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Partial Characterization of Sialoglycopeptides Produced by Cultured Human Melanoma Cells and Melanocytes†

V. P. Bhavanandan,* Anne W. Katlic, John Banks, Jeffrey G. Kemper, and Eugene A. Davidson

ABSTRACT: The sialoglycopeptides produced by HM7 human melanoma and fetal uveal melanocyte cultures grown in the presence of [^{3}H]glucosamine and [^{35}S]sulfate were isolated from the Pronase digests of cells, spent media, and intracellular material. From the melanoma culture, six sialoglycopeptides, accounting for 43% of the total ^{3}H radioactivity in the nondiffusible cell-associated glycopeptides, were purified. A major glycopeptide (GPIb) having an apparent molecular weight in the range 12000–15000 showed specific sialic acid dependent interaction with wheat germ agglutinin (WGA). It was found to contain mainly O-glycosidically linked oligosaccharides having the structure (AcNeu)$\rightarrow_{0\text{-}2}$[Gal$\rightarrow$GalNAc]; some N-glycosidically linked saccharides were also present. A second WGA-binding glycopeptide (GPIa) was smaller and less anionic and had a higher proportion of N-glycosidically linked saccharides than GPIb. The normal fetal cultures yielded either no (iris) or markedly reduced (melanocytes) quantities of the WGA-binding glycopeptides. The four WGA-nonbinding sialoglycopeptides purified from melanoma were shown to have complex (*N*-acetyllactosaminyl type) oligosaccharides linked via *N*-acetylglucosamine to asparagine with either no or insignificant amounts of O-glycosidically linked saccharides. The corresponding glycopeptides from melanocytes were of smaller molecular size and lower anionic charge, reflecting an overall lower degree of glycosylation.

Differences in the glycoproteins produced by malignant cells compared to normal cells have been reported (Bramwell & Harris, 1978; Buck et al., 1979; Ceccarini & Atkinson, 1977; Codington et al., 1979; Glick, 1979; Muramatsu et al., 1979; Takasaki et al., 1980; van Beek et al., 1977). A molecular description of these alterations is of importance because it will help to understand the biological properties of malignant cells.

We have investigated in some detail the glycoproteins and glycopeptides isolated from B16 mouse melanoma cells grown in culture and in vivo. The characterization of a mucin-type sialoglycopeptide (Bhavanandan & Davidson, 1976; Bhavanandan et al., 1977; Fareed et al., 1978) and a melanoma-associated antigen (Bhavanandan et al., 1980) have been reported. The characterization of a malignancy-related mucin-type sialoglycopeptide produced by human breast cancer cell lines has also been reported (Chandrasekaran & Davidson, 1979).

We report here the purification and partial structures of the major glycopeptides isolated from cultured human melanoma cells and from the culture media. These are compared to those produced by normal fetal melanocytes in culture. The glycopeptides obtained from the highly tumorigenic (in athymic mice) human melanoma cells are markedly different from those obtained from nontumorigenic human fetal uveal melanocytes. A preliminary report of these results has been presented (Bhavanandan et al., 1979).

Experimental Procedures

Materials. The cells and culture conditions used are described in the following paper in this issue (Bhavanandan, 1981). Neuraminidase (*Vibrio cholerae*) was from Calbiochem. Bio-Gel P-2, P-4, P-6, and P-10 were obtained from Bio-Rad Laboratories (Richmond, CA). DEAE-Sephacel, DEAE-Sepharose CL-6B, Sephadex G-50, and Sephacryl S-200 were purchased from Pharmacia (Piscataway, NJ). β-Galactosidase, β-*N*-acetylhexosaminidase, and endo-α-*N*-acetylgalactosaminidase from *Diplococcus pneumoniae* culture filtrates were prepared in this laboratory. These enzymes were free of contaminating glycosidases as tested with *p*-nitrophenyl glycosides and isotopically labeled natural substrates (fetuin or pig submaxillary mucin glycopeptides) (Umemoto et al., 1977). Endo-β-*N*-acetylglucosaminidase D (Muramatsu et al., 1978) and endo-β-*N*-acetylglucosaminidase H (Tarentino & Maley, 1974) were purchased from Miles (Elkhart, IN). Highly purified exo-β-galactosidase from jack bean (Li et al., 1975) and endo-β-galactosidase from *Escherichia freundii*

