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ISOLATION AND CHARACTERIZATION OF A WHEAT GERM AGGLUTININ-BINDING GLYCOPROTEIN FROM B16 MOUSE MELANOMA CELLS*,†

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ABSTRACT

Cells of B16 mouse melanoma grown in serum-free medium in the presence of [^{3}H]glucosamine secreted or shed labeled glycoproteins. A wheat germ agglutinin-binding glycoprotein was isolated that accounted for 37% of the total [^{3}H]glucosamine incorporated; it had a molecular weight of ~50,000 and was absent in less-tumorgenic wheat germ agglutinin (isolectin I)-resistant variants of the cells. The glycoprotein contained ~25% of serine and threonine plus equimolar amounts of glucosamine and galactosamine, indicating both *N*- and *O*-linked oligosaccharide chains. Neuraminidase treatment released ~60% of the glycoprotein's ^{3}H radioactivity as *N*-acetylneuraminic acid. The sialoglycoprotein did not, but the desialylated species did, bind (97%) to ricin–Sepharose, suggesting the presence of terminal sialic acid and penultimate galactose residues in most of the saccharide units. Alkaline borohydride released 61% of the glycoprotein's radioactivity as oligosaccharide alcohols that were mainly tetrasaccharides (75%) with some branched trisaccharides (10%) from the *O*-linked structures. Hydrazinolysis and analysis of the alkaline borohydride-resistant portion of the glycoprotein indicated the presence of mainly triantennary, complex-type structures (*N*-linked) containing three sialic acids residues plus L-fucose. Serial lectin-affinity chromatography of the hydrazine-released saccharides with concanavalin A–agarose, pea lectin–agarose, L-PHA–agarose, and E-PHA–agarose, indicated the absence of high-mannose or hybrid-type structures and further confirmed the presence of triantennary, complex-type units.

INTRODUCTION

There is considerable information on differences in the membranes of normal and malignant cells[1]. It has also been established that the protein-bound carbo-

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hydrate level of blood plasma is elevated in individuals with malignant disease and the possibility exists that tumor cells may contribute carbohydrate-containing proteins to the circulation[2-6]. Although conclusive evidence is lacking, it has been suggested that transformed cell surfaces have a high proteolytic activity, thus contributing to an increased level of glycoproteins in the circulation[7,8].

Comparisons of the glycoproteins and proteoglycans produced by malignant and normal cells have been reported[9-15]. The characterization of mucin-type sialoglycopeptides isolated from B16 mouse melanoma cells grown in cultures has been previously described from our laboratory[9,10,16-18]. These sialoglycopeptides were shown to have a high sialic acid content, an affinity for wheat germ agglutinin, and the linkage of the saccharides to peptide was mainly *O*-glycosidic, involving *N*-acetylgalactosamine. Antigens associated with B16 melanoma which appear to be secreted or shed by these cells have also been described[19-21].

In this paper, we describe the isolation and structural characterization of a major wheat-germ agglutinin (WGA*)-binding glycoprotein secreted or shed by tumorigenic B16 mouse melanoma cells, which appears to be absent in less-tumorigenic wheat germ agglutinin-resistant variants of the same cell-line[22].

MATERIALS AND METHODS

Cell culture. — B16 mouse melanoma cells were maintained in serum free, modified Ham's F12 medium (Flow Laboratories, Rockville, MD) supplemented with penicillin (10 units/mL), streptomycin (10 μg/mL), 0.11% $NaHCO_3$, and 1% BSA[22]. This medium containing all of these ingredients will be referred to as serum-free medium.

Tritium labeling of saccharide units. — Tritium labeling of the saccharides was performed[22] with D-[6-^{3}H]glucosamine hydrochloride (Amersham Corp., Arlington Heights, IL). The cells were extracted with lithium diiodosalicylate by the method of Marchesi[23a].

Affinity column chromatography. — Wheat germ agglutinin (WGA) was isolated from raw wheat germ obtained from local health food stores, according to the method of Nagata *et al.*[23b]. The lectin was coupled to beads of Sepharose 4B after cyanogen bromide activation[24]; the ligand density was 2 mg of lectin/mL of gel beads. The gel was equilibrated in 50mM Tris buffer, pH 8.0, and bound material eluted with 0.1M GlcNAc in the same buffer. Con A–agarose, pea lectin–agarose, L-PHA–agarose and E-PHA–agarose gels were obtained from Vector Laboratories (Burlingame, CA). The Con A and pea lectin columns were equilibrated in 10mM Tris buffer, pH 8.0, containing 0.15M NaCl, mM $CaCl_2$, and mM $MgCl_2$. The

