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Structural Analysis of the Sugar Chains of Human Urinary Thrombomodulin[1]

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The sugar chains of human urinary thrombomodulin were studied. *N*- and *O*-linked sugar chains were simultaneously liberated by hydrazinolysis followed by *N*-acetylation and were tagged with 2-aminopyridine. Then the structures of the *N*- and *O*-linked pyridylamino (PA-) sugar chains were analyzed by two-dimensional sugar mapping combined with exoglycosidase digestion. The major *N*-linked sugar chains of human urinary thrombomodulin were found to be monosialo- and disialofucosylbiantennary chains, while the major *O*-linked sugar chain was ±Siaα2-3Galβ1-3(±Siaα2-6)GalNAc. Thrombomodulin also contained the reported structure SO_4-3GlcAβ1-3Galβ1-3(±Siaα2-6)Galβ1-4Xyl [H. Wakabayashi, S. Natsuka, T. Mega, N. Otsuki, M. Isaji, M. Naotsuka, S. Koyama, T. Kanamori, K. Sakai, and S. Hase (1999) *J. Biol. Chem.* 274, 5436–5442]. In addition to these sugar chains, a single Glc was linked to Ser 287.

Key words: *N*-linked sugar chain, *O*-linked sugar chain, thrombomodulin.

Thrombomodulin (TM) is a physiologically important anticoagulant (*1*) that is present not only on the surface of endothelial cells but also in the plasma and urine in soluble form (*2–5*). Takahashi *et al.* have purified the major active forms of human urinary thrombomodulin (uTM) and demonstrated their strong cofactor activity for the thrombin-catalyzed activation of protein C as well as potent anticoagulant activity *in vivo* (*6*). They have also demonstrated that uTM improves disseminated intravascular coagulation without excessive prolongation of the activated partial thromboplastin time (*7*). There are five potential *N*-linked glycosylation sites (*8*) based on the amino acid sequence of the protein, and the presence of *O*-linked sugar chains has been suggested by the detection of GalNAc (*9*). Some forms of recombinant TM and some of the TMs obtained from cultured human endothelial cells are reported to contain chondroitin sulfate (*10, 11*). Recombinant TM expressed in Chinese hamster ovary cells was also shown to contain chondroitin 4-sulfate (*12*). We have previously reported that uTM has a sulfated linkage tetrasaccharide but no glycosaminoglycan (*13*). The *N*-linked sugar chain structures of TM have been reported for a form of recombinant TM in which the amino acid sequence of the glycosaminoglycan attachment site was modified (*14*), but no information has been reported on the sugar chains of native TM. To understand the structure and biological activities of TM, detailed knowledge of its sugar moieties is essential. The present paper describes a detailed structural analysis of the sugar chains of uTM.

EXPERIMENTAL PROCEDURES

Materials—uTM was purified from human urine, as reported previously (*6*). Neuraminidase (*Arthrobacter ureafaciens*) was purchased from Nacalai Tesque (Kyoto), β-galactosidase (*Aspergillus* sp.) was obtained from Toyobo (Osaka), and β-galactosidase (*Xanthomonas manihotis*) was purchased from Bio Labs (New England). β-*N*-Acetylhexosaminidase (jack bean) and α-fucosidase (bovine epididymis) were obtained from Sigma (St. Louis), while β-*N*-acetylhexosaminidase (*Streptococcus pneumoniae*), β-galactosidase (*Streptococcus pneumoniae*), and endoproteinase Asp-N were obtained from Roche Diagnostics (Mannheim). α1-2Fucosidase (*Arthrobacter* sp.) and arginylendopeptidase were obtained from Takara Biomedicals (Kyoto), and protease V8 was purchased from Seikagaku (Tokyo).

The standard sugar chains SD1–2 were reported previously (*13*). SD3 was prepared from the corresponding sugar (*15*), SD4, 5, and 7 were prepared from human factor IX

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Abbreviations: Fuc, L-fucose; Gal, D-galactose; GalNAc, *N*-acetyl-D-galactosamine; Glc, D-glucose; GlcA, D-glucuronic acid; GlcNAc, *N*-acetyl-D-glucosamine; Man, D-mannose; Neu5Ac, *N*-acetylneuraminic acid; PA-, pyridylamino; Sia, sialic acid; TalNAc, *N*-acetyl-D-talosamine; TM, thrombomodulin; UEA-I, *Ulex europaeus* lectin-I; uTM, urinary thrombomodulin; Xyl, D-xylose. The structures and abbreviations of the authentic PA-sugar chains used in this study are shown in Table I.

TABLE I. **Structures and elution positions of the authentic PA-sugar chains used in this study.**

