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The Linear Tetrasaccharide, Galβ1-4GlcNAcβ1-6Galβ1-4GlcNAc, Isolated from Radiolabeled Teratocarcinoma Poly-*N*-acetyllactosaminoglycan Resists the Action of *E. freundii* Endo-β-galactosidase

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A novel linear tetrasaccharide, Galβ1-4GlcNAcβ1-6Galβ1-4GlcNAc, was isolated from partial acid hydrolysates of metabolically labeled poly-*N*-acetyllactosaminoglycans of murine teratocarcinoma cells. It was characterized by exo-glycosidase sequencing and by mild acid hydrolysis followed by identification of all partial cleavage products. The tetrasaccharide, and likewise labelled GlcNAcβ1-6Galβ1-4GlcNAc, resisted the action of endo-β-galactosidase (EC 3.2.1.103) from *E. freundii* at a concentration of 125 mU/ml, while the isomeric, radioactive teratocarcinoma saccharides Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc and GlcNAcβ1-3Galβ1-4GlcNAc were cleaved in the expected manner.

Endo-β-galactosidase (EC 3.2.1.103) from *Escherichia freundii* hydrolyzes the β(1-4)-linkage between galactose and 2-acetamido-2-deoxyglucose within the unbranched sequence GlcNAcβ1-3Galβ1-4GlcNAc [1], which occurs commonly in poly-*N*-acetyllactosaminoglycans, keratan sulfates and glycosphingolipids. The enzyme has gained importance in analytical degradations [2-4] although many of these saccharides are hydrolyzed only partially [2, 5, 6]. The partial cleavage has been attributed to a relative inability of the enzyme to hydrolyze the β(1-4)-bond in the branched sequences GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAc [7, 8], the bonds involving a sulfated galactose residue [1], and the internal galactosidic linkages in close proximity to fucosylated residues [9, 10].

The present report adds the linear sequence GlcNAcβ1-6Galβ1-4GlcNAc to the list of groups resistant to *E. freundii* endo-β-galactosidase. This finding emphasizes the role of the

Abbreviations: WGA, wheat germ agglutinin; BSA, bovine serum albumin; [^{3}H]GlcNAcβ1-4-GlcNAcβ1-4GlcNAcOL, *N,N′,NN″*-triacetylchitotriose reduced with NaB^3H_4.

"6-branches" in the resistant backbone sequences of poly-*N*-acetyllactosaminoglycans; the "3-branches" in the branching galactose units may be of much lesser importance in generating the endo-β-galactosidase resistance.

The tri- and tetrasaccharides used in the present experiments originated from radiolabeled poly-*N*-acetyllactosaminoglycan of murine teratocarcinoma cells; the trisaccharides have been described before [11], while the tetrasaccharides are characterized here for the first time. An essential part of the characterization process was the generation and isolation of the middle disaccharide unit, a GlcNAcβGal sequence, from both tetrasaccharides. This disaccharide was then identified as GlcNAcβ1-6Gal in one of the tetrasaccharides and as GlcNAcβ1-3Gal in the other.

Materials and Methods

Reference Saccharides

Radiolabeled markers, [^{14}C]Galβ1-4GlcNAc, GlcNAcβ1-3[^{14}C]Gal, GlcNAcβ1-3[^{14}C]Galβ1-4GlcNAc, GlcNAcβ1-6[^{14}C]Gal and GlcNAcβ1-6[^{14}C]Galβ1-4GlcNAc were obtained as described [11]. [^{14}C]Galβ1-4GlcNAcβ1-3[^{14}C]Gal was obtained by endo-β-galactosidase digestion of radioactive poly-*N*-acetyllactosaminoglycan of PC13 cells [12]. [^{14}C]Galβ1-4GlcNAcβ1-6Gal was synthesized by galactosylating GlcNAc-β1-6Gal with UDP-[U-^{14}C]Gal (Amersham Internat., Amersham, U.K.) and *N*-acetyllactosamine synthase (EC 2.4.1.90) (Sigma, St Louis, MO, USA) as described elsewhere (O. Renkonen *et al.*, manuscript in preparation). [^{3}H]Galβ1-3GlcNAcβ1-3-[^{3}H]Gal was obtained by endo-β-galactosidase digestion of radioactive poly-*N*-acetyllactosaminoglycans of human teratocarcinoma cells of line PA1 (O. Renkonen *et al.*, unpublished data). [^{3}H]GlcNAcβ1-4GlcNAcβ1-4GlcNAcOL was prepared by treating *N,N´,N´´*-triacetylchitotriose with NaB^3H_4. [^{14}C]Galactose was from Amersham. Unlabeled markers, threose, lyxose, 2-acetamido-2-deoxyglucose, GlcNAcβ1-6Gal, Galβ1-3GlcNAc, Galβ1-6GlcNAc,and *N,N´,N´´*-triacetylchitotriose were purchased from Sigma. Galactose was from Merck (Darmstadt, W. Germany) and maltotriose from Boehringer (Mannheim, W. Germany).

