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Donor specificity in the glycosylation of Tamm-Horsfall glycoprotein: Conservation of the Sd^a determinant in pairs of twins

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The content of the Sd^a determinant in urinary human Tamm-Horsfall glycoprotein (THp) has been reported to be donor-specific. This feature was further addressed by investigating THp from genetically identical individuals. To this end, THp was isolated from the urine of two monozygotic pairs of twins (A and B). The four samples (THp A1, A2, B1, and B2) were subjected to endo-β-galactosidase from *Bacteroides fragilis* leading to the liberation of the Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal and Neu5Ac(α2-3)[GalNAc(β1-4)]-Gal(β1-4)GlcNAc(β1-3)Gal (Sd^a epitope) motifs, both located at the nonreducing termini of complex type N-glycans. The isolated mixtures of oligosaccharides were analyzed for the absolute and relative amounts of the two oligosaccharides. The obtained data clearly indicate that in THp A1 and A2, and in THp B1 and B2, the molar ratios of the tetra- and Sd^a pentasaccharide are identical for a pair of twins. This conservation of molar ratios points to an identical relative expression of β-1,4-*N*-acetylgalactosaminyltransferase activity involved in the biosynthesis of the Sd^a determinant. Apparently, the degree of conversion of the tetrasaccharidic Sd^a precursor into the final pentasaccharidic Sd^a form can be considered to result from a very closely related pattern of glycosylation for genetically homogeneous individuals.

Key words: donor specificity/Sd^a determinant/Tamm-Horsfall glycoprotein

Introduction

Tamm-Horsfall glycoprotein (THp) is the most abundant protein constituent of normal human urine, being excreted in quantities of up to 100 mg/day (Tamm and Horsfall, 1950). It is synthesized in the kidney as a glycosylphosphatidylinositol (GPI)-anchored membrane glycoprotein (Rindler *et al.*, 1990), where it is localized in the thick ascending limbs of the loop of Henle and the early distal convoluted tubule of the nephron. The GPI-anchored form is cleaved by a highly specific protease yielding the urinary form (Cavallone *et al.*, 2001; Fukuoka and Kobayashi, 2001). Over the years a variety of biological functions for THp have been proposed, for example, natural inhibition of urinary tract bacterial infections (Pak *et al.*, 2001), regulation of water/electrolyte transport (Mattey and Naftalin, 1992), a role in kidney stone formation (Scurr and Robertson, 1986), and immunomodulation (Horton *et al.*, 1990; Moonen and Williamson, 1987; Muchmore and Decker, 1985; Su and Yeh, 1999; Thomas *et al.*, 1993; Toma *et al.*, 1994).

THp has a very heterogeneous glycosylation pattern, distributed over seven N-glycosylation sites (van Rooijen *et al.*, 1999) and one or more O-glycosylation sites (Easton *et al.*, 2000). Detailed structural studies of the N-glycosylation pattern of THp from individual donors by ^{1}H-nuclear magnetic resonance (NMR) spectroscopy have resulted in the elucidation of 63 complex-type (Hård *et al.*, 1992; van Rooijen *et al.*, 1998a,b) and 4 oligomannose-type (Dall'Olio *et al.*, 1988; Smagula *et al.*, 1990; van Rooijen *et al.*, 1999) N-glycans. Di-, tri-, and tetraantennary structures (including dimeric *N*-acetyllactosamine sequences) are present, which can be fucosylated, sialylated, and/or sulfated. Structural studies of the O-glycosylation pattern of THp from individual donors by mass spectrometry (MS) have shown the presence of sialylated or fucosylated core 1-type O-glycans (Easton *et al.*, 2000).

One of the interesting terminal epitopes present in THp N-glycans is the Sd^a determinant (Donald *et al.*, 1983; Hård *et al.*, 1992). The Sd^a pentasaccharide Neu5Ac(α2-3)[GalNAc(β1-4)]Gal(β1-4)GlcNAc(β1-3)Gal can be released by endo-β-galactosidase digestion of the glycoprotein, together with the tetrasaccharide Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal and the trisaccharide Gal(β1-4)GlcNAc(β1-3)Gal (Donald *et al.*, 1983). In an earlier investigation on THp probes from four male donors, we demonstrated that the molar ratio of Sd^a pentasaccharide to tetrasaccharide and thus the Sd^a-related glycosylation is a donor-specific feature (van Rooijen *et al.*, 1998a). The donor specificity holds also for the total content of Sd^a pentasaccharide plus tetrasaccharide (van Rooijen *et al.*, 1998a). Having found this, the question arose whether in genetically identical individuals an identical donor specificity will be observed.