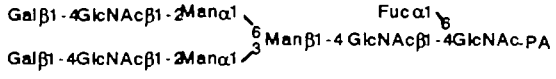
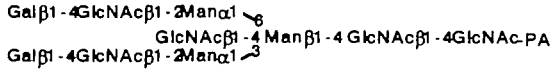
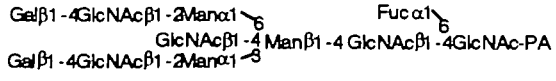
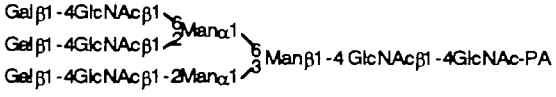
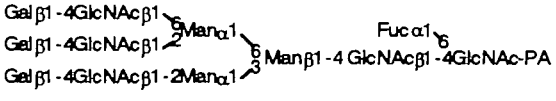
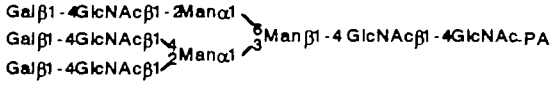
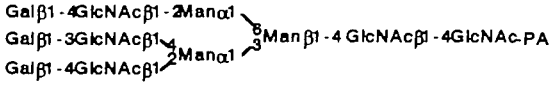
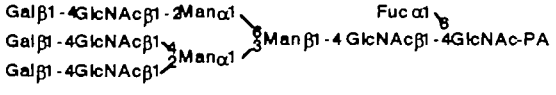
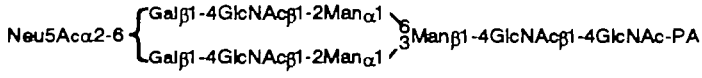
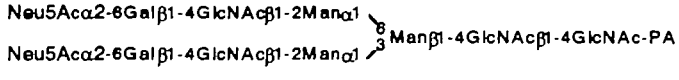
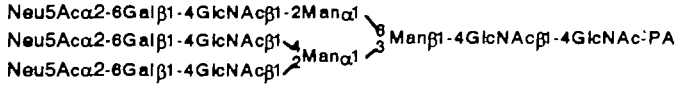
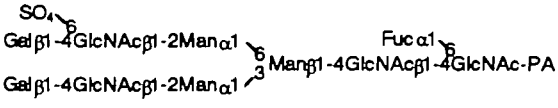
SD	Structure	Reversed-phase[a]		Size fractionation[b]
		RP-1	RP-2	SF-1
1	SO_4-3GlcAβ1-3Galβ1-3Galβ1-4Xyl-PA	0.48	–	–
2	Neu5Acα2, 6 / SO_4-3GlcAβ1-3Galβ1-3Galβ1-4Xyl-PA	0.33	–	–
3	Galβ1-3GalNAc-PA	1.00	–	1.7
4	Neu5Acα2, 6 / Galβ1-3GalNAc-PA	–	–	–
5	Neu5Acα2-3Galβ1-3GalNAc-PA	–	–	–
6	Neu5Acα2-3Gal-PA	–	–	–
7	Neu5Acα2, 6 / Neu5Acα2-3Galβ1-3GalNAc-PA	–	–	–
8	Manα1, 6 / Manα1, 3 / Manβ1-4GlcNAcβ1-4GlcNAc-PA	–	0.81	4.4
9	GlcNAcβ1-2Manα1, 6 / Manα1, 3 / Manβ1-4GlcNAcβ1-4GlcNAc-PA	–	1.00	5.3
10	Manα1, 6 / GlcNAcβ1-4Manα1, 3 / Manβ1-4GlcNAcβ1-4GlcNAc-PA	–	0.89	5.0
11	GlcNAcβ1-2Manα1, 6 / GlcNAcβ1-2Manα1, 3 / Manβ1-4GlcNAcβ1-4GlcNAc-PA	–	0.92	6.3
12	GlcNAcβ1-2Manα1, 6 / GlcNAcβ1-4 / GlcNAcβ1-2Manα1, 3 / Manβ1-4GlcNAcβ1-4GlcNAc-PA	–	1.16	6.5
13	GlcNAcβ1, 6 / GlcNAcβ1, 2 Manα1 / GlcNAcβ1-2Manα1, 3 / Manβ1-4GlcNAcβ1-4GlcNAc-PA	–	0.77	7.1
14	GlcNAcβ1-2Manα1, 6 / GlcNAcβ1, 4 / GlcNAcβ1, 2 Manα1, 3 / Manβ1-4GlcNAcβ1-4GlcNAc-PA	–	1.05	6.8
15	Galβ1-4GlcNAcβ1-2Manα1, 6 / Galβ1-4GlcNAcβ1-2Manα1, 3 / Manβ1-4GlcNAcβ1-4GlcNAc-PA	–	1.00	7.9
16	Galβ1-4GlcNAcβ1-2Manα1, 6 / GlcNAcβ1-2Manα1, 3 / Fucα1, 6 / Manβ1-4GlcNAcβ1-4GlcNAc PA	–	1.12	7.4
17	GlcNAcβ1-2Manα1, 6 / Galβ1-4GlcNAcβ1-2Manα1, 3 / Fucα1, 6 / Manβ1-4GlcNAcβ1-4GlcNAc PA	–	1.12	7.5

[a]Expressed as the relative elution time to SD3 (RP-1) or to SD15 (RP-2). [b]Expressed as glucose units. [c]See Table IV.

(*16*), SD6 was prepared from recombinant macrophage colony-stimulating factor (*17*), and SD29 was prepared from bovine lung (Murakami, T., Makino, Y., Natsuka, S., and Hase, S., manuscript in preparation). SD8–11, 13–15, 18, and 22–28 were purchased from Takara Biomedicals, and SD16–17 were obtained from Seikagaku. SD12 and SD19–20 were prepared from the corresponding sugar chains, which were purchased from Seikagaku (SD12) or Oxford GlycoScience (Abingdon, UK) (SD19–20). SD21 was prepared by digestion of SD22 with α-fucosidase.