Partial Acid Hydrolysis

Partial acid hydrolysis was carried out by dissolving the labeled saccharide in 0.20 ml of 0.1 M trifluoroacetic acid in a 10 ml tube, and by incubating the solution 100°C for 40 min. At the end of the reaction the solution was quickly cooled in ice, transferred to a rotary evaporator with 3 ml water and evaporated to dryness.

Periodate Oxidation of GlcNAcβ1-3[U-^{14}C]Gal

Periodate oxidation of the labeled disaccharide at pH 3.6, and subsequent acid hydrolysis of the oxidation product were carried out by a procedure adapted from a description by Hough [13]: The dry, labeled disaccharide was dissolved in 200 µl of 0.015 M sodium metaperiodate in 0.2 M sodium acetate buffer, pH 3.6 and incubated in darkness at room temperature overnight. Excess periodate was destroyed by adding 100 µl of 0.3 M aqueous ethylene glycol and incubating at 50°C for 2 h. The reaction mixture was then passed through

a mixed bed of Dowex AG 1 (AcO^-) and Dowex AG 50 (H^+), and the eluate was lyophilized. The desalted reaction product was hydrolyzed with 200 µl of 1 M HCl at 100°C for 4 h, and the acid was removed by passage through a column of Dowex AG 1 (AcO^-). The hydrolysate was finally analyzed by paper chromatography.

Chromatographic Methods

Bio-Gel P-10 chromatography was carried out as described [5]. Chromatography on agarose-bound wheat germ agglutinin (WGA) was carried out as described elsewhere [11]. An immobilized mixture of *Griffonia simplicifolia* I isolectins (Pharmacia, Sweden) was used for chromatography, essentially as described by Blake and Goldstein [14].

Chromatography on Whatman No. 3 paper was carried out with the upper phase of *n*-butanol/acetic acid/water, 4/1/5 by vol, (Solvent A); with *n*-butanol/ethanol/water, 10/1/2 by vol (Solvent E), or with ethyl acetate/pyridine/water 2/1/2 by vol, (Solvent C); the running times varied from 16 h for monosaccharides in Solvent A to 12 days for tri-saccharides in Solvent E. Borate impregnated papers were prepared and run as described [15]. The radioactivity in paper chromatograms was analyzed by cutting the 3 cm wide lanes into strips of 3 x 1 cm that were counted in a cocktail containing 5.5 g Permablend (United Technologies Packard, Downers Grove, IL, USA). in 1 l of toluene; after counting, the strips were washed with petroleum ether, dried and extracted with water to recover the saccharides.

HPLC using system I of Blanken *et al.* [16] was carried out as described earlier [11].

Exoglycosidase Digestions

Cleavage with jack bean β-galactosidase (EC 3.2.1.23) (Sigma) was carried out in 50 µl reaction mixtures containing 2.5 U/ml of the enzyme and 0.05 M sodium citrate, pH 4.0. Incubations were per-formed at 37°C for 5 h. Under these conditions the control Galβ1-4[^{3}H]GlcNAc was cleaved almost completely, but [^{3}H]GlcNAcβ1-3Galβ1-4[^{3}H]GlcNAc remained intact.

Cleavage with *D. pneumoniae* β-galactosidase (Boehringer) was carried out essentially according to the method of Hughes and Jeanloz [17]. The labeled saccharide was dissolved in 50 µl of 0.1 M sodium citrate buffer, pH 6.2, and 2 mU of the enzyme in 2 µl of 20 mM sodium cacodylate, 10 mM sodium azide, 0.1% BSA were added, followed by a drop of toluene. The mixture was incubated at 37°C overnight. Under these conditions [U-^{14}C]Galβ1-4GlcNAc was completely cleaved, but the trisaccharide[^{3}H]Galβ1-3GlcNAcβ1-3[^{3}H]Gal remained intact.