In the present study, THp was isolated from the urine of each individual of a female (pair A) and a male (pair B) monozygotic pairs of twins, and the endo-β-galactosidase released oligosaccharides were analyzed for their Sd^a pentasaccharide/tetrasaccharide contents and their molar ratios.

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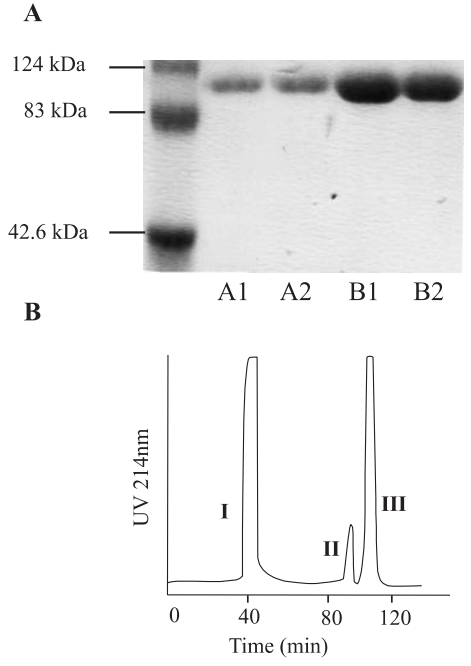

Fig. 1. (**A**) SDS–PAGE of purified Tamm-Horsfall glycoprotein from two monozygotic pairs of twins; THp A1 and A2 are isolated from individual urines of the female pair, THp B1 and B2 are isolated from individual urines of the male pair. (**B**) Separation pattern on Superdex-75 after treatment of THp A2 with endo-β-galactosidase. Fraction II contains the Sd^a pentasaccharide and precursor tetrasaccharide.

Results

Isolation of the oligosaccharide mixture from endo-β-galactosidase-treated THp

For the isolation of THp, morning urines from two pairs of twins were collected. The four THp samples obtained, A1 and A2 for the female twins pair and B1 and B2 for the male twins pair, appeared as a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) having an apparent molecular mass of 94 kDa (Figure 1A). Monosaccharide analysis (Table I) revealed the presence of Fuc, Man, Gal, GalNAc, GlcNAc, and Neu5Ac in accordance with literature data (Hård *et al.*, 1992). The four probes—A1, A2, B1, and B2—were subjected to endo-β-galactosidase digestion (van Rooijen *et al.*, 1998a), and the digestion products were fractionated by size-exclusion chromatography on Superdex-75, yielding similar peak profiles. A typical pattern showing three fractions, denoted I, II, and III, is depicted in Figure 1B. Fraction I contains partially deglycosylated THp glycoprotein, fraction II the released oligosaccharides, and fraction III salts.

The presence of the requested oligosaccharide material in fraction II was confirmed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS in the negative-ion mode (Figure 2A) and ^{1}H-NMR spectroscopy (Figure 3). MALDI-TOF analysis gave pseudomolecular ions at *m/z* 1036.82 ($[M–H]^-$) and *m/z* 834.24 ($[M–H]^-$), corresponding with the Sd^a pentasaccharide Neu5Ac(α2-3)[GalNAc(β1-4)]Gal(β1-4)GlcNAc(β1-3)Gal (**II.a**) and the precursor tetrasaccharide Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal (**II.b**), respectively. The ^{1}H-NMR assignments of the Sd^a pentasaccharide and the tetrasaccharide (Table II) are based on literature data (van Rooijen *et al.*, 1998a). The presence of **II.a** and **II.b** is reflected by their typical structural-reporter-group signals (**II.a**: Neu5Ac H-3e, δ 2.671; Neu5Ac H-3a, δ 1.923; Gal-c

Table I. Monosaccharide analysis data of THp samples isolated from two monozygotic pairs of twins.

	Molar ratio			
Monosaccharide	A1	A2	B1	B2
Fuc	0.5	0.4	0.5	0.5
Man	3.0	3.0	3.0	3.0
Gal	2.8	2.8	2.7	2.9
GalNAc	0.4	0.3	0.6	0.6
GlcNAc	4.8	4.9	4.7	5.0
Neu5Ac	3.4	3.7	3.0	3.2

Mannose is taken as 3.0.