*The abbreviations used are BSA, bovine serum albumin; PBS, phosphate buffered saline; WGA, wheat germ agglutinin; GlcNAc, 2-acetamido-2-deoxy-D-glucose; Man, mannose; NeuAc, *N*-acetylneuraminic acid; Gal, galactose; GalNAcOH, 2-acetamido-2-deoxygalactitol; Con-A, Concanavalin A; E-PHA, *Phaseolus vulgaris* erythroagglutinin; L-PHA, *Phaseolus vulgaris* leukoagglutinin; PPO, 2,5-diphenyloxazole; and VCN, *Vibrio cholerae* neuraminidase.

bound material was specifically eluted, first with 10mM methyl α-D-glucoside and then with 500mM methyl α-D-mannoside in the foregoing buffer. E-PHA and L-PHA columns were equilibrated in PBS–NaN_3 buffer and chromatography was carried out as described[25]. The recovery of radioactivity from the columns was generally >85%. The distribution of radioactivity in the peaks is reported as percentages of the total recovered.

Electrophoretic studies. — Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed according to the methods described by Laemmli[26] and Hass and Kennett[27] in 1.5-mm thick, 7% polyacrylamide running gels with 4% stacking gels. The [^{3}H]glycoproteins on the destained gels were detected after treatment with Me_2SO–PPO and fluorography[22]. Radiolabeled samples in 5% polyacrylamide tube-gels were analyzed after slicing the gel in 2-mm segments, solubilizing the slices overnight at 37° in 30% H_2O_2, and counting in ACS II scintillation cocktail by liquid-scintillation spectrometry.

Gel filtration. — Columns of BioGel P2 (minus 400 mesh), P4, and P6 (200–400 mesh) were equilibrated and eluted with 0.1M pyridinium acetate buffer, pH 5.0. All gels were obtained as dry powders from BioRad, Richmond, CA.

Enzyme digestions. — Digestion by *Vibrio cholerae* neuraminidase (Calbiochem–Behring, La Jolla, CA) was carried out as follows: the sample in 0.2M sodium acetate buffer containing 2mM Ca^{2+} (pH 5.6) was treated with 10 μL of the enzyme (500 units/L). The mixture was incubated in a water bath for 24 h at 37° with shaking, and another 10-μL aliquot of the enzyme was added. The incubation was continued for an additional 24 h and the reacion stopped by boiling the mixture for 5 min at 100°.

Determination of [^{3}H]sialic acid and [^{3}H]hexosamines. — [^{3}H]Sialic acid in isotopically labeled glycoproteins was determined by acid hydrolysis (0.1M H_2SO_4, 1 h, 80°) or by treatment with *Vibrio cholerae* neuraminidase followed by fractionation on a column of BioGel P2.

[^{3}H]Hexosamines in the labeled glycoconjugates were estimated after acid hydrolysis (4M HCl, 8 h, 100°). Standard hexosamines were added to the dried hydrolyzates and the mixture was analyzed on a Beckman amino acid analyzer equipped with a stream splitter.

Amino acid analysis. — Amino acid analysis was performed with a Dionex amino acid analyzer after hydrolysis of the material *in vacuo* for 24 h at 110° in 6M HCl.

Assay for sialic acids. — *N*-Acetylneuraminic acid and *N*-glycolylneuraminic acid, obtained from Sigma Chemical Company, St. Louis, MO, were used as standards. Descending paper chromatography of sialic acids[28] on Whatman No. 1 paper used the solvent system 3:2:1 1-butyl acetate–acetic acid–H_2O for 38 h; sugars were detected by spraying with thiobarbituric acid[29].

N-*Terminal analysis with dansyl chloride.* — The WGA-bound material (0.5–2 nmol) was treated with a 1:5 mixture of 88% formic acid and 30% hydrogen peroxide for 30 min at room temperature and the solution was lyophilized. The

residue was then treated with 1% sodium dodecyl sulfate for 5 min at 100°. *N*-Ethylmorpholine (50 μL) and dansyl chloride (25 mg/mL in *N,N*-dimethylformamide; 75 μL) were added and the mixture was incubated for 2 h at 37°. The proteins in the mixture were precipitated with an equal volume of cold trichloroacetic acid (20%) for 1 h at 0°. The pellet was washed thoroughly with ether, subjected to acid hydrolysis, and the dansylated amino acids detected by t.l.c. on polyamide sheets according to the method of Gray[30].

