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GLYCOPROTEINS OF HUMAN TERATOCARCINOMA CELLS (PA1) CARRY BOTH ANOMERS OF *O*-GLYCOSYL-LINKED D-GALACTOPYRANOSYL-(1→3)-2-ACETAMIDO-2-DEOXY-α-D-GALACTOPYRANOSYL GROUP*

ANNE LEPPÄNEN, ARMI KORVUO, KIRSTI PURO, AND OSSI RENKONEN
Department of Biochemistry, Laboratory of Lipid Research, University of Helsinki, Unioninkatu 35, SF-00170 Helsinki (Finland)

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ABSTRACT

Two disaccharide alcohols, α-D-Gal*p*-(1→3)-GalNAcol and β-D-Gal*p*-(1→3)-GalNAcol, together with a GalNAcol-containing tetra- or penta-saccharide alcohol, were released from human embryonal carcinoma cells of line PA1 by reductive β-elimination. The disaccharides were identified by exoglycosidase digestions and by periodate oxidation. The results were confirmed by affinity chromatography of the disaccharide alcohols on immobilized *Bandeirea simplicifolia* lectin and by chromatography of the parent glycopeptides on immobilized peanut lectin.

INTRODUCTION

The proteins of undifferentiated mouse teratocarcinoma cells yield high-molecular-weight glycopeptides (M_r >7000) upon exhaustive Pronase digestion[1–4]. These molecules may be involved in the process of differentiation as they are not found in differentiated cells[1]. Undifferentiated human teratocarcinoma cells yield also analogous fractions of high-molecular-weight glycopeptides[5–8]. Structural analysis has suggested the presence of polylactosaminoglycans in the teratocarcinoma glycopeptides[1–10].

The biosynthetic pathways leading to the glycan chains of glycoproteins[11,12] and proteoglycans[13] suggest that the high- and low-molecular-weight sugar chains of the teratocarcinoma glycopeptides might share common, proximal structures. Therefore, we have analyzed a fraction of small glycopeptides obtained by Pronase treatment of human teratocarcinoma cells of line PA1 (ref. 14). Two different *O*-glycosyl-linked anomers of D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-D-galactose were isolated and identified.

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EXPERIMENTAL

Preparation of the glycopeptides. — PA1 cells were labeled metabolically with 2-amino-2-deoxy-D-[1-^{14}C]glucose, D-[1-^{14}C]galactose, 2-amino-2-deoxy-D-[6-^{3}H]glucose, or D-[1-^{3}H]galactose, and digested with Pronase as described[6,7]. Gel filtration of the labeled glycopeptides was performed as described[6].

Reference-standard glycopeptides and saccharides. — Antifreeze glycoprotein No. 7 from *Trematomus borchgrevinki* (ref. 15) was labeled[16] with D-galactose oxidase–NaB^3H_4 as described[17]. 2-Acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-D-galactitol was obtained by reductive β-elimination[18] of the labeled antifreeze glycoprotein. Maltotriose and maltopentaose were purchased from Boehringer Mannheim (FRG). *N*-Acetylserinol was obtained by *N*-acetylating[19] DL-serinol hydrochloride (Sigma, St. Louis, MO, U.S.A.). Authentic α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-D-Gal*p* and β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-D-Gal*p* were obtained from Dr. Michiko N. Fukuda.

2-Acetamido-2-deoxy-L-arabinitol was prepared from chondroitin sulfate from PA1 cells that had been labeled metabolically with 2-amino-2-deoxy-D-[1-^{14}C]glucose as follows: Treatment with chondroitin ABC lyase (EC 4.2.2.4) gave unsaturated, acidic disaccharides that were isolated by chromatography on a DEAE-Sephacel column and by chromatography on cellulose-coated aluminium plates as described[7]. 2-Acetamido-2-deoxy-3-*O*-(α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose 4-sulfate was then reduced with $NaBH_4$ and subjected to periodate oxidation according to Spiro[20]. The reaction product was hydrolyzed with 4M HCl at 100° for 4 h and, finally, the liberated aminoalcohol was *N*-reacetylated[19].