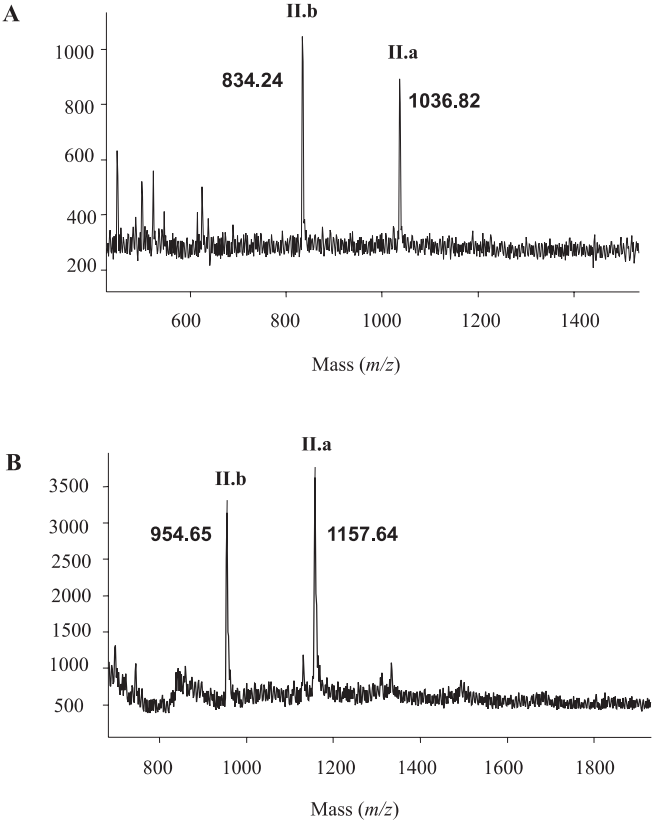

Fig. 2. MALDI-TOF spectra (negative-ion mode) of the mixture of oligosaccharides, obtained after digestion of THp A2 with endo-β-galactosidase and subsequent fractionation on Superdex-75 (fraction II). **II.a**: Neu5Ac(α2-3)[GalNAc(β1-4)]Gal(β1-4)GlcNAc(β1-3)Gal; **II.b**: Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal. (**A**) Free oligosaccharide mixture; (**B**) 2-AB-labeled oligosaccharide mixture.

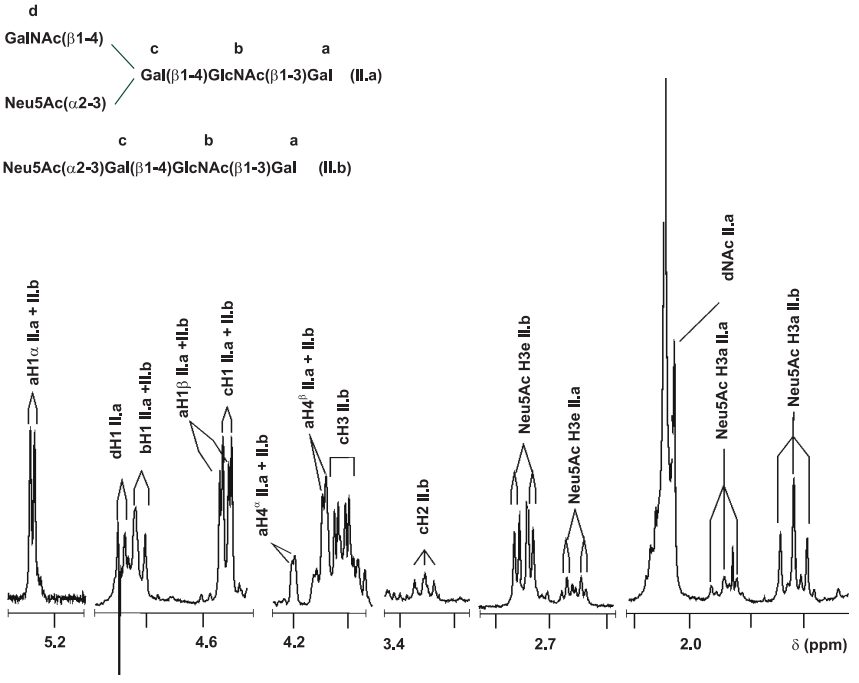

Fig. 3. Resolution-enhanced 500-MHz 1D ^{1}H-NMR spectrum of oligosaccharides, obtained after digestion of THp A2 with endo-β-galactosidase and subsequent fractionation on Superdex-75 (fraction II). **II.a**: Neu5Ac(α2-3)[GalNAc(β1-4)]Gal(β1-4)GlcNAc(β1-3)Gal; **II.b**: Neu5Ac(α2-3)Gal(β1-4)-GlcNAc(β1-3)Gal.

