivative obtained from the reducing-end PA xylose.

in X1 was shifted, indicating that Neu5Ac was linked to C6 of Gal-2. Taking these results together with those given above, the structure of X2 is SO₄⁻³GlcAβ1–3Galβ1–3Neu5Acα2–6Galβ1–4Xyl-Pa.

Binding Analysis of HNK-1 Antibody to X1 and X2—Double-reciprocal plots of the concentrations of the bound oligosaccharide (X1) versus those of the free oligosaccharide in equilibrium dialysis gave the binding constant of HNK-1 antibody to X1. The apparent association constant was 2.5 × 10⁶ M⁻¹. The binding constant of X2, 1 × 10⁶ M⁻¹, was smaller than that of X1. These analyses showed that Neu5Ac in X2 did not contribute to the binding to the antibody used.

DISCUSSION

Analysis of sugar chains from human uTM revealed the presence of oligosaccharides with xylose at the reducing end. On the basis of the results obtained with X1 and X2, the structures were determined to be linkage tetrasaccharides of proteoglycans with SO₄⁻³GlcA at the nonreducing end and with partial substitution of sialic acid. These are novel structures and to our knowledge are the first reported instance of a proteoglycan linkage tetrasaccharide with SO₄⁻³GlcA and sialic acid residues solely linked to a glycoprotein. Because X1 and X2 were liberated and their structures determined by chemical means, the possibility of another chemically labile substituent(s) cannot be excluded. uTM does not contain chondroitin sulfate (2–4), although TM produced in cultured human endothelial cells and recombinant TMs were expressed in both cases with a high molecular weight TM containing chondroitin sulfate and a low molecular weight TM lacking this modification (10). Platelet factor 4 binds to the glycanated form of TM but not to the chondroitin sulfate-lacking TM (12). Acceleration of the inhibition of thrombin by antithrombin III by TM is dependent upon the presence of chondroitin sulfate linked to TM (10). Hence, the binding of chondroitin sulfate seems to be important for several of the versatile functions of TM and to affect its cell-surface anticoagulant potential. Chondroitin sulfate is linked to Ser-474 in the high molecular weight TM of recombinant TM, but Ser-474 is also modified with a small substituent in the low molecular weight TM (9, 10). By way of explanation of the fact that TM is expressed in two distinct forms, Gerlitz et al. (9) postulated a model involving glycosyltransferase competition between xylosyltransferase and N-acetylgalactosaminyltransferase for Ser-474, whereas Lin et al. (10) suggested that occupation of the adjacent O-linked glycosylation may be important because of the steric hindrance for xylosyltransferase of the cell line.

If the new structures determined in the present paper link to a similar position, the following possibilities arise. The key step in producing the low molecular weight TM is the first GalNAc transfer reaction to the proteoglycan linkage tetrasaccharide regulated by sialation at 3C of GlcA, which leads to regulation of the cell-surface anticoagulant potential. This is compatible with the finding that GalNAc transferase catalyzing chondroitin chain elongation cannot transfer GalNAc to 3-O-sulfated GlcA (30). This sulfation may be accomplished either with a similar sulfotransferase, as reported for the detection of SO₄⁻³GlcAβ1–4Xylβ4-methylumbelliferone from the substrate, 4-methylumbelliferone β-Xyl (31), or with the sulfotransferase involved in the biosynthesis of the HNK-1 carbohydrate epitope (32, 33). These results suggest that glycosaminoglycan chain elongation of human TM seems to be abolished at the linkage tetrasaccharide core structure by addition of a sulfate group, indicating that 3-O-sulfation is a stop signal leading to TM lacking chondroitin sulfate.

Acknowledgments—We are grateful to Dr. K. Lee (Osaka University, Graduate School of Science) for operation of the Varian 750-MHz NMR spectrometer of the Venture Business Laboratory, Osaka University and Koutarou Hoshida for operation of the mass spectrometer.

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