Endo-β–galactosidase Digestion

Teratocarcinoma oligosaccharides were digested in 60 µl reaction mixtures containing 125 mU/ml of endo-β-galactosidase from *E. freundii* (Seikagaku, Tokyo), 0.1 M sodium acetate buffer, pH 5.8, 0.07 M NaCl and 30 mM O-galactono-1,4-lactone. One drop of toluene was added and the mixture was incubated at 37°C for 24 h. The teratocarcinoma tetrasaccharide, Galβ1-4[^{3}H]GlcNAcβ1-6Galβ1-4[^{3}H]GlcNAc, was incubated further by adding 60 µl of a

Table 1. Paper Chromatographic mobilities of markers and key saccharides from degradation experiments.

Saccharide	Origin of saccharide	R_{Gal}[a] Solv.A(Solv.E)	R_{Lact}[a] Solv.A(Solv.E)	R_{MT}[a] Solv.A(SolvE)	R_{MTet}[a] Solv.A	R_{MP}[a] Solv.A
Radiolabeled markers						
Galβ1-4GlcNAc	PC13EC[b]	0.86(0.50)	1.43(1.83)			
GlcNAcβ1-3Gal	—"—	0.72	1.22(1.42)	1.65(2.97)		
GlcNAcβ1-6Gal	—"—	0.60	1.05(0.91)	1.42(1.88)		
GlcNAcβ1-3Galβ1-4GlcNAc	—"—		1.06(0.74)	1.44(1.53)		
GlcNAcβ1-6Galβ1-4GlcNAc	—"—		0.89(0.49)	1.21(1.01)		
Galβ1-4GlcNAcβ1-3Gal	PC13EC[c]		0.70	0.94		2.40
Galβ1-4GlcNAcβ1-6Gal	*In vitro* synthesis[d]		0.57	0.77	1.26	
Unlabeled markers						
GlcNAc	Sigma	1.41				
Galβ1-3GlcNAc	Sigma	(0.58)	(2.12)			
Galβ1-6GlcNAc	Sigma	(0.37)	(1.32)			
GlcNAcβ1-6Gal	Sigma	0.61	1.00(0.90)	1.37(1.91)		
Saccharides related to Galβ1-4GlcNAcβ1-6Galβ1-4GlcNAc						
A. [^{14}C]Galβ1-4GlcNAcβ1-6Galβ1-4GlcNAc	PC13EC		0.51	0.68	1.12	1.70
B. [^{3}H]Galβ1-4GlcNAcβ1-6Galβ1-4GlcNAc	—"—		0.47	0.66		1.66
C. [^{14}C]GlcNAcβ1-6Galβ1-4GlcNAc	β-Galactosidase on glycan A.		0.88	1.24		
C-2.[^{14}C]GlcNAcβ1-6Gal	Partial acid hydrolysis of glycan C.		(0.90)	(1.87)		
C-3.[^{14}C]Galβ1-4GlcNAc	Partial acid hydrolysis of glycan C.		(1.76)			
D. [^{3}H]GlcNAcβ1-6Gal1β1-4GlcNAc	β-Galactosidase on glycan B.		0.90	1.23		3.1
D-2.[^{3}H]Galβ1-4GlcNAc	β-N-acetylhexosaminidase on glycan D.		0.86(0.48)	1.45(1.76)		
E. [^{14}C]Galβ1-4GlcNAcβ1-6Gal	Partial acid hydrolysis of glycan A.		0.59	0.80		
F. [^{14}C]GlcNAcβ1-6Galβ1-4GlcNAc	—"—		0.91	1.22		
G. [^{14}C]Galβ1-4GlcNAc	—"—	0.86(0.49)	1.39(1.88)			
H. [^{14}C]GlcNAcβ1-6Gal	β-Galactosidase on glycan E.	0.61	1.02	1.41		

Saccharides related to Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc						
I. [^{14}C]Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc	PC13EC			0.79	1.31	2.00
J. [^{3}H]Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc	—"—			0.79		1.97
K. [^{14}C]GlcNAcβ1-3Galβ1-4GlcNAc	β-Galactosidase on glycan I.	0.65	1.05	1.43		
L. [^{3}H]GlcNAcβ1-3Galβ1-4GlcNAc	β-Galactosidase on glycan J.	0.64	1.07	1.45		
M. [^{3}H]Galβ1-4GlcNAc	β-N-Acetylhexosaminidase on glycan L.	0.85(0.46)	1.43(1.68)			
N. [^{14}C]Galβ1-4GlcNAcβ1-3Gal	Endo-β-galactosidase on glycan I.			0.98		
O. [^{3}H]Galβ1-4GlcNAcβ1-3Gal	Endo-β-galactosidase on glycan J.		0.67	0.94		
P. [^{14}C]GlcNAcβ1-3Gal	β-Galactosidase on glycan N.	0.72	1.23(1.40)	(2.90)		
Q. [^{3}H]Galβ1-4GlcNAc	Partial acid hydrolysis on glycan O.	(0.47)	(1.65)			
Saccharides from Endo-β-galactosidase digests						
R. [^{14}C]GlcNAcβ1-6Galβ1-4GlcNAc	Endo-β-galactosidase digest of [^{14}C]GlcNAcβ1-6Galβ1-4GlcNAc		(0.49)	(1.04)		
S. [^{3}H]GlcNAcβ1-3Gal	Endo-β-galactosidase digest of [^{3}H]GlcNAcβ1-3Galβ1-4GlcNAc	0.73	1.23			