Reductive β-elimination. — Treatment of glycopeptides with 0.05M NaOH–M $NaBH_4$ (ref. 18) was carried out as described[21]; the liberated oligosaccharide alcohols were desalted with AG 50W (H^+) cation-exchange resin.

Affinity chromatography on peanut lectin–Sepharose. — Peanut lectin (Sigma) was coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions (10 mg of lectin/1 g of Sepharose). Chromatography on the immobilized lectin was carried out essentially according to Farrar *et al.*[22].

Affinity chromatography on Bandeirea simplicifolia *lectin–Agarose.* — Immobilized *Bandeirea simplicifolia* lectin (Pharmacia P-L Biochemicals, Uppsala, Sweden) was packed into a column (0.7 × 18 cm) and equilibrated with a phosphate-buffered saline solution[23] containing 0.15M NaCl, 0.1M NaK_2HPO_4–KH_2PO_4 buffer (pH 7.0), 0.1mM $CaCl_2$, and 0.04% NaN_3. Chromatography was carried out with the same buffer; the elution rate was 2 mL/h and fractions (0.5 mL) were collected.

Other chromatographic procedures. — DEAE-cellulose chromatography was performed with 0.7 × 16 cm or 0.7 × 4 cm columns of DEAE-Sephacel (Pharmacia), equilibrated with 50mM pyridine formiate[24] (pH 5.0).

Separation of 2-acetamido-2-deoxyglucitol and 2-acetamido-2-deoxygalactitol on borate-impregnated paper was carried out as described[25]. Ordinary paper chromatography of desalted oligosaccharides was carried out on Whatman No. 3 paper by use of the upper phase of 4:1:5 (v/v) butanol–acetic acid–water (solvent *A*), 6:4:3 (v/v) butanol–pyridine–water (solvent *B*), or 10:1:2 (v/v) butanol–ethanol–water (solvent *C*). Unlabeled saccharides were detected with the aniline phthalate or with the alkaline $AgNO_3$ reagent, and radioactive compounds were located by liquid-scintillation counting of 1 × 3 cm pieces cut out from the chromatograms; these pieces are called fractions in the figures of the present report. In analytical experiments, the radioactivity of the paper pieces was counted in Triton X-114–xylene–Permablend® as described[7], but in preparative chromatograms the radioactivity counting was performed in toluene–Permablend. All the relevant markers were run each time on the same paper with the samples under study.

Thin-layer chromatography of 4′,5′-unsaturated disaccharides from chondroitin sulfate was carried out as described[7].

β-D-Galactosidase treatment. — Incubation with β-D-galactosidase (EC 3.2.1.23) from *E. coli* (Sigma) was performed as described[26] by use of 50 units of the enzyme and 0.1 mL of the buffer; after a 96-h incubation at 37°, fresh enzyme (50 units) was added in 0.05 mL of buffer and the incubation was continued for another 96 h. The reaction was terminated by boiling for 3 min and the mixture was desalted by passage through coupled columns of AG 50W (H^+) and Dowex AG 1 (AcO^-) ion-exchange resins.

α-D-Galactosidase treatment. — Treatment with α-D-galactosidase (EC 3.2.1.22) from green coffee beans (Sigma) was carried out essentially as described[27]. For partial degradation (21%) of the BS-2 disaccharide alcohol, the enzyme (2.5 units) in 3.2M $(NH_4)_2SO_4$ suspension was added to the sample that was dissolved in citrate–phosphate buffer (pH 6.6). The total reaction volume was 80 μL. The incubation was at 37° for 4 h. The reaction was terminated and the reaction mixtures were desalted as described above. Authentic α-D-Gal-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-D-Gal*p* was degraded under these conditions, but authentic β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-D-Gal*p* was not. For complete degradation of the BS-2 disaccharide alcohol, 1.1 unit of the enzyme in 50 μL of the reaction mixture was used for 22 h. Authentic β-D-Gal*p*-(1→3)GalNAcol was completely resistant under these conditions.

Periodate oxidation. — The disaccharide alcohols were treated with HIO_4 and subsequently reduced with $NaBH_4$ and hydrolyzed with acid according to Spiro[20]. The resulting amino alcohols were *N*-reacetylated[19] and analyzed by paper chromatography.