A Cosmosil 5C18-AR300 column (0.6 × 15 cm) and a Cosmosil 5C18-P column (0.15 × 25 cm) were purchased from Nacalai Tesque; a Shodex NH2P-50 column (0.46 × 5 cm, 0.2 × 15 cm) was obtained from Showa Denko (Tokyo); and Toyopearl HW-40F, a TSK gel Sugar AXI column (0.46 × 15 cm) and a DEAE-5PW column (0.75 × 7.5 cm) were pur-

TABLE I (continued)

SD	Structure	Reversed phase[a] RP-1	Reversed phase[a] RP-2	Size fractionation[b] SF-1
18		–	1.16	8.2
19		–	1.24	8.0
20		–	1.44	8.4
21		–	0.78	9.3
22		–	0.94	9.7
23		–	1.15	9.3
24		–	1.16	9.3
25		–	1.33	9.5
26		–	–	–
27		–	–	–
28		–	–	–
29		–[c]	–[c]	–[c]

chased from Tosoh (Tokyo).

Preparation of PA-Sugar Chains from uTM—*N*- and *O*-linked sugar chains were simultaneously liberated from uTM (2 mg) by hydrazinolysis with 0.2 ml of anhydrous hydrazine (60°C, 50 h) followed by *N*-acetylation with 0.4 ml of a $NaHCO_3$ saturated solution and 16 μl of acetic anhydride. After the reaction, excess reagents were removed by use of Dowex 50W X2 (H^+), as reported elsewhere (*15*). After pyridylamination with 20 μl of a pyridylamination reagent (90°C for 60 min) and 70 μl of a reducing reagent (80°C for 35 min), excess reagents were removed by evaporation with triethylamine:methanol:toluene (1:3:4) as reported previously (*15*). The products were finally purified by chromatography on a HW-40F (1.0 × 45 cm) column using 0.01 M ammonium acetate buffer (pH 6.0).

Preparation of Asialo PA-Sugar Chains—Half of the amount of PA-sugar chains obtained from uTM was digested with 0.2 unit of neuraminidase in 200 μl of 100 mM ammonium acetate buffer, pH 5.0, at 37°C for 16 h. The enzymatic reaction was terminated by heating at 100°C for 3 min. Asialo PA-sugar chains were purified by anion exchange HPLC using a DEAE-5PW column. The flow-through fraction was used as asialo PA-sugar chains, and the fraction eluted with 0.5 M ammonium acetate buffer, pH 6.0, was pooled as the neuraminidase-resistant fraction.

HPLC of PA-Sugar Chains—HPLC was carried out under the following conditions. Elution conditions RP-1 for PA-*O*–linked sugar chains: a Cosmosil 5C18-AR300 column was used at a flow rate of 2 ml/min at 25°C. The eluent was

20 mM ammonium acetate buffer, pH 6.0, containing 0.01% 1-butanol. After injecting a sample, the butanol concentration was increased linearly from 0.01 to 0.52% in 51 min, then to 1.0% in 12 min. Elution conditions RP-2 for PA-*N*–linked sugar chains: a Cosmosil 5C18-AR300 column was used at a flow rate of 2 ml/min at 25°C. The eluent was 20 mM ammonium acetate buffer, pH 4.0, containing 0.03% 1-butanol. After injecting a sample, the 1-butanol concentration was increased linearly from 0.03 to 0.45% in 45 min. Elution conditions RP-3: a Cosmosil 5C18-P column was used, and the eluent was 20 mM ammonium acetate buffer, pH 4.0, containing 0.075% 1-butanol. After injecting a sample, the 1-butanol concentration was increased linearly from 0.075 to 0.4% in 90 min. The flow rate was 0.15 ml/min.

Elution conditions SF-1: a Shodex NH2P-50 column (0.46 × 5 cm) was used at a flow rate of 0.6 ml/min at 25°C. Two eluents (A and B) were used. Eluent A was acetonitrile:water:acetic acid (930:70:3 v/v/v) titrated to pH 7.0 with ammonia water, and Eluent B was acetonitrile:water:acetic acid (200:800:3 v/v/v) titrated to pH 7.0 with ammonia water. The column was equilibrated with Eluent A:B (97:3 v/v). After injecting a sample, linear gradient elution was performed to Eluent A:Eluent B (67:33 v/v) in 1 min, then to Eluent A:Eluent B (29:71 v/v) in 34 min. Elution conditions SF-2: a Shodex NH2 P-50 column (0.2 × 15 cm) was used at a flow rate of 0.1 ml/min at 25°C. Two eluents (C and D) were used. Eluent C was acetonitrile:water:acetic acid (90:10:3 v/v/v) titrated to pH 7.3 with triethylamine, and Eluent D was acetonitrile:water:acetic acid (20:80:3 v/v/v) titrated to pH 7.3 with triethylamine. The column was equilibrated with Eluent C:Eluent D (95:5 v/v). After injecting a sample, linear gradient elution was performed to Eluent C:Eluent D (86:14 v/v) in 3 min, followed by Eluent C:Eluent D (73:27 v/v) in 17 min, Eluent C:Eluent D (52:48 v/v) in 59 min, and Eluent C:Eluent D (25:75 v/v) in 6 min.

Separation of sialylated sugar chains was done with a TSK gel DEAE-5PW column at a flow rate of 0.5 ml/min at 25°C. The eluent was 1 mM ammonium acetate buffer, pH 6.0. After injecting a sample, the concentration of ammonium acetate was increased linearly to 0.2 M in 40 min, then to 0.5 M in 10 min.