[a] R_{Gal}, R_{Lact}, R_{MT}, R_{MTet} and R_{MP} give migration rates in relation to those of galactose, lactose, maltotriose, maltotetraose and maltopentaose, respectively. Solv. A and Solv. E refer to Solvent A and Solvent E, respectively.

[b] Renkonen *et al.* [11].

[c] [12].

[d] Renkonen *et al.*, unpublished results.

fresh enzyme solution of the same composition after 24 h and again after 48 h; the reaction was terminated in this case after 72 h. The boiled reaction mixtures were desalted by filtration in water through Dowex AG-1 (AcO^-) and Dowex AG-50 (H^+), and analyzed by paper chromatography.

Results

Isolation and Characterization of Galβ1-4GlcNAcβ1-6Galβ1-4GlcNAc

Two radioactive versions of the title tetrasaccharide were obtained by partial acid hydrolysis of metabolically labeled poly-*N*-acetyllactosaminoglycan of murine embryonal carcinoma cells (line PC13) [12, 18]. The neutral saccharides of the digests were fractionated by consecutive use of chromatography on Bio-Gel P-10 and immobilized *Griffonia simplicifolia* I isolectins, followed by paper chromatography, HPLC and a combined treatment with β-*N*-acetylhexosaminidase and paper chromatography. Alternatively the last step consisted of chromatography on immobilized wheat germ agglutinin (WGA-agarose) according to the method of Renkonen *et al.* [11]. [U-^{14}C]Galβ1-4GlcNAcβ1-6[U-^{14}C]Galβ1-4GlcNAc was prepared from [U-^{14}C]galactose-labeled poly-*N*-acetyl-lactosaminoglycan, and Galβ1-4[6-^{3}H]GlcNAcβ1-6Galβ1-4[6-^{3}H]GlcNAc from 2-acetamido-2-deoxy-[6-^{3}H]glucose-labeled polysaccharide.

[U-^{14}C]Galβ1-4GlcNAcβ1-6[U-^{14}C]Galβ1-4GlcNAc was stable to the action of β-*N*-acetylhexosaminidase, but it was cleaved by β-galactosidase from jack bean to the extent of 95%, releasing an equimolar mixture of [U-^{14}C]galactose and GlcNAcβ1-6[U-^{14}C]Galβ1-4GlcNAc, which were separated by paper chromatography in Solvent A (Table 1). The tetrasaccharide was cleaved in a similar manner also by β-galactosidase from *Diplococcus pneumoniae* (data not shown). Accordingly, there was a β(1-4)-linkage between the non-reducing end galactose and the penultimate GlcNAc residue [19].

The trisaccharide, GlcNAcβ1-6[U-^{14}C]Galβ1-4GlcNAc, obtained from the jack bean β-galactosidase digest, was cleaved by partial acid hydrolysis into two disaccharides, GlcNAcβ1-6[U-^{14}C]Gal and [U-^{14}C]Galβ1-4GlcNAc, that were separated by paper chromatography in Solvent A (Table 1). These disaccharides revealed paper chromatographic mobilities identical to those of authentic markers (Table 1). They were also cleaved by exoglycosidases in the expected manner (data not shown).

[6-^{3}H]GlcNAcβ1-6Galβ1-4[6-^{3}H]GlcNAc was obtained from the Galβ1-4[6-^{3}H]GlcNAcβ1-6Galβ1-4[6-^{3}H]GlcNAc by a jack bean β-galactosidase treatment (Table 1). It was cleaved by β-*N*-acetylhexosaminidase into an equimolar mixture of a labeled *N*-acetylhexosamine and Galβ1-4[6-^{3}H]GlcNAc, which were separated from each other by paper chromatography in Solvent A (Table 1). The former was identified as 2-acetamido-2-deoxy-[^{3}H]glucose by borate paper chromatography, which differentiated it from labeled 2-acetamido-2-deoxy-galactose (data not shown) [15]. The Galβ1-4[6-^{3}H]GlcNAc was differentiated from Galβ1-3GlcNAc and Galβ1-6GlcNAc by paper chromatography in Solvent E [20] (Table 1). It was cleaved completely by β-galactosidase from jack bean, and the liberated 2-acetamido-2-deoxy-[^{3}H]glucose was identified by borate paper chromatography (data not shown).

