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Comparative Study of the Asparagine-Linked Sugar Chains of Human Lipocalin-Type Prostaglandin D Synthase Purified from Urine and Amniotic Fluid, and Recombinantly Expressed in Chinese Hamster Ovary Cells[1]

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Lipocalin-type prostaglandin D synthase (L-PGDS) is a highly glycosylated member of the lipocalin gene family and is secreted into various human body fluids. We comparatively analyzed the structures of asparagine-linked sugar chains of human L-PGDS produced by recombinant Chinese hamster ovary cells and naturally occurring human urine and amniotic fluid. After the sugar chains were liberated by hydrazinolysis followed by *N*-acetylation, they were derivatized with 2-aminobenzamide. All of the sugar chains of three L-PGDSs occur as biantennary complex-type sugar chains. Most of the sugar chains of three samples were fucosylated on the inner most *N*-acetylglucosamine residue. Although the sugar chains of the recombinant L-PGDS do not contain any bisecting *N*-acetylglucosamine residues, 58% and 34% of the fucosylated-sugar chains of amniotic fluid and urine L-PGDSs, respectively, contain bisecting *N*-acetylglucosamine residues. The sialic acid residues occur solely as Siaα2→3Gal groups of the recombinant L-PGDS; the sialic acid residues of other L-PGDS occur as both Siaα2→3Gal and Siaα2→6Gal groups. Variations in L-PGDS glycosylation may prove useful as markers to further elucidate the role of L-PGDS glycoforms in different tissues.

Key words: amniotic fluid, carbohydrate structure, CHO recombinant, prostaglandin D synthase, urine.

Lipocalin-type prostaglandin (PG) D synthase (L-PGDS, EC 5.3.99.2) catalyzes the isomerization of PGH_2, a common precursor of various prostanoids, to produce PGD_2, an endogenous sleep-promoting substance (*1*). L-PGDS is a unique member of the lipocalin superfamily composed of various secretory lipophilic ligand-carrier proteins, because it was the first enzyme to be recognized in this superfamily (*2*, *3*). A possible function as a transporter of hydrophobic molecules, such as retinoids (*4*), thyroids, biliverdin, and bilirubin (*5*), has been suggested. Immunohistochemistry and *in situ* hybridization of L-PGDS have revealed that the enzyme is produced in the leptomeninges of the central nerve system (*6*), pigmented epithelium of the retina (*7*), male genital organs (*8*, *9*), where it is secreted into the cerebrospinal fluid (CSF), interphotoreceptor matrix, and seminal plasma. In these cases, the enzyme is produced at barrier sites, *i.e.*, the blood-brain, blood-retinal and blood-testicular barriers, and secreted into closed compartments separated from the systemic circulation. On the other hand, it is also detectable in various other body fluids, such as serum, amniotic fluid and urine (*10*, *11*). The biosynthesis of L-PGDS in the human heart and atherosclerotic plaques in blood vessels, and its secretion into serum have been demonstrated (*12*). However, the origins of L-PGDS in the amniotic fluid and urine have not yet been identified. The human enzyme was identified as β-trace protein (β-TP) (*13–16*), a major protein in human CSF (*17*).

Among members of the lipocalin family, L-PGDS is unique because it is glycosylated. In addition, all cDNAs for L-PGDS so far isolated from many mammalian species exclusively conserve two *N*-glycosylation sites (*18*). This conservation among mammals is of potential importance. Additionally, it has been suggested that the presence of sugar

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Abbreviations: AAL, *Aleuria aurantia* lectin; 2AB, 2-aminobenzamide; CHO, Chinese hamster ovary; CSF, cerebrospinal fluid; gu, glucose unit; DHBA, 2,5-dihydroxybenzoic acid; αMEM(−), alpha-modified Eagle's medium lacking ribonucleosides and deoxyribonucleosides; MALDI-FTMS, matrix-assisted laser desorption ionization Fourier transform mass spectrometry; MTX, methotrexate; PG, prostaglandin; L-PGDS, lipocalin-type prostaglandin D synthase; β-TP, β-trace protein.

chains might modulate the interaction of L-PGDS with other molecules in ways that differ from the rest of the lipocalins (*19*). Therefore, the structural characteristics of the sugar chains are important to understand their functional aspects. The structure of the carbohydrate of β-TP purified from CSF was elucidated only recently to be of the "brain-type" (*15*, *20*). This means that all biantennary asparagine-linked sugar chains are of the complex type and have a high degree of fucosylation, large amounts of bisecting *N*-acetylglucosamine, and sialic acid in α2→3 or α2→6 linkages. In contrast to these characteristics of β-TP in CSF, partial structures of the carbohydrates of β-TPs from urine and hemofiltrate have been reported (*21*) as bisecting *N*-acetylglucosamine residues detected in 20% of the sugar chains, and sialic acid is found predominantly in α2→3 linkages.

Although organ-specific glycosylation is well known (*22*, *23*), its biological meaning is not clear. Since L-PGDS is present in various organs and tissue fluids, it is an interesting target molecule to reveal the relationship between sugar chains and their functional relevance. In this study, we compared asparagine-linked sugar chains of human L-PGDS produced by recombinant Chinese hamster ovary (CHO) cells and naturally occurring human urine and amniotic fluid. We show that each L-PGDS contains a set of asparagine-linked sugar chains different from those of L-PGDS in human CSF.

MATERIALS AND METHODS

Enzymes—Arthrobacter ureafaciens sialidase and bovine epididymal α-fucosidase were purchased from Nacalai Tesque (Kyoto) and Sigma Chemicals (St. Louis, MO, USA), respectively. NANase I, a recombinant sialidase specific for α2→3 linked *N*-acetylneuraminic acid, was purchased from Glyco (Novato, CA, USA). Diplococcal β-galactosidase and β-*N*-acetylhexosaminidase were purchased from Boehringer Mannheim (Mannheim, Germany). Jack bean β-*N*-acetylhexosaminidase and α-mannosidase, and snail β-mannosidase were purchased from Seikagaku (Tokyo).

Plasmid and Stable Transfection—A plasmid containing full-length human L-PGDS was obtained as follows: L-PGDS cDNA was excised with *Eco*RI from full-length L-PGDS cDNA (*24*) and ligated into the *Eco*RI site of pdKCR-dhfr (*25*). L-PGDS cDNA was transfected into CHO dhfr(−) cells (*26*) by LIPOFECTAMINE^{TR} reagent (GIBCO BRL, Life Technologics, Rockville, MD, USA). Cell lines grown in alpha-modified Eagle's medium lacking ribonucleosides and deoxyribonucleosides [αMEM(−)] supplemented with dialyzed 5% fetal calf serum, 1% proline, 2 mM glutamine were analyzed for L-PGDS expression by Northern blotting and Western blotting with monoclonal antibody 6F5. High expressing cell lines thus obtained were subcloned after the gene expression was amplified with methotrexate (MTX). Finally, a clonal CHO cell line expressing stably transfected human L-PGDS was cultured in αMEM(−) supplemented with dialyzed 5% fetal calf serum, 1% proline, 2 mM glutamine, and 5 μM MTX.