PA-sugar chains were detected by fluorescence, with an excitation wavelength of 320 nm and an emission wavelength of 400 nm for elution conditions RP-2 and RP-3, and an excitation wavelength of 310 nm and an emission wavelength of 380 nm for the other elution conditions.

Reducing-End PA-Monosaccharide Analysis—Analysis of the reducing ends of PA-sugar chains was done according to the reported method after hydrolysis with 4 M hydrochloric acid at 100°C for 8 h followed by *N*-acetylation. The PA-monosaccharides thus obtained were separated and quantified by anion-exchange HPLC (*18*).

Exoglycosidase Digestion—A PA-sugar chain (100 pmol) was digested with 100 milliunits (in the case of AS3, 20 U) of β-galactosidase (*Aspergillus*) in 20 μl of 50 mM ammonium acetate buffer, pH 4.5; with 10 milliunits of α-fucosidase in 50 mM sodium citrate buffer, pH 6.5; with 0.5 milliunits of β-galactosidase (*Streptococcus*) in 50 mM ammonium acetate buffer, pH 6.0; with 50 milliunits of β-*N*-acetylhexosaminidase (jack bean) in 50 mM sodium citrate buffer, pH 5.0; with 6 units of β-galactosidase (*Xanthomonas*), which preferentially hydrolyzes a Galβ1-3 linkage, in 50 mM sodium acetate buffer, pH 4.5; with 5 milliunits of β-*N*-acetylhexosaminidase (*Streptococcus*) in 50 mM sodium citrate buffer, pH 5.0; and with 200 milliunits of α1-2fucosidase in 90 mM sodium phosphate buffer, pH 8.5. One-fifth of each fraction of the sialylated sugar chains separated by the DEAE-5PW column was treated with 40 milliunits of neuraminidase in 50 μl of 100 mM ammonium acetate buffer, pH 5.0.

Lectin Affinity Chromatography—An aliquot of PA-sugar chain (20 pmol) was injected into an ice-cold UEA-I-agarose column (0.8 × 3 cm) equilibrated with 10 mM Tris-HCl buffer, pH 7.4, containing 0.1 M NaCl plus 1 mM $CaCl_2$, $MgCl_2$, and $MnCl_2$. The column was washed with the same solution, then the absorbed PA-sugar chains were eluted with the same solution containing 200 mM Fuc at a flow rate of about 30 μl/min. PA-sugar chains were detected by fluorescence with excitation at 310 nm and emission at 380 nm.

Two-Dimensional Sugar Mapping—The structures of the PA-sugar chains were assessed by two-dimensional sugar mapping. The elution positions of 93 standard PA-*N*–linked sugar chains have already been reported, and the introduction of a reversed-phase scale made it possible to predict the elution positions even if standard PA-*N*–linked sugar chains were not available (*19*). PA-sugar chains were separated by reversed-phase HPLC and size-fractionation HPLC, and the elution position of each chain was compared with those of standard PA-sugar chains on the two-dimensional sugar map. Then each PA-sugar chain was digested sequentially with exoglycosidases, and the structures of the products were analyzed on the two-dimensional sugar map as reported previously (*20, 21*).

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 (PE Biosystems, CA, USA). The matrix was 2,5-dihydroxybenzoic acid (10 mg/ml) for PA-sugar chains or sinapinic acid for peptides (10 mg/ml) in an aqueous solution of acetonitrile containing 0.1% (v/v) of trifluoroacetic acid. Analysis was done in the linear mode.

Peptide Analysis of uTM—uTM (5 mg/ml) in 8 M guanidine hydrochloride–0.1 M Tris-HCl buffer, pH 8.0, containing 5 mM EDTA was reduced by incubation with 3.6% (v/v) 2-mercaptoethanol for 2 h, and the products were alkylated with 10.5 mg of iodoacetic acid for 2 h in the dark. The buffer was changed to 50 mM phosphate buffer, pH 8.0, and arginylendopeptidase was added at an enzyme/substrate ratio of 1:50 (w/w). Then the solution was incubated at 37°C for 16 h. The digested peptides were separated by reversed-phase HPLC and some of them were further digested with endoproteinase Asp-N or protease V8. The amino acid sequences of the isolated peptides were analyzed with a Procise 473A or 476A protein sequencer (PE Biosystems).

Sugar Composition Analysis of Peptides—Peptides were hydrolyzed with the vapor of 4 M trifluoroacetic acid at 100°C for 4 h. The hydrolyzates were *N*-acetylated and pyridylaminated, as described previously (*18*), and quantified by the reported method (*22*).

RESULTS

Reducing-End Analysis of PA-Sugar Chains Prepared

From uTM—PA-sugar chains were prepared from uTM as described in "EXPERIMENTAL PROCEDURES." To obtain an overview of the sugar structures of uTM, the reducing-end PA-monosaccharides obtained by acid hydrolysis of the PA-sugar chains were analyzed. PA-GlcNAc, PA-GalNAc, and PA-Xyl were detected, and these PA-monosaccharides seemed to be derived from *N*-linked sugar chains, mucin type sugar chains, and sulfated linkage tetrasaccharides (*13*), respectively (data not shown). In addition, PA-Gal and PA-Glc were detected. PA-Gal was probably a by-product of the liberation of Galβ1-3GalNAc-Ser/Thr type sugar chains, as reported previously (*15*). PA-Glc, which was found as PA-monosaccharide in the PA-sugar chain fraction obtained from uTM, was from the Glc-Ser type of sugar chain, as described below.