Alkaline $NaBH_4$ treatment. — The glycoproteins were treated, under nitrogen in a sealed tube in the dark, with M $NaBH_4$ in 0.1M NaOH. After 72–96 h at 37°, the mixture was cooled in an ice bath and the excess borohydride decomposed by dropwise addition of M acetic acid. The saccharides released were isolated by chromatography on columns of BioGel P6 or P4. The excluded peak, which contained peptide having *N*-linked saccharides, was subjected to hydrazinolysis.

Paper electrophoresis. — Whatman No. 1 paper was used for analytical experiments and Whatman No. 3 MM paper for preparative purpose. High-voltage paper electrophoresis was performed in pyridinium acetate buffer, pH 5.4 (3:1:387 pyridine–acetic acid–water) at a potential of 73 V/cm for 1.5 h for *N*-linked saccharides and 1 h for *O*-linked saccharides. Radioactive components were detected by scanning the paper in a Packard model 7201 radiochromatogram scanner.

Hydrazinolysis. — The procedure of Kobata *et al.*[31] was used and the saccharide products were purified by paper chromatography in 4:1:1 1-butanol–ethanol–water for 2 days. The larger oligosaccharides, which remained at the origin, were recovered by elution with water. The oligosaccharide fraction was then reduced with 3 μmol (1mCi) of NaB^3H_4 (New England Nuclear, Boston, MA) in 200 μL of 0.05M NaOH for 4 h at 30°. For metabolically radiolabeled samples, similar concentrations of unlabeled borohydride were added. Cold $NaBH_4$ was then added (7 mg/70 μL of 0.05M NaOH) and the reaction continued for another 2 h. The reaction was stopped by acidification with M acetic acid and passage through a column (3 mL) of Dowex 50 (H^+ form). The eluate and water washings were combined and lyophilized. The boric acid-freed residue was then subjected to paper chromatography for 2 days with 5:5:1:3 ethyl acetate–pyridine–acetic acid–water to remove contaminants. The radioactive oligosaccharides that remained at the origin were recovered by elution with water.

The oligosaccharide fraction thus obtained was further fractionated by high-voltage paper electrophoresis and by high-performance liquid chromatography (l.c.). Oligosaccharides released from ceruloplasmin, α_1 acid glycoprotein, fetuin (*N*-linked), and transferrin were used as standards.

High-performance liquid chromatography. — Chromatographic separations were performed with amine-bearing columns (4 mm × 30 cm), MicroPak AX-5 and MicroPak AX-10 (Varian Associates, Florham Park, NJ), or a column (4 mm × 25 cm) of Lichrosorb-NH_2 (partical size 5 μM, Merck) and a Waters Liquid Chromatograph, Model 6 UK. All solvents were filtered through a 0.22μM Millipore filter before use.

Separation of acidic oligosaccharides on MicroPak AX-10. — The mobile phase consisted of 25–500mM KH_2PO_4, pH 4.0. The flow rate was maintained at 1 mL/min and fractions of 300 μL were collected and counted to determine radioactivity[32].

Separation of neutral oligosaccharides on MicroPak AX-5. — The initial solvent composition was 13:7 acetonitrile–water and the acetonitrile content was decreased at a rate of 0.5% per min. The flow rate was maintained at 1 mL/min and fractions of 300 μL were collected and analyzed for radioactivity[33].

Separation of acidic, O-linked oligosaccharides on Lichrosorb-NH_2. — The mobile phase consisted of a mixture of acetonitrile and deionized distilled water containing 15mM potassium phosphate (pH 5.2). The separation of sialylated oligosaccharides was performed by starting with an isocratic elution with a 4:1 (v/v) mixture of the foregoing solutions for 30 min, after which a linear gradient of increasing water content at a rate of 0.6% per min was applied. The flow rate was maintained at 2 mL/min. Fractions of 1 mL (0.5 min) were collected and analyzed for radioactivity.

RESULTS

The labeled glycoproteins secreted or shed to the medium by the mouse melanoma cells were fractionated into wheat germ agglutinin-bound and -unbound components on the basis of their interaction with WGA-Sepharose (Fig. 1). The results show that 37% of the radioactivity was bound and eluted with 0.1M GlcNAc. This bound material was recovered by dialysis against distilled water for 3 days and lyophilized.