Purification of L-PGDS—An immunoaffinity column was prepared as described previously (*11*). In brief, 100 mg of monoclonal anti-PGDS antibody (6F5) in 20 ml of 0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3, was applied to a column packed with 5 ml of HiTrap NHS-activated (Amersham Pharmacia Biotech, Tokyo). The antibody solution was recirculated through the column overnight using a peristaltic pump. The column was washed with PBS until the absorbance at 280 nm fell to baseline. The remaining active sites were blocked by incubation with 0.5 M ethanolamine, 0.5 M NaCl, pH 8.3. The column was then washed extensively with phosphate-buffered saline (PBS), pH 7.4.

Human amniotic fluid specimens were collected from patients with polyhydramions at term by Dr. Yoshihiro Tokugawa (Osaka University Medical School). Two hundred milliliters of human amniotic fluid diluted with 500 ml of PBS was loaded onto the anti-PGDS immunoaffinity column constructed above, and the column was washed sequentially with PBS containing 1 M NaCl (30 ml), PBS containing 0.05% Triton X-100 (30 ml), and PBS (50 ml). The absorbed proteins were eluted with 0.5 M sodium citrate, pH 3.0 (20 ml). The eluate was titrated to pH 7.4 with 1 M Tris-HCl, pH 9.0 (11 ml). The sample containing L-PGDS was concentrated and then fractionated on a Superdex 200 HR 10/30 gel-filtration column (10 × 300 mm, Amersham Pharmacia Biotech) using PBS at a flow rate of 0.5 ml/min. Fractions containing L-PGDS were pooled and concentrated. Urinary L-PGDS was purified from 500 ml of human urine by the same procedures as for amniotic L-PGDS. CHO recombinant human L-PGDS was purified from 100 ml of culture medium by immunoaffinity chromatography alone.

The purity of each L-PGDS was confirmed by SDS-PAGE performed according to Laemmli (*27*). Samples were incubated in sample buffer at 100°C for 3 min and loaded onto a Mini-PROTEAN II Dual Cell (Bio-Rad Laboratories, Richmond, CA, USA) with a 2.5% stacking gel and 12.5% separating gel (5 cm). The gel was electrophoresed at a constant 150 V for 1 h. After electrophoresis, the protein in the gel was visualized with Silver Stain II Kit Wako (Wako Pure Chemical Industries, Osaka).

Release of Asparagine-Linked Sugar Chains from L-PGDS—Purified L-PGDS (500 μg) was thoroughly dried over P_2O_5 *in vacuo* and subjected to hydrazinolysis at 100°C for 9 h. The liberated oligosaccharides were *N*-acetylated and purified as described previously (*28*). This procedure releases the asparagine-linked sugar chains of glycoproteins quantitatively as oligosaccharides. Following *N*-acetylation, the sample was separated by paper chromatography using 1-butanol:ethanol:water (4:1:1, v/v) for 18 h. To isolate the asparagine-linked oligosaccharides, the area of the paper from the origin to the migration position of authentic lactose was extracted with water.

2-Aminobenzamide (2AB) Derivatization of Oligosaccharides—The total asparagine-linked oligosaccharides obtained above were labeled with 2-aminobenzamide (2AB) as described previously (*29*, *30*). To detect the 2AB-labeled oligosaccharides, fluorescence emission was monitored at 420 nm with excitation at 330 nm.

Oligosaccharides—Galβ1→4GlcNAcβ1→2Manα1→6-(Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc-2AB (Gal_2·$GlcNAc_2$·Man_3·GlcNAc·GlcNAc-2AB, NA2), Galβ1→4GlcNAcβ1→2Manα1→6(Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc-2AB (Gal_2·$GlcNAc_2$·Man_3·GlcNAc·Fuc·GlcNAc-2AB, NA2F) and Galβ1→4GlcNAcβ1→2Manα1→6(Galβ1→4GlcNAcβ1→2Manα1→3)(GlcNAcβ1→4)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc-2AB (Gal_2·$GlcNAc_2$·Man_2·

GlcNAc·Man·GlcNAc·Fuc·GlcNAc-2AB, NA2FB) were purchased from Oxford GlycoSciences (Rosedale, NY, USA). Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc-2AB (Man_3·GlcNAc·GlcNAc-2AB, M3), and Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc-2AB (Man_3·GlcNAc·Fuc·GlcNAc-2AB, M3F) were obtained by digestion of NA2 and NA2F with a mixture of diplococcal β-galactosidase and β-*N*-acetylhexosaminidase, respectively.

Analytical Methods—Anion-exchange chromatography of 2AB labeled asparagine-linked oligosaccharides was carried out on an HPLC apparatus equipped with a Mono Q HR5/5 column. The column was eluted with water for 10 min, then with a linear gradient from 0 to 600 mM ammonium acetate (pH 4.0) during 35 min at a flow rate of 1 ml/min at room temperature.

Neutral- and asialo-oligosaccharides were subjected to HPLC using an LA-AAL (*Aleuria aurantia* lectin) column (Seikagaku). After elution of the unbound oligosaccharides with 10 ml of 10 mM ammonium acetate buffer, pH 7.3, the bound oligosaccharides were eluted with the same buffer containing 5 mM L-fucose at a flow rate of 0.5 ml/min at 25°C.

Reversed-phase HPLC was carried out on a Cosmosil $5C_{18}$-AR column (Nacalai Tesque) equilibrated with 100 mM ammonium acetate buffer, pH 4.0, and eluted with a gradient of 1-butanol (0.25–1%) during 120 min at a flow rate of 1 ml/min at 55°C.

Normal-phase HPLC was carried out on a GlycoSep N column (Oxford GlycoSciences) by elution with a 250 mM ammonium acetate–acetonitrile gradient solvent system at a flow rate 1 ml/min at 30°C. The 250 mM acetate–acetonitrile ratio was changed linearly from 20:80 to 53:47 (v/v) during 132 min. The column was calibrated using 2AB-labeled glucose oligomer, the elution positions of which were used to obtain the glucose unit (gu) values for each glycan.

Determination of the Molecular Mass of 2AB Labeled Oligosaccharides by Matrix-Assisted Laser Desorption Ionization Fourier Transform Mass Spectrometry (MALDI-FTMS)—2,5-Dihydroxybenzoic acid (DHBA) was purchased from Sigma Aldrich and used as the matrix. It was dissolved to a concentration of 10 mg/ml of 30% aqueous ethanol. The dried 2AB labeled oligosaccharide was dissolved in the matrix solution (2 pmol/μl). Aliquots of the resulting mixtures (5 μl) were placed onto probe tips and dried at room temperature. The molecular masses of the oligosaccharides were determined using a BioAPEX 47e (4.7-T, Bruker Daltonics GmbH, Bremen, Germany) FTMS.