Table II. ^{1}H-chemical shifts of structural-reporter-group protons of the constituent monosaccharides of the liberated Sd^a pentasaccharide **II.a** and the precursor tetrasaccharide **II.b** (Superdex-75 fraction II) derived from THp

		Chemical shift (δ, ppm)		
Residue	Reporter group	Fraction II	Ref. **II.a**	Ref. **II.b**
Gal-**a**	H-1α	5.225	5.229	5.225
	H-1β	4.566	4.568	4.562
	H-4$^\alpha$	4.202	4.202	4.202
	H-4$^\beta$	4.146	4.148	4.147
GlcNAc-**b**	H-1	4.715	4.721	4.721
	NAc	2.033	2.04	2.04
Gal-**c**	H-1	4.551	4.556	4.556
	H-2	3.357 (**II.a**)	3.366	3.572
	H-3	4.156 (**II.a**)	4.155	4.116
		4.115 (**II.b**)		
Neu5Ac	H-3e	2.671 (**II.a**)	2.668	2.757
		2.758 (**II.b**)		
	H-3a	1.923 (**II.a**)	1.926	1.798
		1.796 (**II.b**)		
	NAc	2.031	2.031	2.031
GalNAc-**d**	H-1	4.75 (**II.a**)	4.746	—
	NAc	2.013 (**II.a**)	2.015	—

II.a, Neu5Ac(α2-3)[GalNAc(β1-4)]Gal(β1-4)GlcNAc(β1-3)Gal. **II.b**, Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal. Chemical shifts are given at 300 K and were measured in ^2H_2O relative to internal acetone (δ 2.225).

H-3, δ 4.156; GalNAc H-1, δ 4.75; GalNAc NAc, δ 2.013. **II.b**: Neu5Ac H-3e, δ 2.758; Neu5Ac H-3a, δ 1.796; Gal-c H-3, δ 4.115).

Molar ratios of Sd^a pentasaccharide and tetrasaccharide

The molar ratios of the Sd^a pentasaccharide Neu5Ac(α2-3)-[GalNAc(β1-4)]Gal(β1-4)GlcNAc(β1-3)Gal (**II.a**) and the precursor tetrasaccharide Neu5Ac(α2-3)Gal(β1-4)-GlcNAc(β1-3)Gal (**II.b**) released from THp of each of the four donors (A1, A2, B1, B2) were estimated along two routes. Mixtures of 2-aminobenzamide (2-AB)-labeled oligosaccharides were analyzed by high-performance liquid chromatography with fluorescence detection (HPLC-FD) on GlycoSepC and GlycoSepN, and mixtures of free oligosaccharides were analyzed by high-pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on CarboPac PA-1. The results are presented in Table III.

In Figure 4 the HPLC-FD fractionation patterns of Superdex-75 fraction II after labeling with 2-AB containing the Sd^a pentasaccharide (**II.a**) and the tetrasaccharide (**II.b**), related to the female pair of twins A (GlycoSepC, Figure 4A; GlycoSepN, Figure 4C) and the male pair of twins B (GlycoSepC, Figure 4B; GlycoSepN, Figure 4D), are presented. Following such a protocol, it is important to check the completeness of the derivatization reaction. As shown in Figure 2B, MALDI-TOF analysis in the negative-ion mode gave pseudo-molecular ions at *m/z* 1157.64 ([M–H]$^-$) and *m/z* 954.65 ([M–H]$^-$), corresponding to 2-AB-labeled **II.a** and **II.b**, respectively. No indications for non-2-AB-labeled components were found, indicating a complete derivatization.

Table III. Molar ratio determinations of Sd^a pentasaccharide (**II.a**) and precursor tetrasaccharide (**II.b**), released from THp samples A1, A2, B1, and B2 of two monozygotic pairs of twins

	Molar ratio (%)			
	HPAEC-PAD		HPLC-FD	
Donors	**II.a**	**II.b**	**II.a**	**II.b**
Female twin A1	27	73	38	62
Female twin A2	25	75	37	63
Male twin B1	40	60	47	53
Male twin B2	41	59	48	52
Male HR	50	50		
Male CF	35	65		
Male PM	41	59		
Male YB	28	72		

II.a, Neu5Acα(2-3)[GalNAc(β1-4)]Gal(β1-4)GlcNAc(β1-3)Gal. **II.b**, Neu5Acα(2-3)Gal(β1-4)GlcNAc(β1-3)Gal. Values have been determined using HPAEC-PAD of free oligosaccharides (no molar adjustment factors) and using HPLC-FD of 2-AB-labeled oligosaccharide derivatives. For comparison, HPAEC-PAD literature data of four male donors have been included (van Rooijen *et al.*, 1998a).