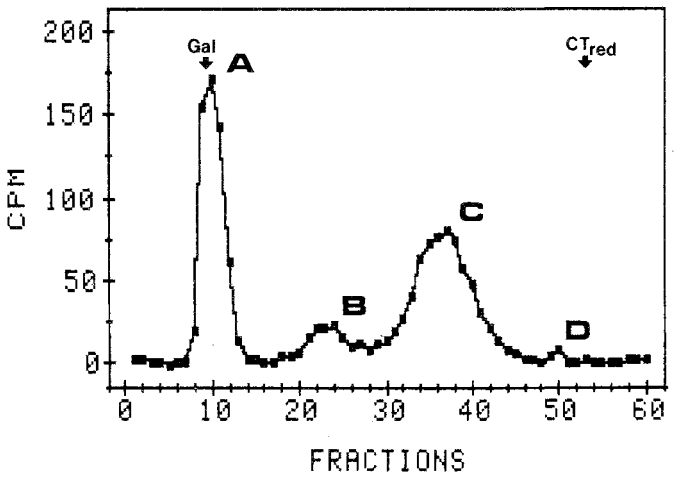

Figure 1. WGA-Agarose chromatography of a partial acid hydrolysate of [U-^{14}C]Galβ1-4GlcNAcβ1-6[U-^{14}C]Galβ1-4GlcNAc from PC13EC cells. A sample of 2150 cpm of the tetrasaccharide was partially hydrolyzed with 0.1 M trifluoroacetic acid and the desalted hydrolysate was chromatographed on a column (0.7 x 12 cm) of WGA-agarose (1.86 mg WGA per ml of 4% beaded agarose from Pharmacia/P.L.Biochemicals) in 10 mM sodium phosphate buffer, pH 7.1, containing 0.15 M NaCl and 0.02% NaN_3. The flow rate was 0.1 ml/min; fractions of 0.55 ml were collected and assayed for radioactivity as described elsewhere [5]. Tubes 1-40 were eluted with the equilibrating buffer, and tubes 41-70 with the same buffer containing 0.2 M 2-acetamido-2-deoxyglucose. Paper chromatography in Solvent A revealed that Peak A contained labeled galactose and Galβ1-4GlcNAc; Peak B contained GlcNAcβ1-6Galβ1-4GlcNAc and Galβ1-4GlcNAcβ1-6Gal; Peak C contained Galβ1-4GlcNAcβ1-6Gal and Galβ1-4GlcNAcβ1-6Galβ1-4GlcNAc; Peak D was assumed to contain GlcNAcβ1-6Gal. The arrows marked Gal and CT_{red} show the positions of the markers galactose and reduced N,N´,N´´-triacetylchitotriose, respectively.

Partial acid hydrolysis of [U-^{14}C]Galβ1-4GlcNAcβ1-6[U-^{14}C]Galβ1-4GlcNAc (2150 cpm) gave a mixture of six saccharides that were separated by WGA-agarose chromatography (Fig. 1) and subsequent paper chromatography in Solvent A. The saccharides isolated in pure form were:1, The starting tetrasaccharide (511 cpm); 2, [U-^{14}C]Galβ1-4GlcNAcβ1-6[U-^{14}C]Gal (167 cpm); 3, GlcNAcβ1-6[U-^{14}C]Galβ1-4GlcNAc (69 cpm); 4, GlcNAcβ1-6-[U-^{14}C]Gal (11 cpm); 5, [U-^{14}C]Galβ1-4GlcNAc (278 cpm); and 6, [U-^{14}C]Gal (243 cpm).

No other saccharides were detected in the partial acid hydrolysate. The glycans 1-3 and 5-6 above the expected behaviour on paper chromatography (see Table 1); in addition, all cleavage products migrated on WGA chromatography like the corresponding markers (Cf. Fig. 1 and [11]). Further, glycan 2 was cleaved by jack bean β-galactosidase to the extent of 95% releasing a 1/1/3 mixture of GlcNAcβ1-6[U-^{14}C]Gal and [U-^{14}C]Gal, which were separated from each other by paper chromatography in Solvent A (See Table 1). Treatment of the disaccharide with β–*N*-acetylhexosaminidase liberated [U-^{14}C]galactose that was identified by paper chromatography in Solvent A (data not shown). The intact disaccharide was run on HPLC using the system I of Blanken *et al.* [16]; it revealed an elution time

equivalent to 67% of that of the maltotriose marker (not shown). It was thus similar to the GlcNAcβ1-6Gal standard and different from GlcNAcβ1-4Gal and GlcNAcβ1-3Gal.