Glycosidase Digestion—Oligosaccharides were incubated with one of the following mixtures for 18 h at 37°C: (i) *A. ureafaciens* sialidase (25 mU) in 30 μl of 500 mM ammonium acetate buffer, pH 5.0; (ii) NANase I (10 mU) in 20 μl of 50 mM ammonium acetate buffer, pH 6.0; (iii) diplococcal β-galactosidase (12.5 mU) in 50 μl of 300 mM citrate phosphate buffer, pH 6.0; (iv) diplococcal β-*N*-acetylhexosaminidase (5 mU) in 50 μl of 300 mM citrate phosphate buffer, pH 6.0; (v) jack bean β-*N*-acetylhexosaminidase (0.5 U) in 40 μl of 300 mM citrate phosphate buffer, pH 5.0; (vi) α-mannosidase (1 U) in 50 μl of 50 mM sodium acetate buffer, pH 4.5; (vii) β-mannosidase (10 mU) in 50 μl of 50 mM citrate phosphate buffer, pH 4.0; (viii) α-fucosidase (10 mU) in 40 μl of 200 mM citrate phosphate buffer, pH 6.0. One drop of toluene was added to all reaction mixtures to inhibit bacterial growth during incubation. Digestions were terminated by heating the reaction mixture in a boiling water bath for 3 min. Digested samples were desalted by HPLC using a Cosmosil $5C_{18}$-AR column for neutralized 2AB-labeled oligosaccharides. As for samples treated with *A. ureafaciens* sialidase or NANase I, ammonium acetate was removed by extensive evaporation.

RESULTS

SDS-PAGE of Purified Human L-PGDS—The human recombinant L-PGDS purified from CHO cells showed considerable heterogeneity with a major protein band at about M_r 28,000 and two minor bands at about 23,000 and 19,500 (Fig. 1, lane 1). Since human L-PGDS has two potential *N*-glycosylation sites (*2*, *18*), we estimated that the sample from recombinant CHO cells contained three isoforms; non-, mono-, and diglycosylated forms. It is noteworthy that β-TP in CSF from patients with congenital glycoprotein-Ia (CDG-Ia) disorders due to phosphomannomutase II deficiency show three protein bands corresponding to the di- (24 kDa), mono- (22 kDa), and non-glycosylated (20 kDa) isoforms (*31*). On the other hand, the L-PGDSs purified from amniotic fluid and urine migrated on SDS-PAGE as a single band of about 28 kDa representing the diglycosylated isoform (Fig. 1, lanes 2 and 3).

Fractionation of Oligosaccharides by Anion-Exchange Column Chromatography—The 2AB-labeled oligosaccharide mixtures obtained from human L-PGDS produced by recombinant CHO cells and naturally occurring human urine and amniotic fluid by hydrazinolysis were separated into one neutral (N) and two acidic (A1 and A2) fractions by anion-exchange chromatography on a Mono Q HR5/5 column (Fig. 2). Fractions A1 and A2 were eluted at the same positions as 2AB-derivatized monosialo- and disialo-biantennary sugar chains obtained from human transferrin, suggesting that fractions A1 and A2 contain one and two sialic acids, respectively. The percentage molar ratio of oligosaccharides in fractions N, A1, and A2, calculated on the basis of their fluorescence intensity is summarized in Table I.

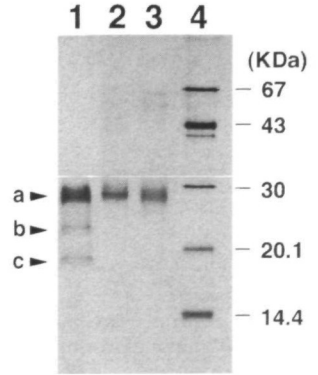


Fig. 1. **SDS-PAGE analysis of purified human L-PGDS.** Recombinant L-PGDS, human amniotic fluid L-PGDS, and urine L-PGDS were purified as described in "MATERIALS AND METHODS." Lane 1, recombinant L-PGDS in transfected CHO cells; lane 2, L-PGDS from amniotic fluid; lane 3, L-PGDS from urine; lane 4, molecular mass standards. The migration positions of L-PGDS are indicated by arrows; a, diglycosylated isoform; b, monoglycosylated isoform; c, nonglycosylated isoform. The migration positions of the molecular mass standards are indicated on the right side.

By exhaustive *A. ureafaciens* sialidase digestion, all acidic fractions were completely converted into neutral oligosaccharides, indicating that only sialic acids are included among the acidic residues in these oligosaccharides. The neutral oligosaccharide fractions obtained from A1 and A2 were named A1N and A2N, respectively. In order to discriminate the sialyl linkages of oligosaccharides in fractions A1 and A2, the following experiments were performed. NANase I is very useful for determining the sialic acid linkages, because it cleaves Siaα2→3Gal linkages, but not the Siaα2→6Gal linkages (*32*). When fraction A1 and fraction A2 of recombinant L-PGDS were incubated with NANase I, most of both fractions was completely converted into neutral oligosaccharides (Table II), indicating that only Siaα2→3Gal linkages are included in recombinant L-PGDS. This is consistent with previous analyses of CHO cell–derived glycoproteins. On the other hand, when fraction A1 of amniotic fluid and urine L-PGDSs was incubated with NANase I, 47.2 and 43.9% remained unchanged (Table II). In the case of fraction A2 of the three L-PGDSs, 9.7 and 12.2% remained unchanged, 40.3 and 37.0% was converted into the monosialooligosaccharide fraction, and the remaining 50.0 and 50.8% was neutralized (Table II).

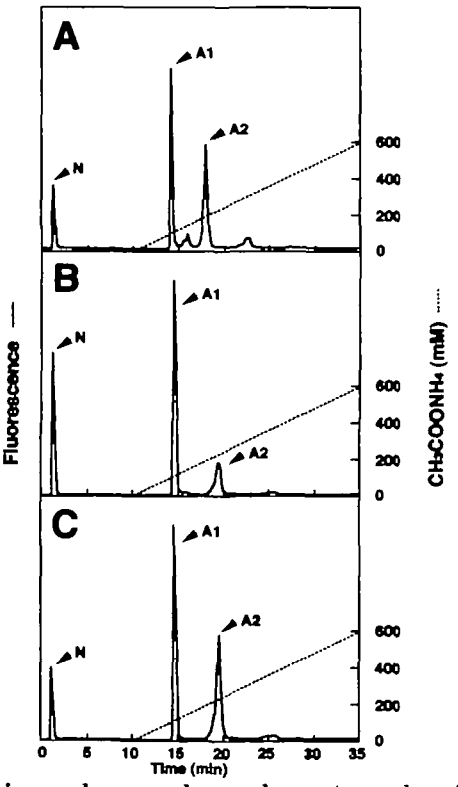


Fig. 2. **Anion-exchange column chromatography of 2AB-labeled asparagine-linked sugar chains from L-PGDS.** 2AB-labeled oligosaccharides were subject to HPLC on a Mono Q HR5/5 column. After elution of the neutral oligosaccharides with 10 ml of water, the acidic oligosaccharides were eluted with a 0–600 mM gradient of ammonium acetate, pH 4.0, at a flow rate of 1 ml/min at room temperature (dotted line). (A) Oligosaccharides from recombinant L-PGDS in transfected CHO cells; (B) oligosaccharides from human amniotic fluid L-PGDS; (C) oligosaccharides from human urine L-PGDS. The molar ratio of each fraction is shown in Table I. N, A1 and A2 indicate neutral, monosialo, and disialo oligosaccharide fractions, respectively.