As is evident from the GlycoSepC patterns in Figure 4A and 4B, the derivatized oligosaccharides partly overlap, with retention times of 12.1 and 12.5 min for components **II.a** and **II.b**, respectively, and are therefore not suited for the determination of the molar ratios. The GlycoSepN patterns show a much better resolution, allowing a quantitative analysis of the molar ratios of **II.a** and **II.b**. The retention time of **II.b** is 21 min (4.2 glucose units [gu]) and that of **II.a** 25 min (4.7 gu) (Figure 4C and 4D). The assignments are in agreement with empirical rules when using a glucose units ladder of hydrolyzed dextran (β1-4-linked GalNAc: 0.59 gu in Wing *et al.*, 2001). Based on integrated peak areas of three injections, the following percentages were found for the Sd^a pentasaccharide: pair of twins A, $37.7\% \pm 0.8\%$ (A1) and $37.3\% \pm 0.8\%$ (A2); pair of twins B, $46.8\% \pm 0.8\%$ (B1) and $47.7\% \pm 0.8\%$ (B2). These data clearly show identical molar ratios of Sd^a pentasaccharide and precursor tetrasaccharide for THp A1 and THp A2, as well as for THp B1 and THp B2.

In Figure 5 the HPAEC-PAD fractionation patterns of Superdex-75 fraction II, containing the Sd^a pentasaccharide **II.a** and the tetrasaccharide **II.b** (van Rooijen *et al.*, 1998a), related to the female pair of twins (Figure 5A) and the male pair of twins (Figure 5B), are presented. Based on integrated peak areas of three injections relative

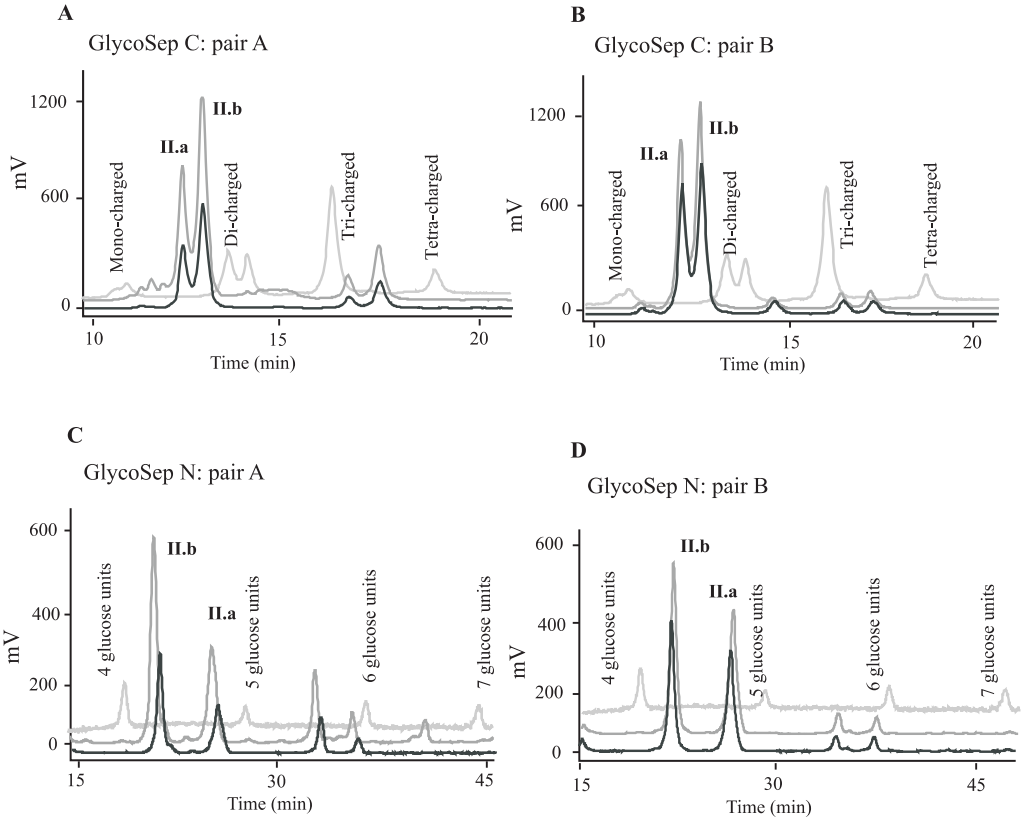

Fig. 4. HPLC Fractionation patterns of Superdex-75 fractions II after derivatization with 2-AB on GlycoSepC and GlycoSepN with fluorescence detection. (**A**) GlycoSepC: THp A1 (gray) and A2 (black) derived samples; (**B**) GlycoSepC: THp B1 (gray) and B2 (black) derived samples; (**C**) GlycoSepN: THp A1 (gray) and A2 (black) derived samples; (**D**) GlycoSepN: THp B1 (gray) and B2 (black) derived samples. For GlycoSepC chromatography, fetuin oligosaccharides were used as standard (light gray) for calibration according to their sialic acid charges. For elution details, see *Materials and methods*. **II.a**: Neu5Ac(α2-3)[GalNAc(β1-4)]Gal(β1-4)GlcNAc(β1-3)Gal; **II.b**: Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal.