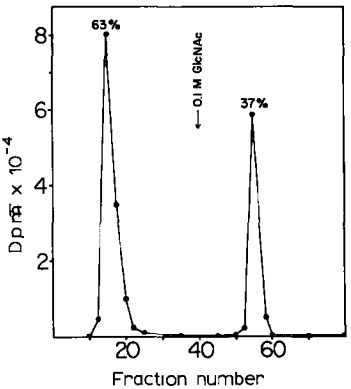


Fig. 1. Affinity chromatography of B16 medium glycoproteins on WGA–Sepharose. The melanoma cell culture was labeled with [^{3}H]glucosamine and the dialyzed, lyophilized medium chromatographed on a WGA–Sepharose column (0.7 × 15 cm) containing 2 mg of WGA per mL of gel beads. The column was eluted with 50mM Tris buffer, pH 8.0, followed by 0.1M GlcNAc in the same buffer. Fractions (1 mL) were collected and aliquots analyzed for radioactivity. The material specifically eluted with 0.1M GlcNAc was recovered by dialysis followed by lyophilization.

Characterization of the WGA-binding glycoprotein(s). — SDS-polyacrylamide gel electrophoresis of the WGA-bound glycoprotein(s) from the spent medium of B16 melanoma cells is shown in Fig. 2. One glycoprotein having an approximate molecular weight of 50,000 is identifiable and is apparently present as a component of the cell membranes of these cells (Fig. 2A lanes 6, 7, and 8). Fig. 2B shows the SDS-cylindrical gel pattern of the WGA-bound material from the medium of B16 cells. The major peak, which accounts for 71% of the total radioactivity applied, has a molecular weight of ~50,000.

Serine and threonine comprised ~25% of the total amino acid residues of the glycoprotein. The glycoprotein was also rich in glutamic acid (glutamine), glycine, alanine, and aspartic acid (asparagine). The *N*-terminal analysis showed the pre-

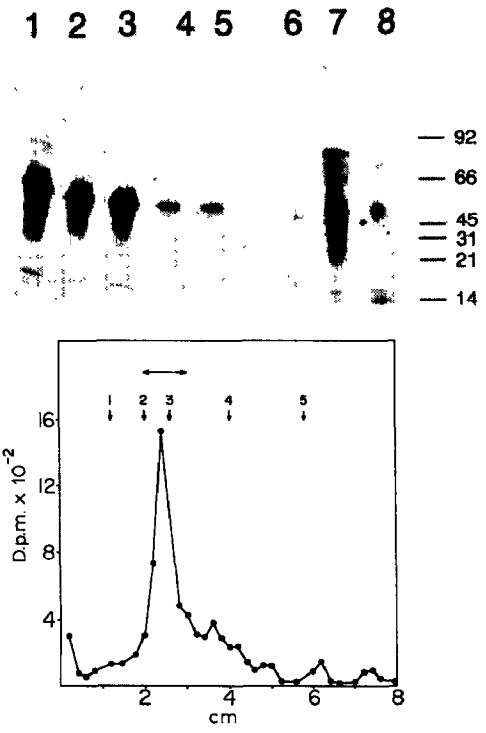


Fig. 2. (A) SDS-Polyacrylamide gel electrophoresis of ^{3}H-labeled WGA-bound material from the B16 mouse melanoma. The material eluted with 0.1M GlcNAc was subjected to electrophoresis followed by fluorography. Lane 1, 43,000 c.p.m.; lane 2, 31,000 c.p.m.; lane 3, 25,000 c.p.m.; lane 4, 5,300 c.p.m. (in the presence of 2-mercaptoethanol); lane 5, 5,300 c.p.m. (in the absence of 2-mercaptoethanol); lanes 6, 7, and 8, lithium diiodosalicylate-extracted cells; lane 9, molecular-weight markers, phosphorylase B (92,000), BSA (66,000), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,000), and lysozyme (14,000). (B) WGA-bound material from the medium of B16 cells (5,000 c.p.m.) was subjected to electrophoresis in 6% cylindrical polyacrylamide gels containing SDS. The gel was cut into 0.2-mm slices and counted for ^{3}H radioactivity after dissolving in 30% H_2O_2. Standards were 1, phosphorylase B (92,000); 2, bovine serum albumin (66,000); 3, ovalbumin (45,000); 4, carbonic anhydrase (31,000); and 5, lysozyme (14,000). The region indicated by ↔ contained 70% of the total ^{3}H label in the gel.

sence of one product that corresponded to dansylated arginine or to dansylated internal lysines.

Hexosamine analysis showed the presence of both glucosamine and galactosamine residues, suggesting that the glycoprotein contains both *N*-linked and *O*-linked saccharide chains[34].