† From the Department of Biological Chemistry and The Specialized Cancer Research Center, The Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, Pennsylvania 17033. *Received November 10, 1980.* This research was supported by Research Grants CA15483 and CA17686 from the U.S. Public Health Service.

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(Nakagawa et al., 1980) were kindly provided by Dr. Y.-T. Li of Tulane University (New Orleans, LA). Wheat germ agglutinin, concanavalin A, *Ricinus communis* agglutinins of molecular weights 120000 and 60000 (RCA_{120} and RCA_{60}),[1] and soybean agglutinin were isolated and conjugated as described (Bhavanandan & Katlic, 1979a; Bhavanandan et al., 1977). [[^{3}H]AcNeu]glycophorin and its Pronase and tryptic glycopeptides were prepared as described (Bhavanandan & Katlic, 1979a). Fetuin glycopeptide fractions A, B, and C were isolated and labeled by reductive methylation with H^{14}CHO (Umemoto et al., 1977; Bhavanandan & Katlic, 1979a). Cyanogen bromide fragment A from [[^{3}H]AcNeu]glycophorin was prepared according to Blumenfeld & Puglia (1979).

Methods. Column Chromatography. Columns of Sephadex G-50 (fine), Bio-Gel P-2 (minus 400 mesh), P-4, P-6, and P-10 (200–400 mesh) were equilibrated and eluted with 0.1 M pyridine/0.1 M acetic acid, pH 5.0. DEAE-Sepharose CL-6B and DEAE-Sephacel columns were generated in the acetate form, packed in columns, and extensively washed with 0.1 M pyridine/0.1 M acetic acid, pH 5.0. The columns were finally equilibrated in 0.01 M pyridine/0.01 M acetic acid, pH 5.0, prior to application of the sample and elution with a linear gradient of 0.01 M pyridine/acetic acid, pH 5.0, in the mixing chamber and an equal volume of 1.0 M or 0.5 M pyridine–acetic acid, pH 5.0, in the reservoir. Affinity chromatography on lectin–Sepharose columns was done as described (Bhavanandan & Katlic, 1979a). The WGA–Sepharose columns used had about 2 mg of lectin per mL of Sepharose. The recovery of radioactivity and standards from the columns was greater than 80% unless otherwise specified. The distribution of radioactivity in the peaks is reported as percentages of the total recovered.

Paper chromatography was carried out on Whatman No. 1 paper by the descending technique. The following solvent systems were employed: (A) 1-butyl acetate/acetic acid/water (3:2:1 v/v), (B) 1-butanol/pyridine/water (6:4:3 v/v), (C) pyridine/ethyl acetate/water/acetic acid (5:5:3:1 v/v) with pyridine/ethyl acetate/water (11:40:6 v/v) in the bottom of the chromatography tank, and (D) 1-butanol/acetic acid/1 M NH_4OH (2:3:1 v/v).

Marker neutral sugars, hexosamines, oligosaccharides, and oligosaccharide alcohols were located by the silver nitrate staining procedure (Trevelyan et al., 1950) or by the periodate–benzidine technique (Gordon et al., 1956). Sialic acids were detected by the thiobarbituric acid spray (Warren, 1960) and the *N*-acetylhexosamines and Gal→GalNAc by the Morgan–Elson reagent (Partridge, 1948).