RESULTS

Metabolic labeling and isolation of protein-bound disaccharides. — Human

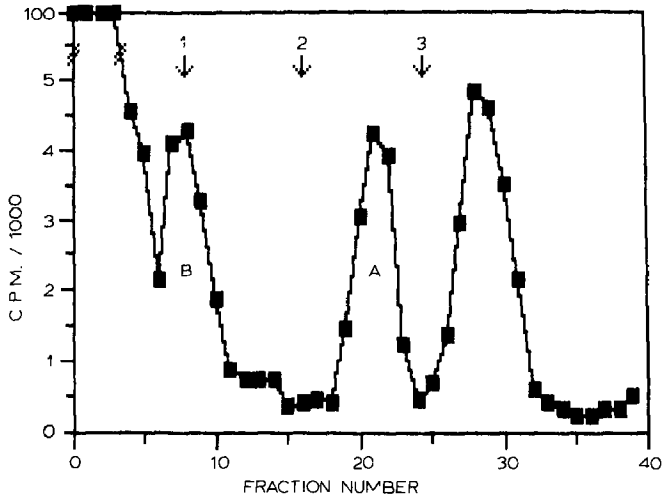

Fig. 1. Paper chromatography of neutral oligosaccharides liberated from PA1 cell glycoproteins. Low-molecular-weight glycopeptides ($800 < M_r < 2000$) obtained by Pronase digestion of 2-amino-2-deoxy-D-[1-^{14}C]glucose-labeled PA1 cells were treated with NaOH–$NaBH_4$ according to Carlson[18], and the liberated oligosaccharide alcohols desalted and separated into neutral and acidic fractions, and finally the neutral fraction was chromatographed on paper with solvent (*B*) for 21 h. Peak A contained reduced disaccharides and Peak B higher-molecular-weight oligosaccharide alcohols, all of which were originally *O*-glycosyl-linked to teratocarcinoma proteins. Fractions 25–32 contained 2-acetamido-2-deoxygalactitol and 2-acetamido-2-deoxyglucitol, both of which were probably derived from serine- or threonine-linked 2-acetamido-2-deoxyhexose residue[28]. Marker positions shown by arrows are: (1) maltopentaose, (2) maltotriose, and (3) D-galactose.

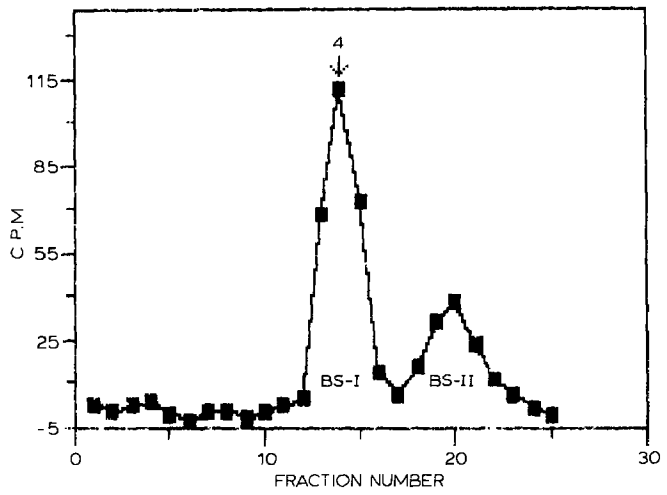

Fig. 2. Chromatography of the ^{14}C-disaccharide alcohols of Peak A in Fig. 1 on immobilized *Bandeirea simplicifolia* lectin. Marker No. 4 is D-xylose.

embryonal carcinoma cells[14] of line PA1 were labeled with radioactive 2-amino-2-deoxy-D-glucose or with D-galactose, digested exhaustively with Pronase, and chromatographed on Bio-Gel P-10 to give labeled glycopeptide fractions of low-molecular-weight ($800 < M_r < 2000$). These glycopeptides were treated with 0.05M NaOH–M $NaBH_4$ in order to release the *O*-glycosyl-linked saccharides in reduced form. The liberated oligosaccharide alcohols were then desalted and chromatographed on a DEAE-cellulose column to separate a fraction of neutral molecules from acidic glycans. The neutral oligosaccharides were finally chromatographed on paper, and the fraction of reduced disaccharides (Peak A, fractions 19–24, Fig. 1) was isolated.