These results indicate that acidic oligosaccharides of amniotic fluid and urine L-PGDSs contain both Siaα2→3- and Siaα2→6Gal linkages; the distribution of the Siaα2→3- and Siaα2→6Gal linkages in each fraction A2 is summarized in Table II.

Fractionation of Neutral Oligosaccharides by Aleuria aurantia Lectin Column (LA-AAL) Chromatography—An AAL column can be used effectively to separate oligosaccharides based on their binding specificities. All complex-type asparagine-linked sugar chains containing an α-fucosyl residue linked at the C-6 position of the proximal *N*-acetylglucosamine residue of their trimannosyl core bind to the AAL column, while those without fucose residues do not (*33, 34*).

When fraction A2N of recombinant CHO L-PGDS was subjected to LA-AAL-column chromatography, 93.5% of the fraction was retained on the column and eluted with 5 mM L-fucose. The retained fraction was named A2NAAL(+F) while the unretained fraction was named A2NAAL(−F). A1N and N were also separated into two fractions on an LA-AAL-column; retained [A1NAAL(+F) and NAAL(+F)], and unretained [A1NAAL(−F) and NAAL(−F)]. Three fractions each obtained from amniotic fluid and urine L-PGDSs were also separated by the same procedures. The percentage of each retained fraction on the LA-AAL-column can be summarized as follows: CHO, NAAL(+F) 96.6%, A1NAAL(+F) 93.4%, A2NAAL(+F) 93.5%; amniotic fluid, NAAL(+F) 82.0%, A1NAAL(+F) 89.1%, A2NAAL(+F) 88.9%; urine, NAAL(+F) 85.4%, A1NAAL(+F) 91.2%, A2NAAL(+F) 87.6%. The results indicated that most of the oligosaccharides in the three samples were fucosylated.

Structural Study of Oligosaccharides in Fractions AAL(−F)—The same results were obtained in structural studies of each fraction AAL(−F). Therefore, the experimental results obtained for fraction A1NAAL(−F) of recombinant L-PGDS will be given below.

When fraction A1NAAL(−F) of recombinant L-PGDS was subjected to chromatography on a Cosmosil $5C_{18}$-AR column, a major component with the same elution position as authentic NA2 was obtained (Fig. 3A). As shown in Fig.

TABLE I. **Fractionation of asparagine-linked sugar chains released from L-PGDS recombinantly expressed in CHO or purified from amniotic fluid and urine by anion-exchange chromatography.**

Fraction	Molar ratio (%)		
	CHO	Amniotic fluid	Urine
Neutral (N)	15.4	28.9	12.1
Monosialo (A1)	38.1	48.3	41.1
Disialo (A2)	46.5	22.8	46.8

TABLE II. **Distribution of sialic acid linkages of asparagine-linked sugar chains from three L-PGDSs.**

Fraction	Linkage	Molar ratio (%)		
		CHO	Amniotic fluid	Urine
A1	α2-3	100	52.8	56.1
	α2-6	—[a]	47.2	43.9
A2	α2-3,α2-3	100	50.0	50.8
	α2-3,α2-6	—	40.3	37.0
	α2-6,α2-6	—	9.7	12.2

[a] Not detected.

3Ba, this component was also eluted at the same position as authentic NA2 on normal phase HPLC (7.3 gu). These results suggest that A1NAAL(−F) is a complex-type nonfucosylated biantennary oligosaccharide as shown in Fig. 3C.

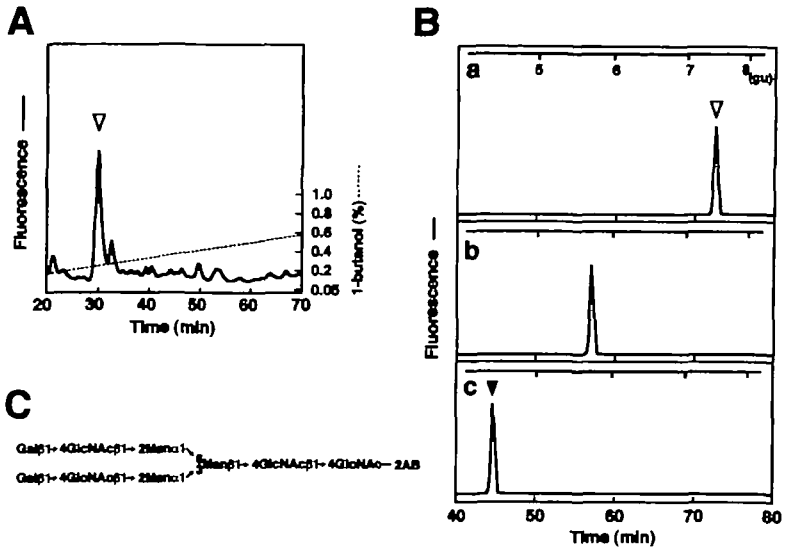


Fig. 3. **Reversed phase and normal phase HPLC of the component in fraction A1NAAL(−F) of recombinant L-PGDS, and its proposed structure.** (A) The A1NAAL(−F) fraction was applied to a Cosmosil $5C_{18}$-AR column equilibrated with 100 mM ammonium acetate buffer, pH 4.0, and eluted with a gradient of 1-butanol (0.25–1%) during 120 min at a flow rate of 1 ml/min at 55°C (dotted line). (B, a) The major fluorescent component in A was applied to a GlycoSep-N column and eluted by a 250 mM ammonium acetate–acetonitrile gradient solvent system at a flow rate 1 ml/min at 30°C. The 250 mM acetate–acetonitrile ratio was changed linearly from 20:80 to 53:47 (v/v) during 132 min; (B, b) the component in (B, a) after digestion with diplococcal β-galactosidase; (B, c) the component in (B, b) after digestion with diplococcal β-*N*-acetylhexosaminidase. Peaks were assigned gu values by comparison with the 2AB-labeled glucose oligomer ladder shown at the top of each panel. (C) Proposed structure of nonfucosylated asparagine-linked sugar chains obtained from three L-PGDSs. The open triangle at the top of A indicates the elution position of the 2AB-labeled authentic oligosaccharide standard, NA2. The open triangle at the top of Ba indicates the elution position of the standard, NA2, and the closed triangle in Bc indicates the position of standard M3.