Determination of [^{3}H]Sialic Acids and [^{3}H]Hexosamines. [^{3}H]Sialic acid in isotopically labeled glycoproteins was determined by acid hydrolysis (0.1 M H_2SO_4, 80 °C, 1 h) or by treatment with *Vibrio cholerae* neuraminidase followed by addition of carrier sialic acid (0.5–1.0 mg) and fractionation on a Bio-Gel P-2 column. Aliquots of the fractions were analyzed for sialic acid and radioactivity. [^{3}H]Hexosamines in the labeled glycoconjugates were estimated after acid hydrolysis (4 M HCl, 100 °C, 8 h). Standard hexosamines and [^{14}C]hexosamines were added to the dried hydrolysates, and the mixture was analyzed by a stream-splitting technique on an amino acid analyzer (Bhavanandan & Davidson, 1976) or on a column of AG50W-X8 (H+) according to Gardell (1953). The fractions were analyzed for hexosamine by the ninhydrin or the Elson–Morgan assay (Levvy & McAllan, 1959) and for radioactivity.

Digestions with Enzymes. Vibrio cholerae neuraminidase digestion was done in 0.1 M sodium acetate buffer, pH 5.6, containing 1 mM Ca^{2+} with 1.0 unit of enzyme in a total digest volume of 200 μL. Incubations with jack bean or *D. pneumoniae* β-galactosidase were done in 50 mM citrate buffer, pH 4.0, or 50 mM citrate/phosphate buffer, pH 7.0, with 0.2 unit of the enzyme at 37 °C for 24 h in a total of 200 μL. Digestion with *D. pneumoniae* β-hexosaminidase was done in 50 mM citrate/phosphate buffer, pH 5.3, with 0.2 unit of the enzyme. Treatment with endo-α-*N*-acetylgalactosaminidase from *D. pneumoniae* was done in 50 mM Tris/maleate buffer, pH 7.6, at 37 °C for 24 h in a total volume of 50 μL (Umemoto et al., 1977). Digestion with endoglycosidase D or H was done in 50 mM citrate/phosphate buffer (D, pH 6.5; H, pH 5.0) at 37 °C for 20 h (Koide & Muramatsu, 1974). Incubation with *E. Freundii* endo-β-galactosidase was in 0.05 M sodium acetate buffer, pH 5.8, at 37 °C for 24 h (Nakagawa et al., 1980). All digestions were terminated by heating at 100 °C for 2–5 min. The mixtures were centrifuged and the supernatants fractionated on a Bio-Gel P-2 column (0.9 × 70 cm) calibrated with known saccharides. In some experiments, appropriate carrier sugars (GlcNAc, Gal, and AcNeu) were added to the supernatants prior to chromatography.

Alkaline Borohydride Treatment of Glycopeptides. Alkaline borohydride treatment of the glycopeptides was done with 1.0 M $NaBH_4$ in 0.1 M NaOH for 72–96 h at 37 °C under nitrogen in sealed tubes in the dark. The reaction mixture was cooled in an ice bath, the excess borohydride destroyed, and the mixture neutralized by careful addition of 1 M acetic acid. The eliminated products were analyzed by chromatography on calibrated columns of Bio-Gel P-4 and P-6.

Other methods used in this study are described in the following paper in this issue (Bhavanandan, 1981).

Results

Several experiments were carried out on the melanoma cells over a period of about 4 years by using early (<20) and late passage (>100) cells. There was no significant difference between the results from individual experiments; the results presented are representative.