Further fractionation of the reduced disaccharides. — When the reduced disaccharides were fractionated on immobilized *Bandeirea simplicifolia* lectin, ~70% of the total radioactivity (Fraction BS-1) was not retarded (Fig. 2). The rest (Fraction BS-2) was retarded by the lectin.

The reduced disaccharides of Peak A (fractions 19–24, Fig. 1) were also chromatographed on paper for a long running time (97 h) with solvent (*C*). The two disaccharide alcohols were separated from each other, BS-2 migrating with a rate of $R_{Lactose}$ 1.75 and R_{Gal} 0.52, and BS-1 with a rate of $R_{Lactose}$ 2.08 and R_{Gal} 0.60.

Identification of BS-2 disaccharide alcohol. — Acid hydrolysis of the BS-2 disaccharide alcohol, labeled metabolically with 2-amino-2-deoxy-D-[^{14}C]glucose, released radioactive 2-amino-2-deoxy-D-galactitol that was identified by borate paper chromatography of the *N*-reacetylated saccharide (not shown). Acid hydrolysis of galactose-labeled, BS-2 disaccharide alcohol released radioactive galactose

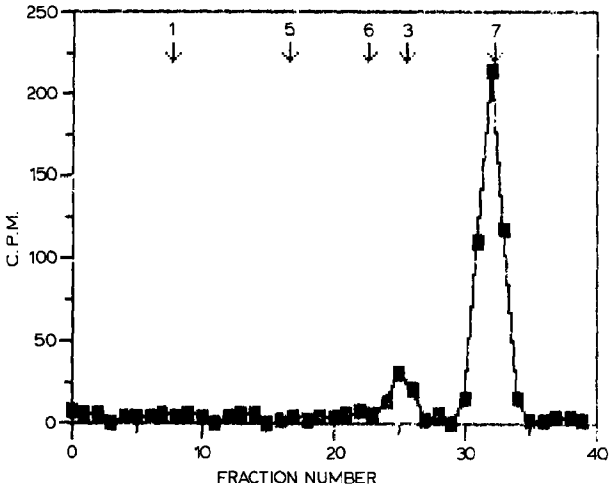

Fig. 3. BS-2 disaccharide alcohol, metabolically labeled with 2-amino-2-deoxy-D-[^{14}C]glucose, was treated with α-D-galactosidase, desalted and subjected to paper chromatography in solvent (*B*) for 24 h. The markers run on the same paper are: (1) Maltopentaose, (3) D-galactose, (5) lactose, (6) β-D-Gal*p*-(1→3)-GalNAcol, and (7) D-GalNAcol.

that was identified by paper chromatography with solvent (*A*) (not shown). β-D-Galactosidase did not cleave the BS-2 disaccharide alcohol, labeled metabolically with 2-amino-2-deoxy-D-[1-^{14}C]glucose, but α-D-galactosidase from green coffee beans cleaved it completely into 2-acetamido-2-deoxygalactitol and D-galactose (Fig. 3). Even the liberated galactose (fractions 24–26, Fig. 3) revealed a small amount of label because of metabolic interconversions at the monosaccharide level that occurred during the labeling of the cells. The α configuration of the D-galactosyl group in the BS-2 disaccharide alcohol was confirmed also by the observation that the saccharide was retarded in the chromatography on *Bandeirea simplicifolia* lectin[23].

The disaccharide nature of the BS-2 fraction was established by its composition (see above) and chromatographic mobility (see Fig. 1). Additional evidence was provided by partial α-D-galactosidase treatment. A cleavage that released 21% of the ^{14}C-labeled D-galactose failed to produce any saccharide moving between the original BS-2 peak and free D-galactose in paper chromatography (not shown). This finding is compatible with BS-2 being a disaccharide, but incompatible with a trisaccharide structure, as the partial digest would have contained a trisaccharide, a disaccharide, and free D-galactose.