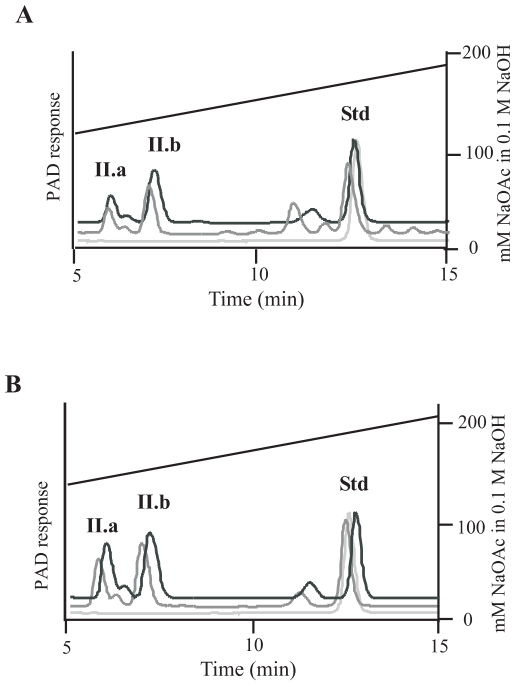

Fig. 5. HPAEC Fractionation patterns of Superdex-75 fractions II on CarboPac PA-1 with PAD. (**A**) THp A1 (gray) and A2 (black) derived samples, Std = internal standard galacturonic acid (light gray); (**B**) THp B1 (gray) and B2 (black) derived samples, Std = internal standard galacturonic acid (light gray). For elution details, see *Materials and methods*. **II.a**: Neu5Ac(α2-3)[GalNAc(β1-4)]Gal(β1-4)GlcNAc(β1-3)Gal; **II.b**: Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal.

to an internal standard the following percentages were found for the Sd^a pentasaccharide: pair of twins A, 26.8% ± 1.5% (A1) and 24.9% ± 1.5% (A2); pair of twins B, 39.6% ± 1.5% (B1) and 41.2% ± 1.5% (B2). These data also clearly indicate the equality of THp A1 and A2, and of THp B1 and B2, when focused on the molar ratio of the Sd^a pentasaccharide and the precursor tetrasaccharide.

Comparison of the quantitative data from the HPLC-FD and the HPAEC-PAD approaches show systematic differences, which, however, do not influence the final conclusions. In view of the type of detection used in HPAEC-PAD, absolute quantification of oligosaccharides based on peak areas should be considered with care. Different oligosaccharides can have completely different molar responses (Townsend *et al*., 1988). In the present study no molar adjustment factors for corrections were available for the individual components in the determination by HPAEC-PAD. Fluorescently labeled structures are quantified on the basis of the label absorbance, whatever the original molecule may be. This means that the values obtained by HPLC-FD can be considered as more reliable than those obtained by HPAEC-PAD.

Discussion

Extensive studies on the THp glycosylation have shown an impressive panel of N- and O-glycan structures (Easton *et al*., 2000; Hård *et al*., 1992; van Rooijen *et al*., 1998a,b, 1999). The occurrence of donor specificity in male THp has been demonstrated for dimeric *N*-acetyllactosamine sequences with or without the Sd^a epitope (van Rooijen *et al*., 1998a) and for oligomannose-type N-glycans (see van Rooijen *et al*., 2001, and references cited therein). For females a pregnancy-associated variability has been reported for the total amount and type of oligomannose-type N-glycans (Smagula *et al*., 1990; van Rooijen *et al*., 2001), whereas O-glycans undergo a change from core 1-type to core 2-type (Easton *et al*., 2000). Furthermore glycosylation changes have been reported in relation to age (Goodall and Marshall, 1980; Reinhart *et al*., 1991), malignancy (Olczak *et al*., 1999), and renal deficiency (Storch *et al*., 1992; Torffvit *et al*., 1998).