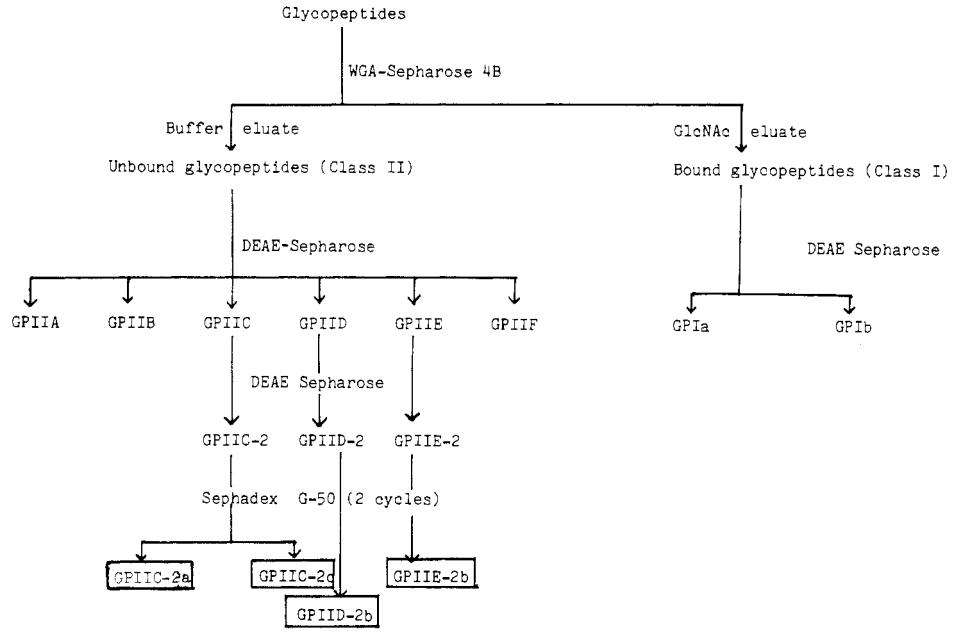
The processing of the labeled glycoconjugates from the cells, spent media, and EGTA supernatants and the cetylpyridinium chloride fractionation are described in the following paper (Bhavanandan, 1981). The glycopeptides were recovered from the cetylpyridinium chloride supernatant. The total radioactivity from [^{3}H]glucosamine incorporated into glycopeptides by the melanoma culture was 93.8 × 10^6 dpm per 10^7 cells compared to 57.8 × 10^6 dpm for 10^7 cells of the melanocyte culture (see Table I, Bhavanandan, 1981).

Gel Filtration Analysis of the Glycopeptides. The mixtures of glycopeptides obtained from melanoma and melanocytes were examined on Sephadex G-50 (not illustrated) and Bio-Gel P-10 (Figure 1) columns. On both columns, the bulk of the melanocyte glycopeptides were included, and only 1–2% eluted in the void volume. In contrast, the melanoma glycopeptides eluting at the void volume of the Bio-Gel P-10 column represented 5 times as much material.

Affinity Chromatography of the Glycopeptides on a WGA–Sepharose Column. The results illustrated in Figure 2 show striking differences in the proportions of the glyco-

[1] Abbreviations used: glyceryl-CPG, glyceryl controlled pore glass beads; WGA, wheat germ agglutinin; RCA_{120} and RCA_{60}, *Ricinus communis* agglutinins of M_r 120000 and 60000, respectively; class I and class II glycopeptides refer to WGA-binding and WGA-nonbinding glycopeptides, respectively; DEAE, diethylaminoethyl; EGTA [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; AcNeu, *N*-acetylneuraminic acid; Tris, tris(hydroxymethyl)aminomethane.