Separation of Asialo PA-Sugar Chains by DEAE-HPLC—Half of the amount of each PA-sugar chain obtained was digested with neuraminidase, and the product was separated by DEAE-HPLC (data not shown). The flow-through fraction (AS) and the adsorbed fraction (SR) were obtained. Reducing-end PA-monosaccharide analysis of these fractions indicated that Fraction AS contained 85% of

TABLE II. **Structural analysis of the PA-sugar chains in Fractions AS and SR by two-dimensional HPLC mapping and exoglycosidase digestion.**

Fraction	Exoglycosidase	Reducing-end PA-sugar	Identification of PA-sugar chains on the 2-dimensional sugar map	Elution Position		
				Reversed-phase[a]		Size fractionation[b]
				RP-1	RP-2	
AS3	No treatment	PA-GalNAc	SD3	0.96		1.7
	Aspergilus β-galactosidase		PA-GalNAc	1.35		-[d]
AS4	No treatment			1.18		1.5
AS7	No treatment	PA-ManNAc		2.15	0.87	8.2
AS8	No treatment	PA-GlcNAc	SD22	2.32	0.93	9.6
	α-fucosidase		SD21		0.80	9.3
	β-galactosidase (*Streptococcus*)		SD13		0.77	7.1
	β-*N*-acetylhexosaminidase (Jack bean)		SD8		0.81	4.4
AS10	No treatment	PA-GlcNAc		2.59	1.10	8.3
	β-galactosidase (*Aspergilus*) digest of AS10				1.04	7.5
	β-galactosidase (*Xanthomonas*) digest of AS10		SD16, SD17		1.10	7.4
AS11	No treatment	PA-GlcNAc	SD18	2.71	1.15	8.2
	α-fucosidase		SD15		1.00	7.9
	β-galactosidase (*Streptococcus*)		SD11		0.93	6.3
	β-*N*-acetylhexosaminidase (Jack bean)		SD8		0.81	4.4
AS12	No treatment	PA-GlcNAc		2.85	1.25	8.6
	α1-2fucosidase		SD18		1.16	8.2
	β-galactosidase (*Streptococcus*)				1.22	7.8
	β-*N*-acetylhexosaminidase (Jack bean)				1.30	6.9
	α-fucosidase				1.04	6.1
	β-galactosidase (*Streptococcus*)		SD9		0.99	5.3
AS13	No treatment	PA-GlcNAc	SD25	2.94	1.33	9.5
	α-fucosidase		AS13a + AS13b		1.15+1.17	
AS13a	(α-fucosidase digest of AS13)		SD23		1.15	9.2
	β-galactosidase (*Streptococcus*)		SD14		1.06	6.9
	β-*N*-acetylhexosaminidase (Jack bean)		SD8		0.81	4.4
AS13b	(α-fucosidase digest of AS13)		SD24		1.17	9.2
	β-galactosidase (*Streptococcus*)				1.10	7.7
	β-*N*-acetylhexosaminidase (Jack bean)				0.92	5.9
	β-galactosidase (*Xanthomonas*)		SD10		0.89	5.0
AS14	No treatment	PA-GlcNAc	SD20	3.29	1.43	8.3
	α-fucosidase		SD19		1.22	8.0
	β-galactosidase (*Streptococcus*)		SD12		1.16	6.6
	β-*N*-acetylhexosaminidase (*Streptococcus*)		SD8		0.81	4.4
SR1	No treatment	PA-Xyl	SD1	0.48		-[c]
SR2[e]		PA-GlcNAc	SD29			

[a]Expressed as the relative elution times to SD3 (RP-1) or to SD15 (RP-2). [b]Expressed as glucose units. [c]SR1 was not eluted from the NH2P-50 column because of its negatively charged sulfate group and GlcA. [d]Not done. [e]See Table IV.

total PA-GlcNAc, 91% of total PA-GalNAc, and 92% of total PA-Glc, while Fraction SR contained 12% of PA-GlcNAc and 69% of PA-Xyl (loss during fractionation was not considered). Detection of PA-GlcNAc in Fraction SR indicated that some of the PA-*N*–linked sugar chain(s) contained acidic groups other than sialic acid.

Separation of PA-Sugar Chains by Reversed-Phase HPLC—Fractions AS and SR were each further fractionated by reversed-phase HPLC (Fig. 1). Reducing-end PA-monosaccharide analysis of each peak indicated that the underlined fraction contained PA-sugar chains, so the structures of the other peaks were not studied further.

Structural Analysis of Fraction AS—Fraction AS1 was PA-Gal, which was probably a by-product as described above. AS2 was a mixture of PA-Glc and PA-Man; but PA-Man was not reproducibly detected and was thus considered to be a contaminant. AS3 was identified as SD3 based on the results shown in Table II. AS4 was eluted at the

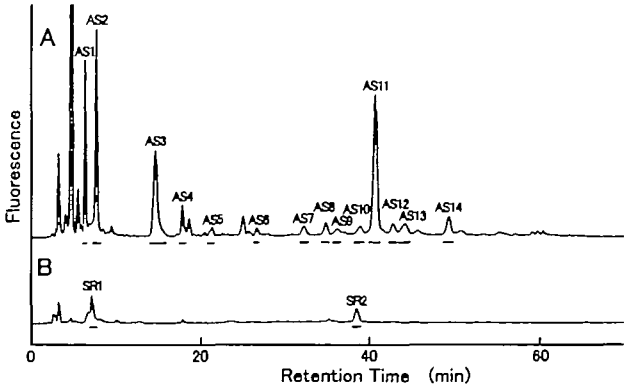


Fig. 1. **Separation of Fractions AS (A) and SR (B) by reversed-phase HPLC.** Elution conditions RP-1 were used. Peaks indicated by the bars were collected for further analysis.