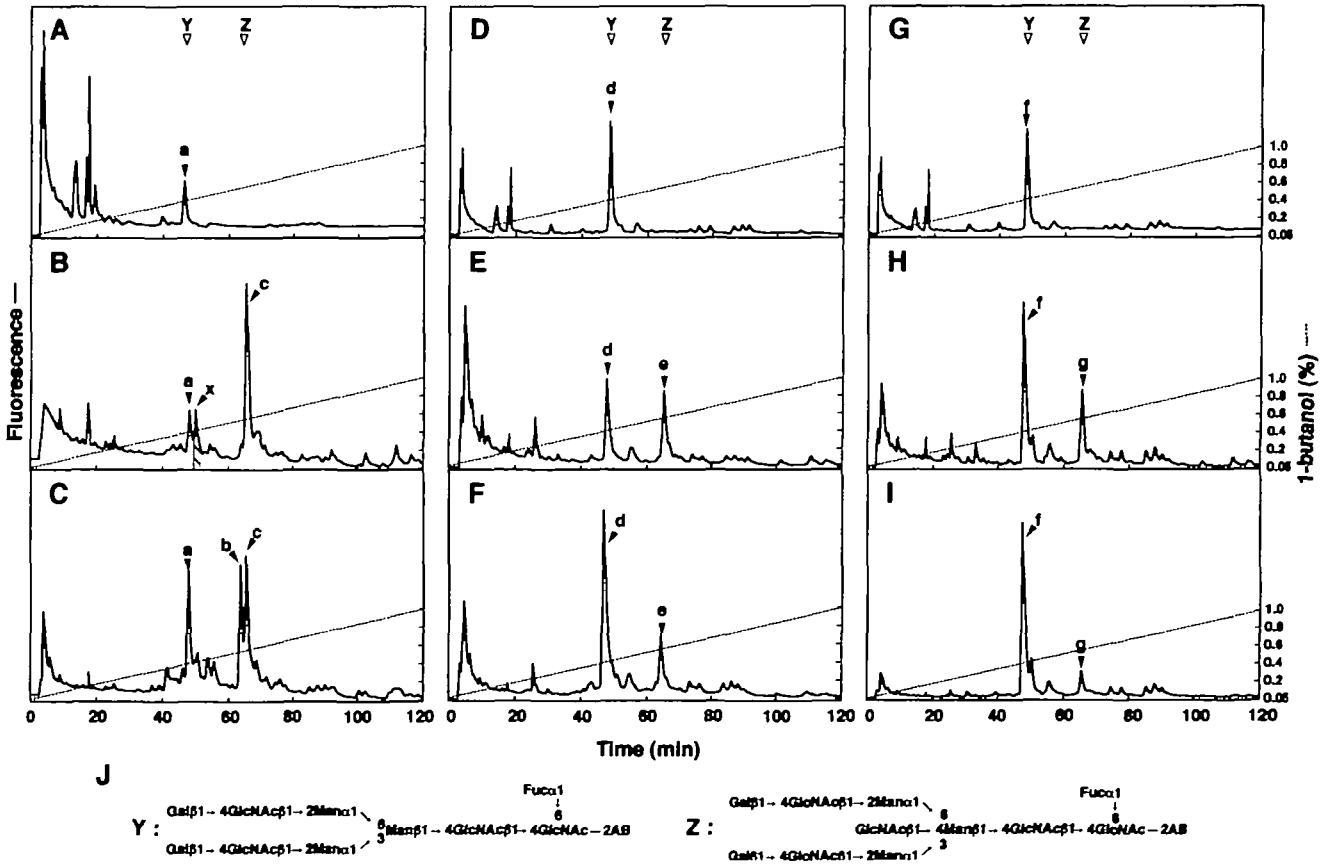


Fig. 4. **Reversed phase HPLC of components in the AAL(+F) fractions.** Nine AAL(+F) fractions were applied to a Cosmosil $5C_{18}$-AR column, which was conditioned as described for Fig. 3A. (A, D, and G) Fractions N, A1N, and A2N from recombinant L-PGDS; (B, E, and H) from amniotic fluid L-PGDS; (C, F, and I) from urine L-PGDS. The open triangles Y and Z at the top of A, D, and G indicate the elution positions of the 2AB-labeled authentic oligosaccharide standards of NA2F and NA2FB, respectively. Their structures are shown in (J).

In order to confirm these findings, the component in Fig. 3Ba was subjected to sequential exoglycosidase digestion and analyzed by HPLC. When it was incubated with diplococcal β-galactosidase, which cleaves only Galβ1→4GlcNAc linkages (*35*), two galactose residues were removed completely from the component in Fig. 3Ba (Fig. 3Bb). The component in Fig. 3Ba was resistant to β-*N*-acetylhexosaminidase digestion (data not shown). When the component in Fig. 3Bb was incubated with diplococcal β-*N*-acetylhexosaminidase, which cleaves only GlcNAcβ1→2Man linkages (*36*), it was converted to a component with the same mobility as authentic M3 (4.5 gu) with the release of two *N*-acetylglucosamine residues (Fig. 3Bc). The component in Fig. 3Bc was confirmed by sequential digestion with jack bean α-mannosidase, β-mannosidase, and jack bean β-*N*-acetylhexosaminidase. The small component eluted at about 33 min in Fig. 3A could not be identified due to the limited amount of the sample.

Since the components obtained from NAAL(−F) and A2NAAL(−F) of recombinant L-PGDS, and the component from each fraction AAL(−F) of the amniotic fluid and urine enzymes gave the same results, they will not be repeated here. Based on these results, the proposed structure of the component of each fraction AAL(−F) is shown in Fig. 3C.

Structural Study of Oligosaccharides in Fraction AAL(+F)—When nine AAL(+F) fractions from three L-PGDSs were chromatographed on a Cosmosil $5C_{18}$-AR column, several components were obtained (Fig. 4). Each AAL(+F) fraction from recombinant L-PGDS yielded a major single component: component *a* from fraction NAAL(+F) (Fig. 4A), component *d* from fraction A1NAAL(+F) (Fig. 4D), and component *f* from fraction A2NAAL(+F) (Fig. 4G). The remaining six AAL(+F) fractions gave three components from fractions NAAL(+F) of amniotic fluid L-PGDS (components *a*, *x*, and *c* in Fig. 4B) and urine L-PGDS (components *a*, *b*, and *c* in Fig. 4C) and two components from fractions A1NAAL(+F) (components *d* and *e* in Fig. 4, E and F) and fractions A2NAAL(+F) (components *f* and *g* in Fig. 4, H and I) of both enzymes. These components were subjected to further structural analysis, and were separated by normal phase HPLC. The elution profiles are shown in Fig. 5, and finally eleven components were