Periodate oxidation of the BS-2 disaccharide alcohol, which had a 2-acetamido-2-deoxy-D-[1-^{14}C]galactitol residue, followed by reduction, acid hydrolysis, and *N*-reacetylation yielded a compound that migrated on paper between *N*-acetyl-

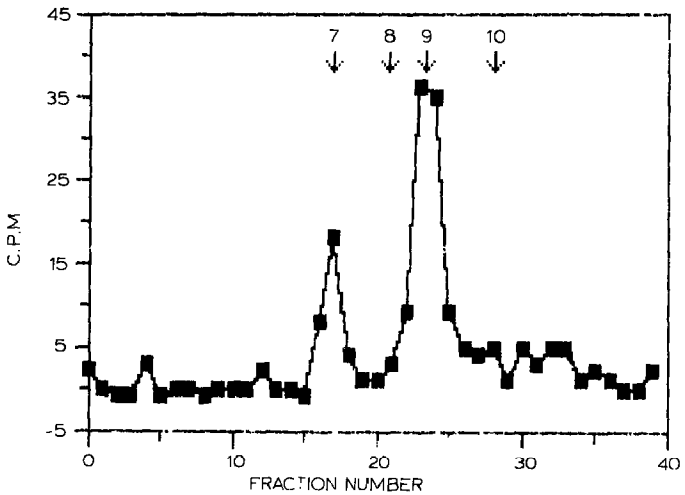

Fig. 4. 2-Acetamido-2-deoxy-D-[^{14}C]galactitol-labeled BS-2 disaccharide alcohol from PA1 cells was oxidized with periodate. The reaction products were reduced, hydrolyzed with acid, *N*-reacetylated, and chromatographed on paper with solvent (*A*) for 15 h. The markers are: (7) 2-acetamido-2-deoxy-D-galactitol, (8) 2-acetamido-2-deoxy-L-arabinitol, and (10) *N*-acetylserinol. Arrow No. 9 shows the position of the reaction product, 2-acetamido-2-deoxy-L-[1-^{14}C]threitol, obtained from radioactive BS-1 disaccharide alcohol.

serinol and 2-acetamido-2-deoxy-L-arabinitol (Fig. 4). This result suggests that the reaction product contained radioactively-labeled 2-acetamido-2-deoxy-L-threitol. Accordingly, 2-acetamido-2-deoxy-D-galactitol appears to be substituted at O-3 in the intact disaccharide.

Taken together, these data show that the BS-2 disaccharide alcohol is α-D-Gal*p*-(1→3)-D-GalNAcol.

Identification of BS-1 disaccharide alcohol. — BS-1 disaccharide alcohol migrated in paper chromatography with solvents (*B*) and (*C*) like standard β-D-Gal*p*-(1→3)-D-GalNAcol (not shown). Like BS-2 disaccharide alcohol, it, too, contained D-galactose and 2-acetamido-2-deoxy-D-galactitol (not shown), but unlike BS-2 disaccharide it was cleaved completely with β-D-galactosidase from *E. coli* (not shown). These findings, together with the observation that BS-1 disaccharide was not retarded in chromatography on *Bandeirea simplicifolia* lectin, suggest that its D-galactosyl group has the β configuration.

Periodate oxidation, sodium borohydride reduction, acid hydrolysis, and subsequent *N*-reacetylation of the BS-1 disaccharide alcohol, which had a 2-acetamido-2-deoxy-D-[1-^{14}C]galactitol residue, gave radioactively-labeled 2-acetamido-2-deoxy-L-threitol (Fig. 4), establishing the structure of the disaccharide as β-D-Gal*p*-(1→3)-D-GalNAcol.

Affinity chromatography of PA1 cell glycopeptides on immobilized peanut lectin. — All low-molecular-weight, PA1-cell glycopeptides that contained the BS-1 disaccharide were retarded in chromatography on immobilized peanut lectin known to bind glycopeptides having a β-D-Gal*p*-(1→3)-D-Gal*p*NAc-(1→ group[29,30]. When