In an earlier study we reported on the donor specificity in relation to the Sd^a epitope expression for THp in four non-genetically related male donors, yielding HPAEC-PAD molar ratios for **II.a**:**II.b** of 50:50, 35:65, 41:59, and 28:72, respectively (van Rooijen *et al*., 1998a). In the present study the Sd^a pentasaccharide (**II.a**):tetrasaccharide (**II.b**) molar ratios in THp samples from a female (pair A) and a male (pair B) monozygotic pair of twins were estimated, being 38:62 (HPLC-FD)/26:74 (HPAEC-PAD) for pair A and 47:53 (HPLC-FD)/40:60 (HPAEC-PAD) for pair B. As discussed, the ratios obtained via the HPLC-FD approach are considered more reliable. Overall it can be concluded that the ratio between the two motifs is conserved for monozygotic pairs of twins.

The final step in the biosynthesis of the Sd^a determinant consists of the transfer of a GalNAc residue from UDP-GalNAc to O-4 of the Gal unit in a Neu5Ac-(α2-3)Gal(β1-4)GlcNAc(β1- sequence and is catalyzed by a specific β-1,4-*N*-Acetylgalactosaminyltransferase. The substrate specificity of the transferase is very high, that is, the presence of a sialic acid residue at O-3 of the Gal unit is a prerequisite for *N*-acetylgalactosaminylation (Serafini-Cessi and Dall'Olio, 1983; Serafini-Cessi *et al*., 1986). β-1,4-*N*-Acetylgalactosaminyltransferase activity has been demonstrated in microsomal preparations of human and guinea pig kidneys (Piller *et al*., 1986; Serafini-Cessi *et al*., 1986), whereas a soluble form was found in human colon carcinoma cells (Serafini-Cessi *et al*., 1995). Conservation of the molar ratio of the Sd^a pentasaccharide and the substrate tetrasaccharide in monozygotic pairs of twins, as found in the present study, reveals that the regulation of the β-1,4-*N*-acetylgalactosaminyltransferase activity results in a very closely related pattern of glycosylation in genetically homogeneous individuals when it comes to the conversion of the Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1- into the Neu5Ac(α2-3)[GalNAc(β1-4)]Gal(β1-4)GlcNAc(β1-3)-Gal(β1- sequence.

Materials and methods

General

For each of the four donors (two pairs of twins), THp was isolated from pooled morning urine as described (Serafini-Cessi *et al*., 1989), and checked for purity by SDS–PAGE and MALDI-TOF MS (matrix: 10 mg/mL

α-cyano-4-hydroxy-cinnamic acid in water) (Beavis and Chait, 1996) using a Voyager-DE PerSeptive Biosystems instrument. Monosaccharide analysis of intact THp samples and quantification of endo-β-galactosidase-released oligosaccharides were performed in triplicate by gas-liquid chromatography (Kamerling and Vliegenthart, 1989).

Endo-β-galactosidase digestion and oligosaccharide isolation

To 50 mg lyophilized THp, dissolved in 50 mL 30 mM sodium acetate buffer pH 5.9, were added 25 mU endo-β-galactosidase (from *Bacteroides fragilis*). The mixture was incubated for 48 h at 37°C then fractionated on a Superdex-75 column (1.6 × 60 cm, Pharmacia) using a FPLC LCC-500 system (Pharmacia, Uppsala, Sweden). The elution was carried out with 50 mM NH_4HCO_3 at a flow rate of 1 mL/min and monitored by UV absorbance at 214 nm (Pharmacia UV-1/214). UV-positive fractions were lyophilized and checked for the presence of carbohydrate by MALDI-TOF MS and 500-MHz 1D ^{1}H-NMR spectroscopy. MALDI-TOF MS measurements were carried out in the negative-ion mode (matrix: 10 mg/mL 2,4,6-trihydroxyacetophenone in water:acetonitrile = 1:1) (Papac *et al.*, 1996) on a Voyager-DE PerSeptive Biosystems instrument operating at an accelerating voltage of 20 kV (grid voltage 90%, ion guide wire voltage 0.03%) and equipped with a VSL-337ND-N_2 laser. ^{1}H-NMR measurements were carried out on a Bruker DRX-500 instrument in D_2O at 300 K with suppression of the residual water signal by applying a water-eliminated Fourier transform pulse sequence (Hård *et al.*, 1992).

Prior to further analysis, the carbohydrate-containing fraction II was redissolved in 0.5 mL water and divided into five 100-μL aliquots.

Determination of the total amount of sialyloligosaccharides in fraction II for each donor was carried out by gas-liquid chromatography. The amounts calculated for the THp A1/A2 and B1/B2 samples are 865/670 and 771/886 μg oligosaccharides/50 mg glycoprotein, respectively, showing only minor differences for the members of each pair of twins, being different from values obtained for other donors (van Rooijen *et al.*, 1998a).