TABLE III. **Proposed structures of neuraminidase-digested PA-sugar chains obtained from uTM.**

Fraction	Structure	Relative amount[a]
O-linked sugar chain		
AS1	PA-Gal	24.0
AS2	PA-Glc	37.9
AS3	Galβ1-3GalNAc-PA	31.4
SR1	SO_4-3GlcAβ1-3Galβ1-3Galβ1-4Xyl-PA	6.8
N-linked sugar chain		
AS8	Galβ1-4GlcNAcβ1, Galβ1-4GlcNAcβ1, Galβ1-4GlcNAcβ1-2Manα1 → 2Manα1 → 3,6Manβ1-4GlcNAcβ1-4GlcNAc-PA; Fucα1-6	4.8
AS10	Galβ1-3 / Galβ1-4 GlcNAcβ1-2Manα1 → 3,6Manβ1-4GlcNAcβ1-4GlcNAc-PA; Fucα1-6	4.0
AS11	Galβ1-4GlcNAcβ1-2Manα1, Galβ1-4GlcNAcβ1-2Manα1 → 3,6Manβ1-4GlcNAcβ1-4GlcNAc-PA; Fucα1-6	64.7
AS12	Fucα1-2Galβ1-4GlcNAcβ1-2Manα1, Galβ1-4GlcNAcβ1-2Manα1 → 3,6Manβ1-4GlcNAcβ1-4GlcNAc-PA; Fucα1-6	5.3
AS13a	Galβ1-4GlcNAcβ1-2Manα1, Galβ1-4GlcNAcβ1 → 2Manα1, Galβ1-4GlcNAcβ1 → 3,6Manβ1-4GlcNAcβ1-4GlcNAc-PA; Fucα1-6	7.1
AS13b	Galβ1-4GlcNAcβ1-2Manα1, Galβ1-3GlcNAcβ1 → 4Manα1, Galβ1-4GlcNAcβ1 → 3,6Manβ1-4GlcNAcβ1-4GlcNAc-PA; Fucα1-6	
AS14	Galβ1-4GlcNAcβ1-2Manα1 → 6, GlcNAcβ1-4, Galβ1-4GlcNAcβ1-2Manα1 → 3 Manβ1-4GlcNAcβ1-4GlcNAc-PA; Fucα1-6	8.7
SR2	SO_4-6Galβ1-4GlcNAcβ1-2Manα1, Galβ1-4GlcNAcβ1-2Manα1 → 3,6Manβ1-4GlcNAcβ1-4GlcNAc-PA; Fucα1-6	5.3

[a]Relative amounts of *O*- and *N*-linked sugar chains are expressed separately based on the peak areas in reversed-phase HPLC.

same position as AS3 by size-fractionation HPLC, and the reducing end PA-monosaccharide (probably PA-TalNAc) was eluted slightly earlier than PA-GalNAc by Sugar AXI column chromatography. Based on these results, AS4 seemed to be the epimerization product of AS3 (*15*). When AS5 and AS6 were treated with 1 M ammonia water at 100°C for 30 min, PA-Glc and PA-Gal were obtained, respectively. Therefore, these fractions were *O*-acetylated PA-sugars that were considered to be by-products.

The reducing end of AS7 was PA-ManNAc, and AS7 was eluted at the same position as AS11 by size-fractionation HPLC. Therefore, AS7 was probably the epimerization product of AS11 during hydrazinolysis, as already reported (*23*). AS9 was further separated into four peaks by reversed-phase HPLC, but these sugar chains were not analyzed because they were present in such small amounts. AS8, AS11, and AS14 were eluted at the same positions as SD22, SD18, and SD20, respectively. Digestion of AS8, AS11, and AS14 with α-fucosidase, β-galactosidase (*Streptococcus*), and β-*N*-acetylhexosaminidase (jack bean or *Streptococcus*) yielded the expected products, as shown in Table II. AS10 was eluted near the elution position of SD18, and MALDI-TOF mass analysis showed a quasi-molecular peak $[M+H]^+$ (*m/z* 1,867.4, calculated for fucosylbiantenna 1,866.8). As one Gal residue of AS10 was hydrolyzed by either β-galactosidase (*Aspergillus*) or β-galactosidase (*Xanthomonas*), which preferentially hydrolyzes β1-3Gal residues, it seemed that one Gal residue was bound by a β1-3 linkage and the other by a β1-4 linkage (Table II). The digest obtained with β-galactosidase (*Xanthomonas*) was eluted at the same position as SD16 and SD17. Based on these findings, the proposed structure of AS10 is shown in Table III. AS12 was not eluted at the position of any standard, and MALDI-TOF mass analysis showed a quasi-molecular peak $[M+H]^+$ (*m/z* 2,013.8, calculated for difucosylbiantenna 2,012.9). When AS12 was hydrolyzed with α1-2fucosidase, the product was eluted at the same position as SD18 (Table II). AS12 bound to the UEA-I-agarose column, which recognized the Fucα1-2Galβ1-4GlcNAc structure. When AS12 was successively digested with β-galactosidase (*Streptococcus*), β-*N*-acetylhexosaminidase (jack bean), α-fucosidase, and β-galactosidase (*Streptococcus*), the product was eluted at the same position as SD9. These results indicated that the Fuc residue was bound to the Gal residue on the Manα1-6 branch by an α1-2 linkage, and the proposed structure of AS12 is shown in Table III. AS13 was eluted at the same position as SD25. However, two peaks (AS13a and AS13b) were obtained by reversed-phase HPLC after digestion with α-fucosidase. The structures of these sugar chains were analyzed separately (as shown in Table II), and the proposed structures of AS13a and AS13b are shown in Table III.