Neuraminidase (VCN) treatment released 60% of the glycoprotein's radioactivity as material that coeluted with NeuAc on BioGel P2, indicating that 60% of the ^3H label residues in sialic acid. No additional sialic acid was released upon mild acid hydrolysis (0.1M H_2SO_4, 1 h, 80°) of the VCN-resistant fraction, suggesting that all the sialic acid of the initial material was VCN-sensitive. Analysis of the sialic acid released by paper chromatography indicated that it is all in the *N*-acetylated form. The sialoglycoprotein did not bind to ricin–Sepharose (<3%), but the desialylated species was bound (97%) by ricin–Sepharose and could be eluted with 0.1M lactose.

Alkaline borohydride treatment followed by gel filtration on BioGel P6 or P4 (Fig. 3) indicated that 61% of the radioactivity was released as small saccharides. On both columns, the major product coeluted with NeuAcGal(NeuAc)GalNAcOH, a tetrasaccharide alditol prepared from glycophorin. There was a minor product that comigrated with a trisaccharide alditol, NeuAcGalGalNAcOH, prepared from fetuin[35]. Acid hydrolysis (4M HCl, 8 h, 100°) followed by stream-split analysis of the total β-eliminated fraction showed the presence of 2-amino-2-deoxygalacitol but not glucosamine. The void material from the BioGel P6 chromatography showed glucosamine as the only hexosamine.

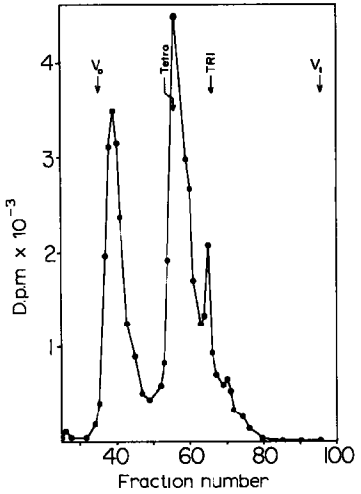


Fig. 3. Chromatography on BioGel P6 of oligosaccharides released by alkaline borohydride treatment. The glycoprotein was treated with M $NaBH_4$ in 0.1M NaOH as described under Materials and Methods. The mixture was applied to a column (0.9 x 105 cm) of BioGel P6 and fractions of 1 mL were collected and assayed for radioactivity. V_o, void volume; V_t, total volume; tetra, glycophorin tetrasaccharide alditol; Tri, fetuin trisaccharide alditol.

Results of high-voltage paper electrophoresis of the β-eliminated saccharides are shown in Fig. 4. Of the radioactivity, 75%, comigrated with the tetrasaccharide alditol NeuAcGal(NeuAc)GalNAcOH and 10% of the radioactivity comigrated with the trisaccharide alditol NeuAcGalGalNAcOH. Acid hydrolysis (0.1M H_2SO_4, 1 h, 80°) followed by high-voltage paper electrophoresis of these fractions gave a peak at the origin, corresponding to neutral oligosaccharides, and sialic acid. The BioGel P6 column-chromatographic data, together with the high-voltage paper electrophoresis, shows that the *O*-linked structures of the glycoprotein of mol.wt. 50,000 are mainly disialylated tetrasaccharides. L.c. of the trisaccharide indicated that it is branched[36].

Hydrazinolysis of the material excluded from the BioGel P6 column (Fig. 3) released saccharides which were analyzed by high-voltage paper electrophoresis [Fig. 5(A)]. The major peak, A-3, which had 48% of the radioactivity released by hydrazine, comigrated with a triantennary complex-type structure, $(NeuAc\text{-}GalGlcNAc)_3$ $Man_3GlcNAcGlcNAcOH$ obtained from fetuin[37]. Fraction A-1 (8% of the total radioactivity), was shown to comigrate with a monosialo, biantennary complex-type structure. The second peak, A-2, which had 15% of the radioactivity, comigrated with a biantennary, disialo complex-type structure. Fraction A-4 (20% of the radioactivity) was shown to co-elute with a tetrasialo, tetraantennary complex-type structure.

Results of l.c. separation of the acidic fraction A-3, obtained from paper electrophoresis are shown in Fig. 6A; standards were obtained from fetuin by similar treatment. The major fraction, A-3, comigrated with the trisialylated, triantennary standard. These results confirm that the major hydrazine-released material from the glycoprotein of mol.wt. 50,000 is structurally similar to the triantennary, complex-type structure obtained from fetuin.