Scheme I: Fractionation of Glycopeptides Isolated from HM7 Human Melanoma Cells



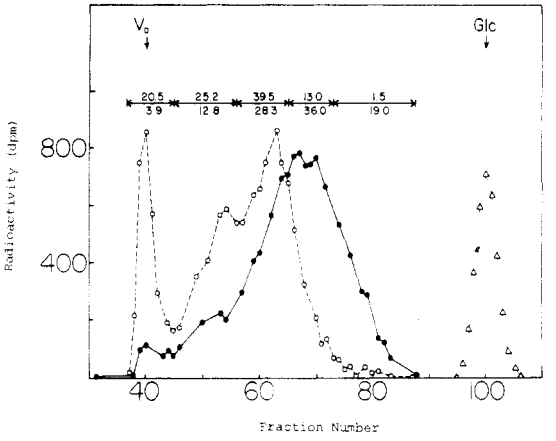


FIGURE 1: Elution profiles of the ^{3}H-labeled glycopeptides from human fetal melanocytes and human melanoma cells on Bio-Gel P-10. Human fetal melanocytes labeled with [^{3}H]glucosamine were processed, digested with Pronase, and treated with cetylpyridinium chloride as described in the text. The ^{3}H-labeled glycopeptides (●) remaining in the cetylpyridinium chloride supernatant were recovered, mixed with [^{14}C]glucose, and chromatographed on a Bio-Gel P-10 column (0.9 × 134 cm). The corresponding ^{3}H-labeled glycopeptides isolated from HM7 human melanoma cells (○) were also mixed with [^{14}C]glucose and chromatographed on the same column. The column was eluted with 0.1 M pyridine/0.1 M acetic acid (pH 5.0) at 20 mL/h, and 1-mL fractions were collected and analyzed for radioactivity. The results are presented by superimposing the elution patterns, using elution of [^{14}C]glucose (△) and the void volume (V_0) as references. The percent of radioactivity of the melanoma and melanocyte glycopeptides eluting in the area indicated by the horizontal arrows are given above and below the arrows, respectively.

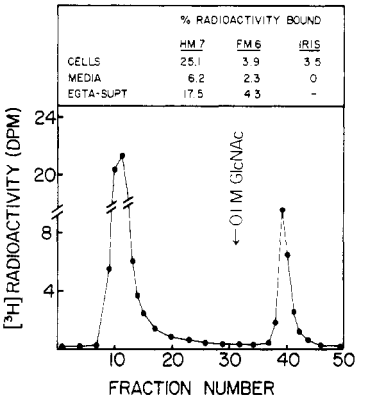


FIGURE 2: Affinity chromatography of glycopeptides on WGA–Sepharose. Glycopeptides isolated from cells, medium, and EGTA supernatant of cell cultures labeled with [^{3}H]glucosamine, as described under Experimental Procedures, were chromatographed on a WGA–Sepharose column (2 × 6.5 cm) containing about 2 mg of WGA covalently bound per mL of gel. The column was eluted with 0.05 M Tris-HCl, pH 8.0, followed by 0.1 M GlcNAc in the same buffer. One-milliliter fractions were collected and analyzed for radioactivity. The results of the elution of melanoma cell-associated glycopeptides are illustrated. The percentage of glycopeptides which specifically bound to WGA when about 50 000 dpm of the glycopeptides from the different cellular compartments of melanoma, melanocytes, and iris were chromatographed is given in the inset. In preparative experiments, the WGA-binding and -nonbinding glycopeptides were recovered by dialysis followed by lyophilization and designated class I and II glycopeptides, respectively.

peptides derived from melanoma, melanocyte, and iris (Banks et al., 1976) cultures specifically binding to WGA. There is a significant reduction of the WGA-binding (class I) glycopeptides in the melanocyte culture and the iris explant when compared to the melanoma culture.

We have previously demonstrated that the binding of sialoglycopeptides to a WGA–Sepharose column depended not only on the number and arrangement of the binding sugar residues (*N*-acetylneuraminic acid or *N*-acetylglucosamine) but also on the density of lectin molecules on the gel beads (Bhavanandan & Katlic, 1979a). Therefore, in the present studies, we have used WGA–Sepharose columns having the same concentration of lectin (about 2 mg/mL). This precaution was important because in subsequent studies we have noticed that on WGA–Sepharose columns having a high density of lectin (10 mg/mL gel), even small serum-type glycopeptides (such as fetuin glycopeptide A) are partially retained (V. P. Bhavanandan, unpublished results).