These findings establish the most likely structure of the teratocarcinoma tetrasaccharide as Galβ1-4GlcNAcβ1-6Galβ1-4GlcNAc.

Isolation and Characterization of Radioactive Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc

The title tetrasaccharide was isolated from partial acid hydrolysates of teratocarcinoma poly-*N*-acetyllactosaminoglycan by using the same set of chromatographic runs as described above. It was only slightly retarded on WGA-chromatography giving a sharp peak at tube 12 under conditions identical to those of Fig. 1 (data not shown).

[U-^{14}C]Galβ1-4GlcNAcβ1-3[U-^{14}C]Galβ1-4GlcNAc resisted the action of β-*N*-acetylhexosaminidase, but was cleaved to the extent of 88% by β-galactosidase from jack bean. The digest contained a 1.2:1 mixture of [U-^{14}C]galactose and GlcNAcβ1-3[U-^{14}C]Galβ1-4GlcNAc, which were separated from each other by paper chromatography in Solvent A (see Table 1). The tetrasaccharide was cleaved in a similar manner also by β-galactosidase from *Diplococcus pneumoniae* which shows that there was a β(1-4)-linkage between the non-reducing galactose and the penultimate *N*-acetylglucosamine residue [19] (data not presented).

[6-^{3}H]GlcNAcβ1-3Galβ1-4[6-^{3}H]GlcNAc was obtained by jack bean β-galactosidase treatment of the [^{3}H]-labeled tetrasaccharide (Table 1). It was cleaved by β-*N*-acetylhexosaminidase to the extent of 75% releasing a 1:1 mixture of *N*-acetyl-[6-^{3}H]hexosamine and Galβ1-4[6-^{3}H] GlcNAc; the cleavage products were separated from each other by paper chromatography in Solvent A (Table 1). Borate paper chromatography showed that the liberated *N*-acetylhexosamine was [6-^{3}H]GlcNAc (data not shown). The liberated disaccharide migrated ion paper chromatography (Solvent E) as Galβ1-4[6-^{3}H]GlcNAc; in this solvent it was clearly differentiated from Galβ1-3GlcNAc and from Galβ1-6GlcNAc (Table 1). The liberated disaccharide was cleaved by jack bean β-galactosidase to the extent of 90%, releasing labeled 2-acetamido-2-deoxyglucose which was isolated by paper chromatography in Solvent A, and identified subsequently by borate paper chromatography.

Galβ1-4[6-^{3}H]GlcNAcβ1-3Galβ1-4-[6-^{3}H]GlcNAc was cleaved by endo-β-galactosidase from *E. freundii* to the extent of 74%, releasing 125 cpm of labeled 2-acetamido-2-deoxyhexose and 170 cpm of Galβ1-4[6-^{3}H]-GlcNAcβ1-3Gal which were separated by paper chromatography in Solvent A (Table 1). The liberated 2-acetamido-2-deoxyhexose was identified as 2-acetamido-2-deoxy-[6-^{3}H]glucose by borate paper chromatography (data not shown).

[U-^{14}C]Galβ1-4GlcNAcβ1-3[U-^{14}C]Galβ1-4GlcNAc, too, was also cleaved by endo-β-galactosidase, releasing [U-^{14}C]Galβ1-4GlcNAcβ1-3[U-^{14}C]Gal was isolated by paper chromatography in Solvent A (see Table 1). This trisaccharide was cleaved by jack bean β-galactosidase from to the extent of 94% releasing a 1:1 mixture of [U-^{14}C]galactose and GlcNAcβ1-3[U-^{14}C]Gal that were separated by paper chromatography in Solvent A (Table 1). The disaccharide migrated like the GlcNAcβ1-3Gal marker in Solvent E (Table 1), and in Solvent C (data not shown); it was also cleaved by β–*N*-acetylhexosaminidase, releasing