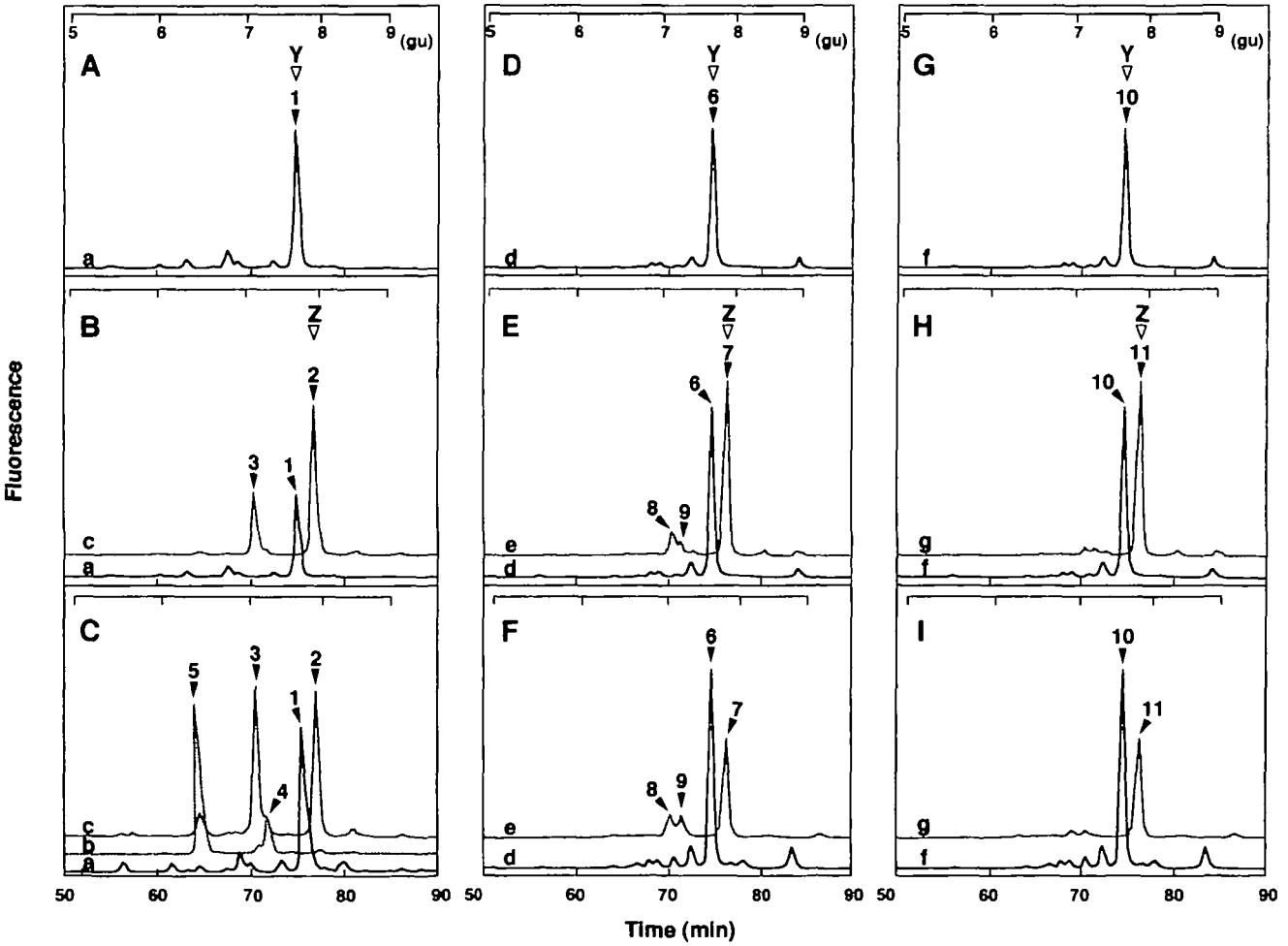


Fig. 5. **Normal phase HPLC of components in the AAL(F+) fractions.** The fluorescent components *a* to *g* in Fig. 4 were applied to a GlycoSep-N column and eluted in a 250 mM ammonium acetate-acetonitrile gradient solvent system at a flow rate 1 ml/min at 30°C. The 250 mM acetate–acetonitrile ratio was changed linearly from 20:80 to 53:47 (v/v) during 132 min. (A, D, and G) Fractions N, A1N, and A2N from recombinant L-PGDS; (B, E, and H) from amniotic fluid L-PGDS; (C, F, and I) from urine L-PGDS. Peaks were assigned gu values by comparison with the 2AB-labeled glucose oligomer ladder shown at the top of each panel. The open triangles Y and Z are the same as in Fig. 4.

obtained. Only component *x* in Fig. 4B could not be determined, although it was eluted at 2.6 gu by normal phase HPLC (data not shown). Panels A, D, and G (Fig. 5) gave fractions NAAL(+F), A1NAAL(+F), and A2NAAL(+F) from recombinant L-PGDS, and panels B, E, and H and panels C, F, and I yielded the corresponding fractions from amniotic fluid L-PGDS and urine L-PGDS, respectively.

As shown in Fig. 5, components 1, 6, and 10 were eluted at the same position as authentic NA2F (7.8 gu, indicated by Y) from normal phase HPLC, suggesting that these components are complex-type fucosylated digalactosyl-biantennary oligosaccharides (Fig. 4J, Y). Components 2, 7, and 11 eluted at the same position as authentic NA2FB (7.9 gu, indicated by Z), suggesting that these are complex-type fucosylated bisected digalactosyl-biantennary oligosaccharides (Fig. 4J, Z).

Information about the molecular weights of these components is helpful for interpreting the HPLC results obtained above and the results of sequential exoglycosidase digestion as described later. Therefore, the molecular weights of components 10 and 11 were determined by MALDI-FTMS. The observed $[M+Na]^+$ ion peaks of components 10 and 11 were at m/z 1,930.8 and 2,133.7, respectively (Fig. 6). The observed molecular mass values were consistent with those expected: 2AB labeled fucosylated digalactosyl-biantennary sugar chains (1,929), and 2AB labeled fucosylated bisected digalactosyl-biantennary sugar chains (2,132).

Those findings were confirmed by sequential exoglycosidase digestion as follows. When component 10 was incubated with diplococcal β-galactosidase, two galactose residues were removed completely (6.2 gu) (Fig. 7A). When the component in Fig. 7A was incubated with diplococcal β-*N*-acetylhexosaminidase, it was converted to a component with the same mobility as authentic M3F (5.0 gu) with the release of two *N*-acetylglucosamine residues (Fig. 7B). The component in Fig. 7B was confirmed by sequential digestion with jack bean α-mannosidase followed by β-mannosidase, jack bean β-*N*-acetylhexosaminidase, and α-fucosidase. The same results were obtained from components 1 and 6 by sequential exoglycosidase digestions. Based on these results, the proposed structures of components 1, 6, and 10 are concluded to be as shown in Table III.