Fractionation of the Glycopeptides from Cell, Spent Medium, and EGTA Supernatant (Refer to Scheme I). The glycopeptides were separated into WGA-binding (class I glycopeptides) and nonbinding (class II glycopeptides) portions by preparative chromatography on WGA–Sepharose (Figure 2).

Characterization of the Melanoma Class I (WGA-Binding) Glycopeptides. The melanoma class I glycopeptides were

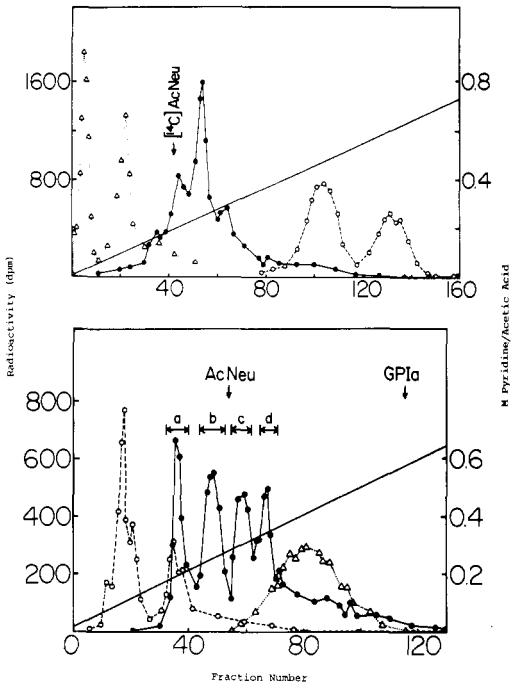


FIGURE 3: Ion-exchange chromatography of glycopeptides on DEAE-Sephacel. (Top) The human melanoma cell-associated glycopeptides [class I (○), class II (●), and asialo class II (△)] isolated from [^{3}H]glucosamine-labeled cultures were each mixed with [^{14}C]AcNeu and chromatographed on a DEAE-Sephacel column (0.9 × 40 cm). The column was eluted with a linear gradient of 0.01–1.0 M pyridine/acetic acid, pH 5.0 (400 mL), at the rate of 20 mL/h. Fractions of 2.0 mL were collected and 1-mL aliquots analyzed for radioactivity. The results are presented by superimposing the elution patterns, using elution of [^{14}C]AcNeu as an internal reference. (Bottom) Elution profiles of the human fetal melanocyte cell-associated glycopeptides [class I (△), class II (●), and asialo class II (○)].

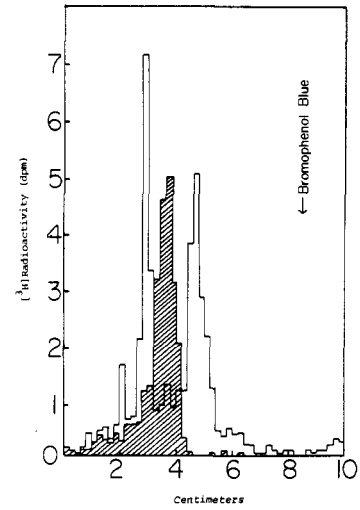


FIGURE 4: Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of human melanoma glycopeptide Ia and glycophorin. The ^{3}H-labeled glycopeptide Ia (shaded area) isolated from human melanoma cells and standard [[^{3}H]AcNeu]glycophorin (unshaded area) were electrophoresed on 6% polyacrylamide gel in the presence of sodium dodecyl sulfate for 3 h at 70 V as described by Weber & Osborn (1969). The gels were sliced into 2-mm segments and solubilized by incubation in glass vials with 0.2 mL of 60% perchloric acid and 0.4 mL of 30% hydrogen peroxide at 60 °C for 4 h. Scintillation counting fluid was added to the solubilized gel, and the radioactivity was determined. The results are superimposed by using the migration position of bromophenol blue as reference. Glycophorin yields two bands corresponding to the monomer (M_r 31 000) and dimer (M_r 62 000).