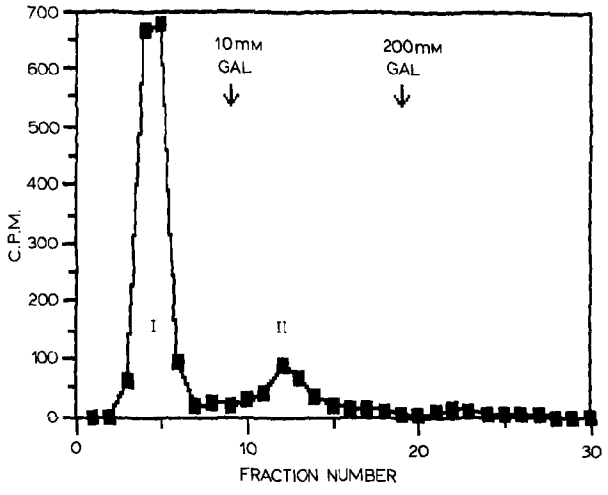

Fig. 5. Chromatography of neutral PA1 glycopeptides ($800 < M_r < 2000$) on peanut lectin–Sepharose. In the first fractions, the eluent was 10mM Tris · HCl buffer, (pH 7.3) which contained 150mM NaCl, mM $CaCl_2$, and 0.02% NaN_3. The arrows show where D-galactose was added to the eluent; I and II refer to glycopeptide fractions named Pn I and Pn II, respectively.

the low-molecular-weight glycopeptides of PA1 cells were chromatographed on peanut lectin–Sepharose columns, 20–25% of the radioactively-labeled compounds were retained, but could be eluted with D-galactose-containing buffers. A major component of the retained glycopeptides (Pn II) was eluted with 10mM D-galactose (Fig. 5); it was eluted at the same position as the principal component of antifreeze glycoprotein[15] No. 7 from *Trematomus borchgrevinki*, known to contain several β-D-Gal*p*-(1→3)-D-Gal*p*NAc groups. Reductive β-elimination of D-galactose-labeled Fraction Pn II released 42% of the radioactive compounds as BS-1 disaccharide alcohol. In contrast, BS-1 disaccharide alcohol was not released from the PA1 glycopeptides not bound to peanut lectin (Fraction Pn I); instead, BS-2 disaccharide alcohol was released. These experiments suggest that the α- and β-D-Gal*p*-(1→3)-D-Gal*p*NAc-(1→ groups may be bound to separate peptides in PA1 cells.

DISCUSSION

Earlier work has shown that high-molecular-weight glycopeptides, obtained from human teratocarcinoma cells of line PA1 by Pronase treatment, contain low-molecular-weight, *O*-glycosyl-linked sugar chains[25] the structures of which had not been established. The present experiments identify two *O*-glycosyl-linked disaccharide groups. One of them, β-D-Gal*p*-(1→3)-D-Gal*p*NAc, is a common group that is found in almost all known *O*-linked carbohydrate structures[11]. In contrast, the other one, α-D-Gal*p*-(1→3)-D-Gal*p*NAc is a rare glycan group which has been identified previously only in the cerebral proteins of rat, rabbit, and hen[31], and in bovine chromogranins, proteins of adrenal neurosecretory granules[32]. Finne and Krusius[31] have suggested that the α-D-Gal*p*-(1→3)-D-Gal*p*NAc group may be specific for neural tissue. The presence of large amounts of this disaccharide group in undifferentiated teratocarcinoma proteins suggests that the α-D-galactosyltransferase required for its synthesis may be specifically preserved during neural development. The present data do not exclude the presence of still other types of disaccharides, *e.g.*, *O*-glycosyl-linked β-D-Glc*p*NAc→D-Gal*p*NAc in PA1 cell proteins.

In the present experiments, we also found, neutral *O*-glycosyl-linked oligosaccharides of medium molecular weight. The tetra- or penta-saccharide-like glycans that were released by reductive β-elimination from PA1 glycopeptides (peak B, fractions 6–10, Fig. 1) were hydrolyzed with acid, and the monosaccharides were *N*-reacetylated. Borate paper chromatography[25] revealed the presence of 2-acetamido-2-deoxy-D-galactitol in the hydrolyzate (not shown). It seems possible that even higher-molecular-weight *O*-glycosyl-linked chains than tetrasaccharides are present, and that one (or both) of the two teratocarcinoma disaccharides represent early stages in the biosynthesis of the medium- and high-molecular-weight glycans.

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