To confirm the findings, components 2, 7, and 11 were subjected to sequential digestion. Component 11 released two galactose residues by diplococcal β-galactosidase digestion (6.4 gu) (Fig. 7C) and three *N*-acetylglucosamine residues by subsequent jack bean β-*N*-acetylhexosaminidase digestion and co-eluted with authentic standard M3F (5.0 gu) (Fig. 7D). On the other hand, the component in Fig. 7C released only one *N*-acetylglucosamine residue by diplococcal β-*N*-acetylhexosaminidase digestion (6.1 gu) (Fig. 7E). These results indicate that the structure of the component in Fig. 7C is GlcNAcβ1→2Manα1→6(GlcNAcβ1→2Manα1→3)(GlcNAcβ1→4)Manβ1→4GlcNAcβ1→4(Fucα1→6)-GlcNAc-2AB. As reported previously (*36*), diplococcal β-*N*-acetylhexosaminidase cleaves only the GlcNAcβ1→2Man

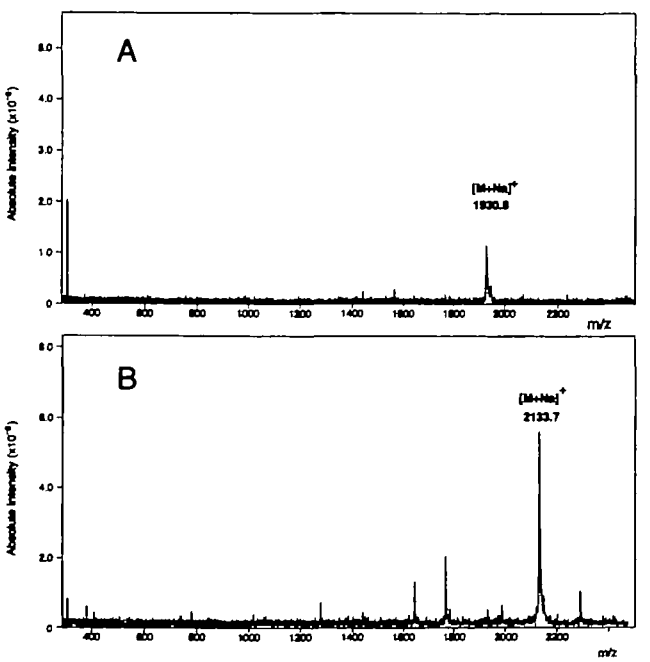


Fig. 6. **MALDI-FTMS analysis of 2AB labeled oligosaccharides.** (A) Component 10; (B) component 11. Both spectra were collected using DHBA as the matrix.

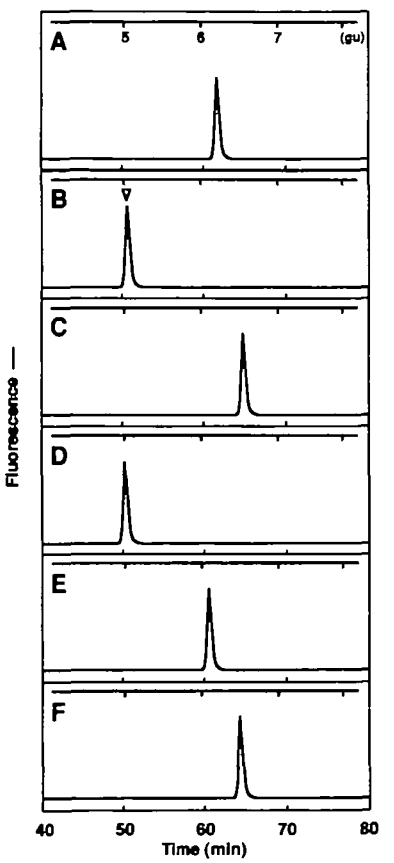
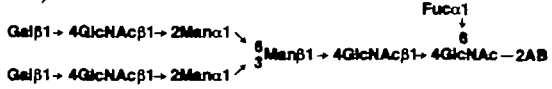
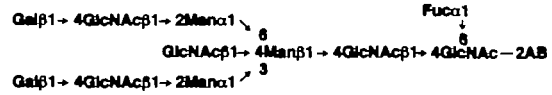
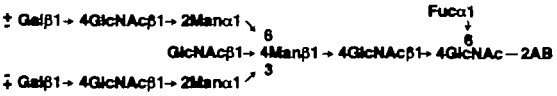
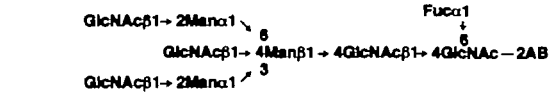
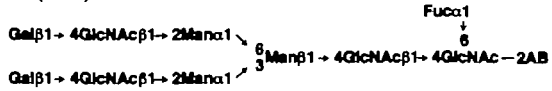
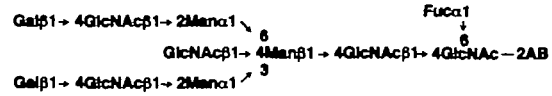
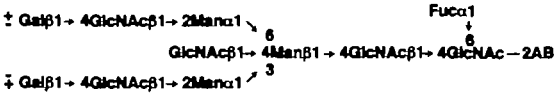
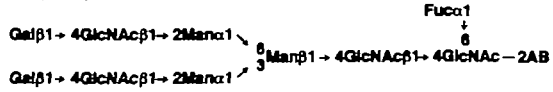
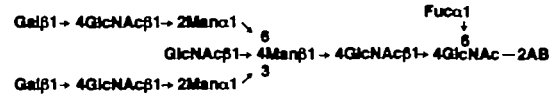


Fig. 7. **Sequential glycosidase digestion of components 10, 11, and 3.** (A) Component 10 in Fig. 5 after digestion with diplococcal β-galactosidase; (B) the component in (A) after digestion with diplococcal β-*N*-acetylhexosaminidase; (C) component 11 in Fig. 5 after digestion with diplococcal β-galactosidase; (D) the component in (C) after digestion with jack bean β-*N*-acetylhexosaminidase; (E) the component in (C) after digestion with diplococcal β-*N*-acetylhexosaminidase; (F) component 3 in Fig. 5 after digestion with diplococcal β-galactosidase. The open triangle at the top of B indicates the elution position of the 2AB-labeled authentic oligosaccharide standard, M3F.

linkage on the Manα1→3 arm from the above oligosaccharide. The same results were obtained for components 2 and 7 by sequential exoglycosidase digestions. Based on these results, the proposed structures of components 2, 7, and 11 are summarized in Table III.

As shown in Fig. 5, components 3 and 8, and components 4 and 9 co-eluted from normal phase HPLC. Component 3 (7.1 gu) released only one galactose residue by diplococcal β-galactosidase digestion (6.4 gu) (Fig. 7F). The results of sequential exoglycosidase digestions of the component in Fig. 7F and component 5 (6.4 gu, in Fig. 5C), which was found only in urine sample, were the same as that of the component in Fig. 7C. In brief, three *N*-acetylglucosamine residues were released by jack bean β-*N*-acetylhexosaminidase digestion, while one *N*-acetylglucosamine residue was released by diplococcal β-*N*-acetylhexosaminidase digestion. The same results were obtained from component 8 by sequential exoglycosidase digestions. Therefore, components 3 and 8 were fucosylated bisected monogalactosyl-biantennary, and component 5 was fucosylated bisected nongalactosyl-biantennary oligosaccharides, as shown in Table III.