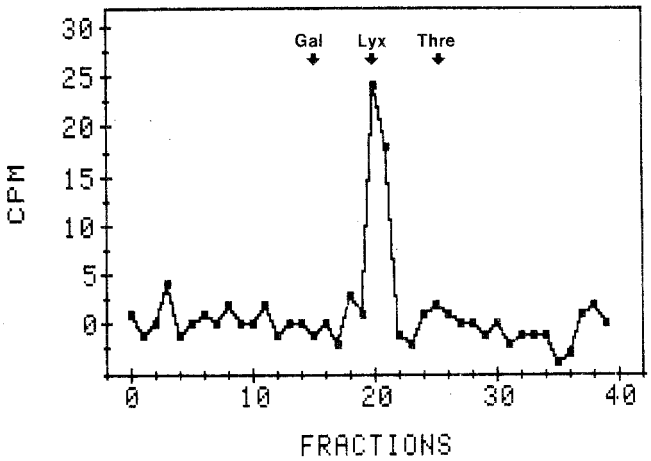

Figure 2. Paper chromatography of periodate cleavage products obtain-ed from the central disaccharide unit of [U-^{14}C]Galβ1-4GlcNAcβ1-3[U-^{14}C]Galβ1-4GlcNAc. The disaccharide was obtained by removing the reducing end *N*-acetylglucosamine of the tetrasaccharide by endo-β-galactosidase from *E. freundii*, and the non-reducing end galactose by β-galactosidase from jack bean. The [U-^{14}C]galactose-labeled disaccharide was then treated with periodate and subsequently hydrolyzed as described in "Materials and Methods" . The desalted reaction product was finally chromatographed on paper in Solvent A for 20 h and counted. The arrows marked Gal, Lyx, and Thre show peak positions of galactose, lyxose and threose, respectively.

[U-^{14}C]galactose which was identified by paper chromatography in Solvent A. The disaccharide was oxidized by periodate and then hydrolyzed according to the method of Hough [13]. This process converted the reducing end [U-^{14}C]galactose unit of the disaccharide into [U-^{14}C]lyxose, which was isolated in a yield of 26% by paper chromatography in Solvent A (Fig. 2); among the oxidation-hydrolysis products was very little label migrating like the threose marker. This experiment showed that the galactose unit of the disaccharide was substituted C-3 not at C-4 [6-^{3}H]GlcNAcβ1-3Gal, which had been obtained from the [^{3}H]-labeled tetrasaccharide by consecutive use of endo-β-galactosidase and β-galactosidase from jack bean, was cleaved by β-*N*-acetylhexosaminidase, releasing 2-acetamido-2-deoxy-[6-^{3}H]glucose, which was isolated by paper chromatography in Solvent A and identified subsequently by borate paper chromatography.

The linkage position between the non-reducing end galactose and the rest of the tetrasaccharide was established by partially cleavage of the endo-β-galactosidase liberated Galβ1-4[6-^{3}H]GlcNAcβ1-3Gal with acid. The digest revealed two tritiated disaccharides on paper chromatography in Solvent E; one of them migrated like Galβ1-4[6-^{3}H]GlcNAc (Table 1) and the other like [6-^{3}H]GlcNAcβ1-3Gal. The former was cleaved by jack bean β-galactosidase, releasing 2-acetamido-2-deoxy-[6-^{3}H]glucose as shown by borate paper chromatography. This experiment confirmed that the tetrasaccharide was linear, and that the non-reducing end consisted of a Galβ1-4GlcNAc sequence.

Taken together our data establish the most likely structure of the tetrasaccharide as Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc.

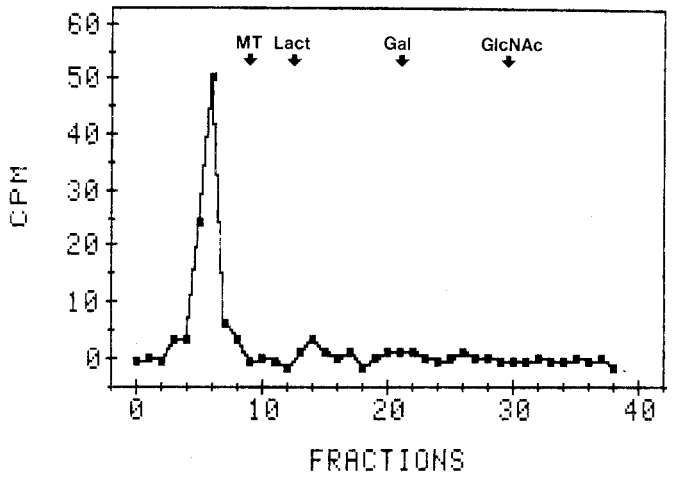

Figure 3. Paper chromatography of an *E. freundii* endo-β-galactosidase digest of Galβ1-4[6-^{3}H]GlcNAcβ1-6Galβ1-4[6-^{3}H]GlcNAc. A sample of 106 cpm of the tetrasaccharide was treated with the enzyme, and the reaction mixture was desalted as described in "Materials and Methods". The digest was then run with Solvent A for 40 h, and the dried chromatogram was counted for radioactivity. The arrows marked GlcNAc, Gal, Lact and MT show the positions of the markers 2-acetamido-2-deoxy-glucose, galactose, lactose and maltotriose, respectively.