separated into two fractions, GPIa and GPIb, by chromatography on DEAE-Sepharose CL-6B (Figure 3, top). The latter fractions were rechromatographed on a DEAE-Sephacel column and yielded single peaks, as did chromatography on a glyceryl-CPG column. Both these fractions were excluded from Sephadex G-50 and Bio-Gel P-10, but on a Sephacryl S-200 column, these glycopeptides apparently interacted irreversibly since greater than 90% of the radioactivity was lost. Affinity chromatography of GPIa or GPIb on immobilized lectin columns resulted in either binding (WGA) or no binding (concanavalin A, RCA_{120}, RCA_{60}, and soybean agglutinin) of the entire fractions. Polyacrylamide gel electrophoresis of GPIa and GPIb in 6% gels in the presence of sodium dodecyl sulfate gave single bands. The result obtained with GPIa is illustrated (Figure 4).

Characterization of the WGA-Binding Glycopeptides GPIa and GPIb. The distribution of ^{3}H radioactivity in sialic acid and hexosamines in the class I glycopeptides is given in Table I. The results of further experiments on the GPIa and GPIb obtained from cells are presented below; analogous studies were also carried out on the fractions isolated from spent medium and EGTA supernatant with substantially similar results.

The class I asialoglycopeptides (mixture of asialo-GPIa and -GPIb) prepared by either mild acid hydrolysis or treatment with neuraminidase did not bind (<5%) to WGA–Sepharose, confirming the role of sialic acid in the interaction with WGA (Bhavanandan et al., 1977; Bhavanandan & Katlic, 1979a,b). The asialoglycopeptides bound to both RCA_{120} and RCA_{60} columns, 79% and 78%, respectively, and could be eluted with 0.1 M lactose. Asialo-GPIa and -GPIb were also separately tested on the RCA_{60} column, and 100% and 83% of the radioactivities were specifically bound, respectively. These results

Table I: Percent Distribution of ^{3}H Activity in Sialic Acid and Hexosamines in the Melanoma Class I (WGA-Binding) Sialoglycopeptides[a]

glycopeptides	sialic acid: acid hydrolysis	sialic acid: neuraminidase	$GlcNH_2$	$GalNH_2$
cell				
class I	28.4	28.0*	35.1†	36.5†
GPIa	26.0	26.0*	45.1†	28.9†
GPIb	40.0	42.0*	10.2†	49.8†
spent medium				
class I	27.4	ND	30.5	42.1
EGTA supernatant				
class I	28.2	ND	33.7	38.1
GPIa	26.5	ND	41.9	31.6
GPIb	44.4	ND	8.9	46.7

[a] The sialoglycopeptides were treated with acid or neuraminidase and chromatographed on Bio-Gel P-2 (see Methods for details), and the percent of radioactivity coeluting with carrier sialic acid was estimated. Sialic acids from experiments marked with an asterisk were recovered and identified as *N*-acetylneuraminic acid by paper chromatography in solvent system A. Percent of radioactivity in the hexosamines was determined by hydrolyzing (4 M HCl, 100 °C, 8 h) a separate aliquot of the glycopeptide followed by analysis on a column of AG50W (H^+) (samples marked †) or by stream-splitting technique on an amino acid analyzer (details and references are given under Methods). Total radioactivity refers to the sum in sialic acid (acid hydrolysate), $GlcNH_2$, and $GalNH_2$. ND = not determined.

indicated the presence of β-galactosyl or β-*N*-acetylgalactosaminyl nonreducing terminals in these asialoglycopeptides (Irimura et al., 1975); however, heterogeneity with respect to the distribution of oligosaccharides on the peptide may be present (V. P. Bhavanandan, unpublished results).