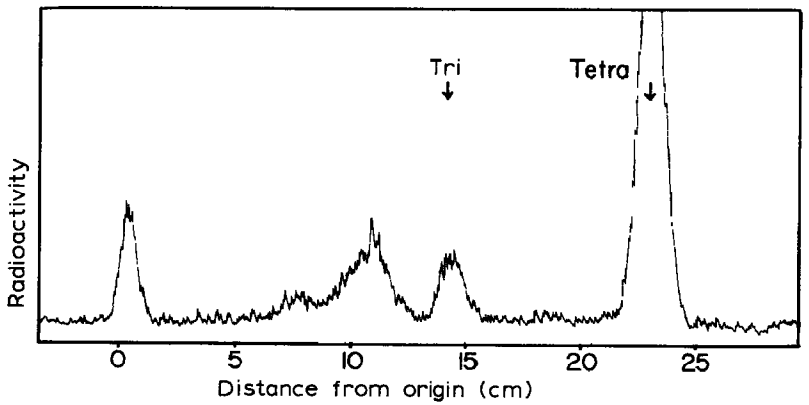


Fig. 4. High-voltage paper electrophoresis of the β-eliminated oligosaccharides. The material released by alkaline borohydride treatment was subjected to paper electrophoresis as described under Materials and Methods. In preparative runs, the material under the peaks was recovered by elution with distilled water. Tetra, glycophorin tetrasaccharide alditol; Tri, fetuin trisaccharide alditol.

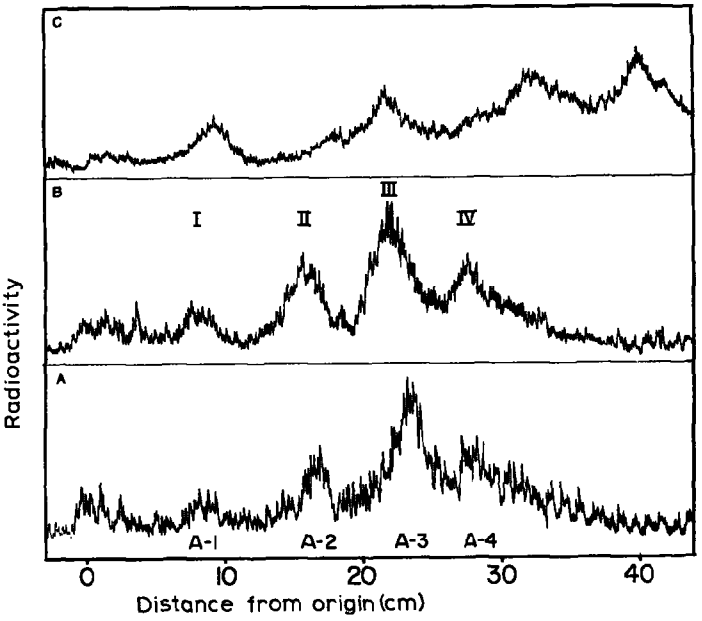


Fig. 5. High-voltage paper electrophoresis of hydrazine-released saccharides. The *N*-linked oligosaccharides released by hydrazinolysis were subjected to paper electrophoresis (A) as described under Materials and Methods. In preparative runs, the material in the peak was recovered by eluting with distilled water. The standards were obtained from fetuin (B) or ceruloplasmin (C) by similar treatment. The structures of the standard oligosaccharides are: I, (NeuAcGalGlcNAc)(GalGlcNAc)Man_3GlcNAcGlcNAcOH; II, (NeuAcGalGlcNAc)$_2Man_3$GlcNAcGlcNAcOH; III, (NeuAcGalGlcNAc)$_3Man_3$GlcNAcGlcNAcOH; and IV, (NeuAcGalGlcNAc)$_4Man_3$GlcNAcGlcNAcOH.

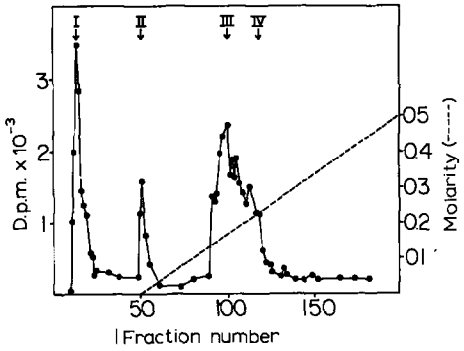


Fig. 6. High-performance liquid chromatographic separation of Fraction A-3 and sialic acid bearing bi-, tri-, and tetra-antennary complex oligosaccharides on a MicroPak AX-10 column. The oligosaccharides were initially preparatively separated by paper electrophoresis and the acidic fractions A-1, -2, -3, and -4 recovered (see Fig. 5). The standard oligosaccharide structures are the same as in Fig. 5.