Fluorescent labeling of oligosaccharides with 2-AB and analysis by HPLC

Oligosaccharides were fluorescently labeled with 2-AB as described (Bigge *et al.*, 1995). Briefly, 31.75 mg $NaCNBH_3$ were added to 23.6 mg 2-AB, dissolved in 500 μL dimethyl sulfoxide containing 30% acetic acid. Lyophilized oligosaccharide samples (10 μL of a 100-μL aliquot of a carbohydrate-positive Superdex-75 fraction) in 6 μL water were mixed with 8 μL of this solution and incubated for 2 h at 65°C. For cleaning up the mixture, two disks of QM-A quartz microfibre filters (Whatman) were placed at the bottom of small syringe-shaped glass holders. The filters were washed with 1 mL water, 1 mL 30% acetic acid, and 1 mL acetonitrile; the carbohydrate-containing solutions were loaded and left to dry for 15 min. After washing with 8 mL acetonitrile, the mixtures of labeled oligosaccharides were eluted with 4 × 0.5 mL water, and the combined eluates were lyophilized and redissolved in 40 μL water.

For checking the quantitative conversion of the labeling procedure, MALDI-TOF MS analysis in the negative-ion mode (matrix: 10 mg/mL 2,5-dihydroxybenzoic acid in water) was performed on a small part of each 2-AB-labeled sialyloligosaccharide mixture.

Molar ratios of endo-β-galactosidase-released, 2-AB-labeled tetra- and Sd[a] pentasaccharides were determined in triplo on GlycoSepC (4.6 × 100 mm) and GlycoSepN (4.6 × 100 mm) columns (Oxford Glycosciences) using a Waters 2690 XE instrument equipped with a Waters 474 fluorescence detector ($\lambda_{exc.max} = 373$ nm, $\lambda_{em.max} = 420$ nm). For weak anion exchange chromatography on GlycoSepC, elutions were carried out using 20% acetonitrile in water (v/v) (solvent A) and 20% acetonitrile:30% water (v/v):50% 500 mM ammonium formate pH 4.4 (solvent B). Gradient conditions were as follows: $t = 0$ min, 100% A and 0% B; $t = 40$ min, 0% A and 100% B; $t = 45$ min, 0% A and 100% B; $t = 46$ min, 100% A and 0% B; $t = 60$ min, 100% A and 0% B. The total run time was 60 min, and the flow rate was 0.4 mL/min. For normal-phase chromatography on GlycoSepN, elutions were carried out using 80% acetonitrile : 20% 50 mM ammonium formate pH 4.4 (v/v) (solvent C), and 50 mM ammonium formate pH 4.4 (solvent D). Gradient conditions were as follows: $t = 0$ min, 93.5% C and 6.5% D; $t = 100$ min, 56.2% C and 43.8% D; $t = 104$ min, 0% C and 100% D; $t = 109$ min, 0% C and 100% D; $t = 111$ min, 93.5% C and 6.5% D; $t = 140$ min, 93.5% C and 6.5% D. The total run time was 140 min, and the flow rate was 0.8 mL/min.

Oligosaccharide analysis by HPAEC

Molar ratios of endo-β-galactosidase-released tetra- and Sd[a] pentasaccharides were determined by HPAEC-PAD. The Dionex LC instrument consisted of a Dionex Bio LC quaternary gradient module, a PAD 2 detector, and a CarboPac PA-1 pellicular anion-exchange column (25 × 0.9 cm, Dionex, Sunnyvale, CA). For the analysis, 5 μL of a 100-μL aliquot of carbohydrate-positive Superdex-75 fraction was used. Elutions were carried out with a linear concentration gradient of sodium acetate in 0.1 M NaOH as shown in the figures, at a flow rate of 4 mL/min. Galacturonic acid (Sigma, St. Louis, MO) in a final concentration of 0.3 mg/mL was added to the samples as an internal standard (Thurl *et al.*, 1996). Molar ratios were determined by comparison of integrated peak areas from three injections (van Rooijen *et al.*, 1998a).

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Abbreviations

2-AB, 2-aminobenzamide; GPI, glycosylphosphatidyl inositol; gu, glucose unit; HPLC-FD, high-performance liquid chromatography with fluorescence detection;

HPAEC-PAD, high-pH anion exchange chromatography with pulsed amperometric detection; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry; NMR, nuclear magnetic resonance; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; THp, Tamm-Horsfall glycoprotein.

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