Endo-β-galactosidase Treatment of Teratocarcinoma Oligosaccharides

Galβ1-4[6-^{3}H]GlcNAcβ1-6Galβ1-4[6-^{3}H]GlcNAc was treated with endo-β-galactosidase from *E. freundii* and the digest was analyzed by paper chromatography in Solvent A. Fig. 3 shows that no labeled 2-acetamido-2-deoxyglucose was liberated, and that the tetrasaccharide had remained intact.

GlcNAcβ1-6[U-^{14}C]Galβ1-4GlcNAc, isolated from a partial acid hydrolysate of teratocarcinoma cell poly-*N*-acetyllactosaminoglycan [11], was treated with endo-β-galactosidase from *E. freundii*, and the digest was analyzed by paper chromatography in Solvent E. The chromatogram revealed that the trisaccharide had remained completely intact during the enzyme treatment (Table 1), and that no label migrating as GlcNAc-β1-6[U-^{14}C]Gal had been formed (data not shown).

[6-^{3}H]GlcNAcβ1-3Galβ1-4[6-^{3}H]GlcNAc was cleaved by endo-β-galactosidase from *E. freundii* to the extent of 81% releasing a 1.2:1 mixture of [6-^{3}H]GlcNAcβ1-3Gal and [6-^{3}H]GlcNAc which were separated by paper chromatography in Solvent A (Table 1).

Both radioactive versions of Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc were cleaved by the endo-β-galactosidase at the internal galactosidic linkage as expected (see under Isolation and Characterization of Radioactive Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc).

Discussion

The present data show that a linear radioactive tetrasaccharide that was identified as Galβ1-4GlcNAcβ1-6Galβ1-4GlcNAc resisted the action of *E. freundii* endo-β-galactosidase when the enzyme was used at a concentration of 125 mU/ml. Under these conditions the enzyme cleaved a labeled isomeric tetrasaccharide, Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc, at the internal galactosidic linkage in the expected manner. The two tetrasaccharides were of comparable specific radioactivities, and were therefore incubated with the enzyme at similar concentrations. The structural characterization of the novel teratocarcinoma tetrasaccharide Galβ1-4GlcNAcβ1-6Galβ1-4GlcNAc, was carried out to a large extent by partial acid hydrolysis, which cleaved the glycosidic bonds in a fairly random manner. The resulting mixture of oligosaccharides was then fractionated by WGA-agarose chromatography, which gives useful separations of *N*-acetyllactosamine oligomers [11, 21]. Eventually all constituent di- and trisaccharide sequences obtainable from the tetrasaccharide could be isolated and identified.

Scudder *et al.* [8] have previously shown that several purified glycolipid substrates are not cleaved at internal Galβ1-4GlcNAc linkages by endo-β-galactosidase from *E. freundii* if the galactose carries *N*-acetylglucosamine substituents at C-6 and at C-3 to form a branch point. Fukuda *et al.* [7] have also reported analogous data by showing that the branched structure found in H_3-glycolipid was hydrolysed much less readily. Supporting these data, the branched radioactive tetrasaccharide, GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAc, resisted the enzyme in our laboratory (A. Seppo *et al* unpublished results). In the light of the present data it appears that the presence of an *N*-acetylglucosamine substituent at C-3 of the branching galactose is not the cause of for the inactivity of the endo-β-galactosidase towards poly- and oligo-*N*-acetyl-lactosaminoglycans. Rather, the resistance of these molecules appears to be caused by the presence of *N*-acetylglucosamine substituents at C-6 of the internal galactose units.

It is not yet clear that linear GlcNAcβ1-6Galβ1-4GlcNAc sequences are present as such in the native teratocarcinoma saccharides. It is possible that they were generated in our present experiments by a loss of the 3-branches during the partial acid hydrolysis step that was used to cleave the original polysaccharide. However, a sequence involving linear GlcNAcβ1-6Gal units was recently identified in human skim milk mucins [22]. The present data suggest that poly-*N*-acetyllactosaminoglycans may be screened for the presence of native linear GlcNAcβ1-6Galβ1-4GlcNAc sequences by analyzing their endo-β-galactosidase resistant oligosaccharides using WGA-agarose chromatography.

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