Structural Analysis of Fraction SR—In Fraction SR, PA-monosaccharides were only detected in the acid hydrolyzates of Fractions SR1 and SR2 (Fig. 1). As the reducing

TABLE IV. **Structural analysis of SR2 by two-dimensional HPLC mapping.** Elution conditions RP-3 were used for reversed-phase HPLC and SF-2 were used for size-fractionation HPLC.

	Reversed-phase[a]	Size fractionation[b]
No treatment		
SR2	1.47	9.5
SD29	1.47	9.5
After digestion with β-galactosidase (*Streptococcus*)		
SR2	1.42	8.5
SD29	1.42	8.5

[a]Expressed as the relative elution time to SD15. [b]Expressed as glucose units.

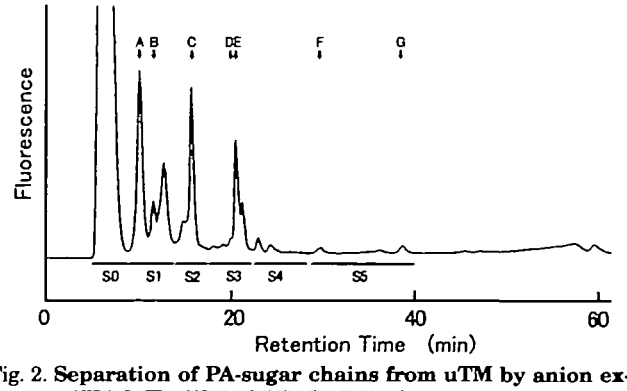


Fig. 2. **Separation of PA-sugar chains from uTM by anion exchange HPLC.** The TSKgel DEAE-5PW column was used. Abbreviations and structures are given in Table I. Arrows indicate the elution positions of standard PA-sugar chains: A, SD26; B, SD4 and SR2; C, SD27; D, SD28; E, SD7; F, SD1; G, SD2.

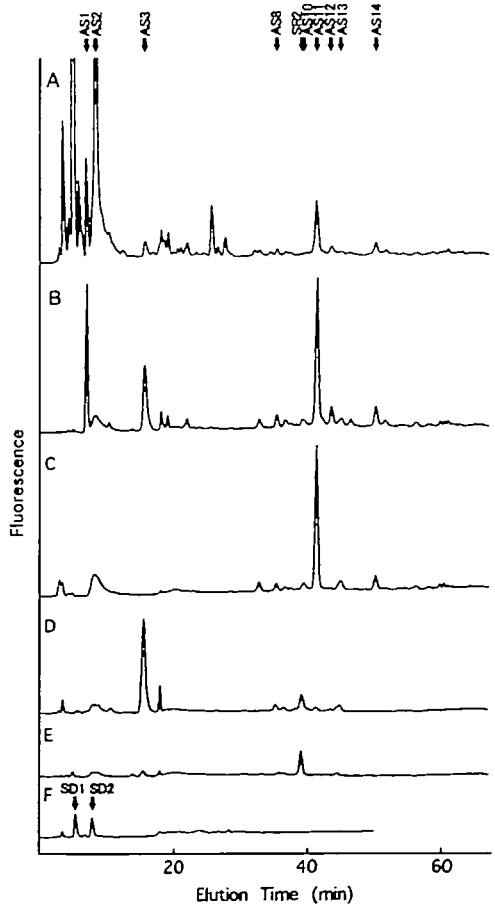


Fig. 3. **Separation by reversed-phase HPLC of S5, and the digests of Fractions S0–S4 with neuraminidase.** A, HPLC profile of S0; B, neuraminidase-digested S1; C, neuraminidase-digested S2; D, neuraminidase-digested S3; E, neuraminidase-digested S4; F, S5. Elution conditions RP-1 were used. Arrows indicate the elution positions of asialo sugar chains prepared from uTM (Table III).

end of SR1 was PA-Xyl, and SR1 was eluted at the same position as SD1 by reversed-phase HPLC, SR1 seemed to be SO_4-3GlcAβ1-3Galβ1-3Galβ1-4Xyl-PA. The reducing end of SR2 was PA-GlcNAc. SR2 and a product obtained by digestion with β-galactosidase (*Streptococcus*) were eluted at the same position as SD29 and its digestion product (Table IV). The expected data were obtained by mass analysis using the negative ion mode $[M-H]^-$ (m/z 1,945.7, calculated 1,944.8) and the positive ion mode $[(M-SO_3)+H]^+$ (m/z 1,867.6, calculated 1,866.8). Based on these results, the proposed structure of SR2 is as shown in Table III.