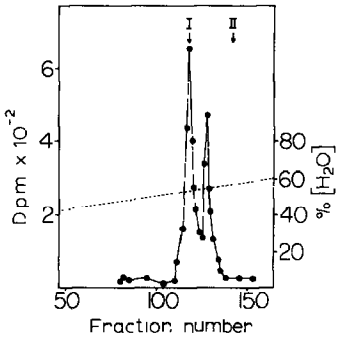


Fig. 7. High-performance liquid chromatographic separation of desialylated, neutral A-3 fraction on a MicroPak AX-5 column. The initial solvent composition was 13:7 acetonitrile–water and final composition was 2:13 acetonitrile–water. Fractions (300 μL) were collected to determine radioactivity. I, $(GalGlcNAc)_3Man_3GlcNAcGlcNAcOH$; II, $(GalGlcNAc)_4Man_3GlcNAcGlcNAcOH$.

Fig. 7 shows the l.c. behavior of desialylated (0.1M H_2SO_4, 1 h, 80°) fraction A-3 on a MicroPak AX-5 column. The major peak (60% of the total radioactivity), coeluted with the neutral standard $(GalGlcNAc)_3Man_3GlcNAcGlcNAcOH$. The minor peak (35% of the total radioactivity), was shown[33] to be a triantennary structure with outer fucosylation $(GalGlcNAc(Fuc))(GalGlcNAc)_2Man_3GlcNAc$-GlcNAcOH.

Serial lectin-affinity chromatography was carried out as described by Cummings and Kornfeld[38]. The acidic fractions A-1, -2, -3, -4, were applied to Con A–agarose; 70% of the radioactivity passed through the column. Methyl α-D-glucoside (10mM) eluted 27% of the counts, whereas <3% of the radioactivity was eluted with 500mM methyl α-D-mannoside, indicating the absence of high-mannose or hybrid type structures[38]. The major acidic fraction, A-3, did not bind to Con A–agarose, confirming that it is larger than a biantennary, complex-type structure[39–41].

E-PHA–Agarose can be used to selectively bind glycopeptides with biantennary, complex-type structures containing a bisecting *N*-acetylglucosamine residue[38]. Likewise, L-PHA selectively interacts with tri- and tetra-antennary complex-type structures that have one α-linked mannose substituted only at O-2 and O-6. These interactions are dependent on the presence of outer galactosyl residues, but are independent of the presence of outer sialic acid or inner fucose residues[25]. Therefore, the material that passed through the pea lectin–agarose column was applied to a L-PHA–agarose column; 86% of the counts passed through; there was no significant binding to E-PHA–agarose.

DISCUSSION

The metabolically labeled glycoproteins were isolated from the spent medium after 3–5 days of culture. Only 4% of the added label was incorporated after 3 days

but this increased to 45% after 5 days, probably because of lowered glucose availability. The possibility that [^{3}H]glucosamine might indirectly interact with BSA was ruled out by a control experiment wherein BSA and [^{3}H]glucosamine were incubated in the absence of cells. After 5 days, only 0.01% of the counts were non-dialyzable, analogous to prior results obtained in our laboratory[42].

The metabolically labeled, non-dialyzable, WGA-bound material contained one major glycoprotein, also a plasma membrane component. *N*-Terminal analysis of the glycoprotein of mol.wt. 50,000 showed a single dansylated amino acid, indicating absence of significant contamination. The presence of glycoproteins of mol.wts. 51,000 and 56,000 on cell surfaces of melanoma cells has been reported[43].

As the desialylated material bound (97%) to ricin–Sepharose, but not to WGA, the presence of (NeuAcGal) terminal sequences is likely. This conclusion is consistent with the observation by other workers that wheat germ agglutinin has a high affinity for clustered sialic acid residues on glycopeptides[44,45].

The detection of only 2-amino-2-deoxy[^{3}H]galacitol after hydrolysis of the alkaline borohydride, β-eliminated products indicated that all galactosamine residues present are involved in the alkali-labile, *O*-glycosidic linkage to Ser/Thr. The major β-eliminated product, which accounted for 75–78% of the alkaline borohydride-released material, was characterized as the tetrasaccharide alditol, NeuAcGal(NeuAc)GalNAcOH. A small proportion (10–12%) of trisaccharide and 9–12% of neutral saccharide are also present. The presence of small, mucin-type, saccharide-containing glycoproteins on B16 mouse melanoma cell surfaces as well in spent culture medium, has been reported[9,17]. Recently, Irimura and Nicolson reported a glycoprotein of mol.wt. 51,000 presumably with *O*-linked tetrasaccharide sugar chains, on mouse melanoma cell surfaces[43].