TABLE III. **Proposed structures and their percent molar ratios of fucosylated asparagine-linked sugar chains obtained from three human L-PGDSs.**

Component	Structures	Molar ratio (%)		
		CHO	Amniotic fluid	Urine
NAAL(+F)				
1		15.9	5.5	2.7
2		-[a]	16.0	2.2
3,4		-	7.0	3.5
5		-	-	3.6
A1NAAL(+F)				
6		37.9	23.5	27.1
7		-	20.3	11.2
8,9		-	4.9	3.4
A2NAAL(+F)				
10		46.2	13.4	36.4
11		-	9.5	9.8

[a] not detected.

Components 4 and 9 were eluted 0.1 gu larger than components 3 and 8, respectively (Fig. 5). Each component also released only one galactose residue by diplococcal β-galactosidase digestion, and eluted at the same position as the component in Fig. 7C. The structure was confirmed by the same procedures described already. Therefore, these results suggest that components 4 and 9 were monogalactosyl bisected biantennary. The reason for the difference in the elution positions between components 3 and 8 and components 4 and 9 in Fig. 5 is unclear, but probably due to the difference in the galactosyl arm on Manα1→3 or Manα1→6. Based on these results, we conclude that components 3, 4, 8, and 9 were fucosylated bisected monogalactosyl-biantennary, and component 5 was fucosylated bisected nongalactosyl-biantennary oligosaccharides, as shown in Table III.

DISCUSSION

In the present study, the asparagine-linked sugar chains of human L-PGDS produced by recombinant CHO cells and naturally occurring human urine and amniotic fluid were elucidated. In summary, all of the sugar chains of the three L-PGDSs occur as biantennary complex-type sugar chains. Most of the sugar chains of the three samples are fucosylated on the inner most *N*-acetylglucosamine residue. Although the sugar chains of recombinant L-PGDS do not contain any bisecting *N*-acetylglucosamine residues, 58 and 34% of the fucosylated-sugar chains of amniotic fluid and urine L-PGDSs, respectively, contain bisecting *N*-acetylglucosamine residues. The sialic acid residues occur solely as Siaα2→3Gal groups in recombinant L-PGDS; the sialic acid residues of other L-PGDS occur as both Siaα2→3Gal and Siaα2→6Gal groups. It should be noted that mono- and non-galactosyl sugar chains were found only in the bisected form and not in the non-bisected form. This supports the notion that the addition of bisecting *N*-acetylglucosamine residues modulates the processing pathway of asparagine-linked sugar chains, including β-galactosylation (*37*), and that the activity of β-galactosyltransferase toward the bisected biantennary is lower than toward the non-bisected one (*38*).

Recently, many glycoproteins have been produced by recombinant techniques and their carbohydrate structures have been analyzed in detail (*39*). Among the cells used as hosts, CHO cells have been used most frequently for the expression of various recombinant glycoproteins. Comparative studies of the sugar moieties of these glycoproteins revealed both qualitative and quantitative differences in their sugar patterns. These differences must be due to the peptide moiety, because CHO cells have the same sets of glycosylation machinery, including the glycosyltransferases and glycosidases that act on glycoprotein biosynthetic intermediates. However, we found the common characteristics of all glycoproteins so far expressed in CHO cells even in this study: a lack of bisecting *N*-acetylglucosamine residues and the exclusive occurrence of Siaα2→3Gal groups. An interesting but obscure phenomenon is that different glycosylated isomers were found in recombinant L-PGDS (Fig. 1). It is stressed here that the ratio in the three isoforms is highly reproducible. It will be important to determine whether or not different glycosylation patterns affect the binding affinity and/or specificity of unidentified hydrophobic molecules that are transferred by L-PGDS. It is also noteworthy that non- and mono-glycosylated β-TPs, in addition to the diglycosylated isoform, were found in the CSF of patients with congenital glycoprotein-Ia (CDG-Ia) disorders due to phosphomannomutase II deficiency, although its relevance to the disease is not yet clear (*31*, *40*).

The carbohydrate structure of fibronectin present in human amniotic fluid has been reported by Krusius *et al.* (*41*) and by us (*42*). The results in these studies differ in several points: (i) the ratio of tri- and bi-antennary sugar chains; and the detection of (ii) bisected sugar chains and (iii) polylactosamine-type sugar chains. Based on the present data, it is suggested that the glycosylation of fibronectin may change during gestation, because Krusius *et al.* isolated fibronectin from second trimester amniotic fluid, while we used term fluid. There has been a report showing that developmental changes take place in the sugar chains of placental fibronectin during gestation (*43*). Since we performed structural studies on L-PGDS obtained from term fluid, it remains to be determined whether the carbohydrate changes in L-PGDS occur during gestation or not. It is worth noting that the biosynthesis of L-PGDS in amniotic fluid has not yet been identified, while its concentration in fluid has been proposed to be associated with fetal abnormalities (*44*).

The urinary concentration of L-PGDS is considered to be a potential diagnostic marker for renal diseases (*21*). However, the origin of urinary L-PGDS has not yet been identified. Although a "brain-type" structure (*20*, *45–47*), truncated, fucosylated, bisected biantennary sugar chain (component 5 in Table III), was found for urinary L-PGDS, it is impossible to conclude that part of the urinary L-PGDS is derived from the CSF (brain). We can not completely exclude the possibility that the partial degradation of sugar chains of urinary L-PGDS occurs during purification, including the collection and preservation of urine. However, such degradation would be rare because galactose-deficient sugar chains were found only in the bisect form. Since organ-specific differences in glycosylation are well known (*22*, *23*), the accumulation of carbohydrate data of L-PGDSs from various organs is required to reach a conclusion.

What is the functional role of the sugar chains on L-PGDS? Since recombinant L-PGDS produced by *Escherichia coli* show full enzymatic activity (*48*, *49*), sugar chains are not necessary for enzymatic activity. One possible function in the transfer of an unidentified hydrophobic molecule, such as retinoid (*50*), thyroids, or bile pigments (*5*), has been suggested. Although the mRNA of L-PGDS could not be detected in neurons, it has been detected immunohistochemically (*51*), suggesting that L-PGDS produced in the leptomenings (*6*) and secreted into the CSF is incorporated into developing neurons. This is of interest because hydrophilic sugar chains are attached to L-PGDS, which may transport the hydrophobic molecules. One of the well-known functions of sugar chains is acting as "tags" to deliver molecules to target tissues or cells (*22*, *23*). If L-PGDS present in different tissues transports different molecules, tissue-specific glycosylation differences may be of functional relevance. Further studies are necessary to identify the hydrophobic molecules transported by L-PGDS.

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