Structural Analysis of Sialylated Sugar Chains—PA-sugar chains were fractionated according to the number of anionic charges using the standard PA-sugar chains, and six fractions (Fractions S0–S5) were obtained (Fig. 2). Each fraction except for S5 was digested with neuraminidase, and the structures of the desialylated PA-sugar chain and S5 were analyzed by reversed-phase HPLC (Fig. 3). The results are summarized in Table V. Sialylated SR2 appeared in Fractions S3 and S4. MALDI-TOF mass analysis revealed a quasi-molecular peak corresponding to monosialo SR2 $[M-H]^-$ (m/z 2,236.3, calculated 2,237.1) in Fraction S3 and a quasi-molecular peak corresponding to disialo SR2 $[M-H]^-$ (m/z 2,527.9, calculated 2,528.3) in Fraction

TABLE V. **Proposed structures for the sugar chains of uTM.**

Structure	Relative amount[a] asialo	mono-sialo	di-sialo	tri-sialo
O-linked sugar chain				
(Gal)[b]	(9.2)[b]	(13.7)[b]	(0.0)[b]	(0.0)[b]
Glc	42.8	0.0	0.0	0.0
Galβ1-3GalNAc	2.6	11.4	16.0	0.0
SO_4-3GlcAβ1-3Galβ1-3Galβ1-4Xyl	1.9	2.4	0.0	0.0
N-linked sugar chain				
Galβ1-4GlcNAcβ1-2Manα1-6 Galβ1-4GlcNAcβ1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc Fucα1-6	11.3	27.7	24.3	0.0
Galβ1-4GlcNAcβ1-2Manα1-6 GlcNAcβ1-4 Galβ1-4GlcNAcβ1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc Fucα1-6	2.5	3.7	2.5	0.0
SO_4-6 Galβ1-4GlcNAcβ1-2Manα1-6 Galβ1-4GlcNAcβ1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc-PA Fucα1-6	0.0	3.5	3.9	0.0
Fucα1-2Galβ1-4GlcNAcβ1-2Manα1-6 Galβ1-4GlcNAcβ1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc-PA Fucα1-6	1.9	3.8	0.0	0.0
Galβ1-3 {GlcNAcβ1-2Manα1-6 Galβ1-4 {GlcNAcβ1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc Fucα1-6	0.0	2.1	1.4	0.0
Galβ1-4GlcNAcβ1-2Manα1-6 Galβ1-4or3GlcNAcβ1-4 Galβ1-4GlcNAcβ1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc Fucα1-6	0.7	2.1	2.3	1.3
Galβ1-4GlcNAcβ1-6 Galβ1-4GlcNAcβ1-2Manα1-6 Galβ1-4GlcNAcβ1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc Fucα1-6	1.0	2.1	1.0	1.0

[a]Relative amounts of *O*- and *N*-linked sugar chains were calculated separately. Values are expressed as percentages based on the total peak areas in reversed-phase HPLC. [b]Gal was probably a by-product (see text).

S4. These results showed that Fraction S3 contained monosialo SR2, while Fraction S4 contained disialo SR2. Asialo SR2, which would be eluted in Fraction S2, was not found in uTM.

Structural Analysis of Glc Type Sugar Chains—On amino acid sequence analysis of peptidase digests of uTM isolated by reversed-phase HPLC, blank cycles were seen for the Asn residues of the *N*-glycosylation sites (Asn 29, Asn 97/Asn 98, Asn 364, and Asn 391). In addition to these glycosylation sites, unknown peaks were detected at the position of Ser 287. Mass analysis of a peptide including Ser 287 (Asp 283–Arg 297) suggested the binding of a hexose [peptide + hexose]$^-$ (m/z 1,974.7, calculated 1,975.1). This hexose was identified to Glc by sugar composition analysis of the peptide. These results indicated that a single Glc was linked to Ser 287, which was consistent with the finding of sugar chain structural analysis that Glc was detected as a monosaccharide.

DISCUSSION

We have already reported on the novel proteoglycan linkage tetrasaccharide sugar chains of uTM (*13*). In the present study, we determined the structures of its other *O*-linked and *N*-linked sugar chains. The major *N*-linked sugar chains were monosialo and disialofucosylbiantennary sugar chains, while the major *O*-linked sugar chain was ±Siaα2-3Galβ1-3(±Siaα2-6)GalNAc. In addition to these typical sugar chains, uTM had some other *N*-linked fucosylbiantennary sugar chains that were modified at the nonreducing end. Such structures have already been found in serum proteins: the Galβ1-3 structure has been found in bovine prothrombin (*24*), the Fucα1-2Gal structure in human factor VIII (*25*), and the Galβ1-4(SO_4-6)GlcNAc structure in human thyroglobulin (*26*). And *N*-linked sugar chains of uTM were all characterized by the presence of (α1,6)-core fucosylation.

uTM also had a single Glc linked to Ser 287 located in the second epidermal growth factor-like domain. This seems to be a kind of $(Xyl)_n$-Glc-Ser type sugar chain like those found in epidermal growth factor-like domains of proteins involved the coagulation system (*27*), which have consensus sequences (C_1XSXPC_2, where *X* represents any amino acid) for the addition of these sugar chains. Binding of a single Glc is also found in murine fetal antigen 1 (*28*), human factor VII (*29*) and mammalian Notch 1 (*30*). Because of the absence of the consensus sequence, uTM was considered to lack $(Xyl)_n$-Glc-Ser type sugar chain (*30*). Our finding that uTM has an *O*-linked Glc suggests that the *O*-linked Glc may be more common in sugar chains in epidermal growth factor–like domain. But there is still too little information available to discuss the functions or biological activities of this sugar chain.

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