Hydrazinolysis of the alkaline borohydride-resistant material, followed by high-voltage paper electrophoresis, showed the presence of several acidic fractions (Fig. 5); the major components were shown to contain one, two, three, and four sialic acids respectively; neither sulfate nor phosphate were present.

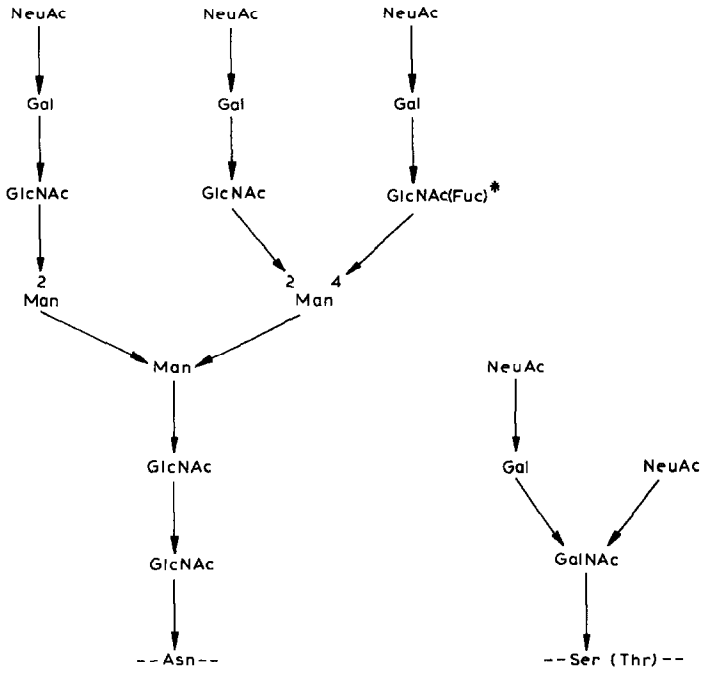
L.c. analysis of the major acidic fraction (A-3) showed a sharp peak that coeluted with a standard triantennary structure containing three sialic acid residues [$(NeuAcGalGlcNAc)_3Man_3$ GlcNAcGlcNAcOH][33,37,46–48]. The desialylated material showed the presence of ~35% outer fucosylation.

The structure of the *N*-linked portion of the glycoprotein was further examined by serial lectin-affinity column chromatography[38–41,49–52]. The results indicate that the majority of the sugar chains are larger than biantennary, with no high-mannose or hybrid structures.

Glycopeptides containing complex-type oligosaccharides can also be fractionated on pea or lentil lectin–Sepharose columns into species with and without core fucose[39]. The Con A–agarose unbound, fraction A-3 material was not retained on a pea lectin column, indicating the absence of core fucose residues. The pea lectin binds only a selected population of triantennary complex molecules with a core fucose residue and α-linked mannose residues[38] substituted only at positions O-2

and O-6. L-PHA–Sepharose selectively interacts with galactosylated tri- and tetra-antennary complex type molecules that have one α-linked mannose substituted at C-2 and C-6 positions[25]. This interaction is independent of the presence of outer sialic acid or inner fucose residues. Therefore, the material that passed through the pea-lectin–agarose column was applied to an L-PHA–agarose column. The passage through the column of 86% of the applied radioactivity confirmed that the saccharide has a triantennary structure with α-mannose substituted at O-2 and O-4; this structure is also present in fetuin[37]. The absence of a bisecting *N*-acetylglucosamine residue was confirmed by using E-PHA–agarose affinity chromatography[25].

The proposed structures of the major *N*- and *O*-linked saccharide chains of the glycoprotein of mol.wt. 50,000 are as shown:



The synthesis and shedding of mucin-type glycoproteins by B16 mouse melanoma cells has been reported[9–11,16], as has evidence for the production and shedding of mucin-type glyucoproteins by other cancerous cell lines. The presence of a mucin-type glycoprotein on the cell surfaces of melanoma cells with presumably tetra- and tri-saccharide units also has been reported[17,43]. In contrast, normal tissues, such as mouse iris melanocytes[53], human fetal iris melanocytes[54], and a

*Fuc may be present or absent.

normal human mammary cell-line[18], either do not produce, or produce in markedly decreased amounts, similar glycoproteins.

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