Treatment of the asialo-GPIa and -GPIb with endo-α-*N*-acetylgalactosaminidase released 25% and 58% radioactivity, respectively, in a product which coeluted on Bio-Gel P-2 columns with Gal1→3GalNAc prepared by treatment of fetuin

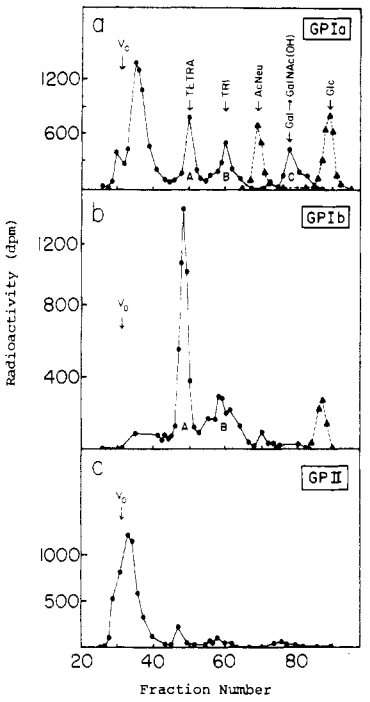


FIGURE 5: Elution profiles on Bio-Gel P-6 of human melanoma glycopeptides after treatment with alkaline borohydride. The human melanoma cell-associated glycopeptides, GPIa (panel a), GPIb (panel b), and GPII, i.e., Class II (panel c), were treated with alkaline borohydride and chromatographed on a Bio-Gel P-6 column (0.9 × 105 cm) as described under Methods. The column was eluted with 0.1 M pyridine/0.1 M acetic acid, 1-mL fractions were collected, and aliquots were analyzed for radioactivity. The elution profiles of internal standards glucose (▲) and *N*-acetylneuraminic acid (▲) and the peak elution positions of blue dextran (V_0), AcNeu→Gal→(AcNeu)→-GalNAc(OH) (tetra), AcNeu→Gal→GalNAc(OH) (tri), and Gal→GalNAc(OH) are also indicated.

asialoglycopeptides (Umemoto et al., 1977) with the same enzyme. The product from asialo-GPIa and -GPIb was isolated by gel filtration on a Bio-Gel P-2 column and desalted by passage through mixed-bed ion-exchange resin. Paper chromatography in solvent system A showed that it had a mobility identical with that of reference Gal1→3GalNAc. The identity of the disaccharide was further confirmed by treatment with $NaBH_4$ followed by acid hydrolysis, after which the only labeled component detected on the amino acid analyzer was [^{3}H]galactosaminitol (Bella & Kim, 1970). The sialo- and the asialoglycopeptides Ia and Ib were not degraded by endo-β-galactosidase from *E. Freundii*.

Alkaline borohydride treatment of GPIa and GPIb followed by gel filtration on a Bio-Gel P-4 or P-6 column indicated that 46% and 88% of the radioactivities, respectively, were released. On both columns, the major products coeluted with [AcNeu→Gal→(AcNeu)→GalNAcOH], [AcNeu→Gal→GalNAcOH], and [Gal→GalNacOH] prepared from fetuin or glycophorin by alkaline borohydride treatment (Spiro & Bhoyroo, 1974) (Figure 5). The major elimination products from GPIa and GPIb were isolated by preparative chromatography on Bio-Gel P-6, repeatedly evaporated with methanol to remove borate (when present), and examined by paper chromatography. In solvent systems A and C, they comigrated with the tetra-, tri-, and disaccharide alcohols isolated from fetuin (Figure 6). Acid hydrolysis of the total β-eliminated oligosaccharides from GPIb followed by stream-split analysis on the amino acid analyzer employing the citrate–borate buffer system (Bella & Kim, 1970) gave only [^{3}H]galactosaminitol, with no detectable [^{3}H]glucosamine or [^{3}H]galactosamine. Mild acid hydrolysis (0.1 N H_2SO_4, 80 °C, 1 h) of the tetrasaccharide followed by chromatography on a Bio-Gel P-6 column gave two sharp peaks coincident with reference AcNeu and Gal1→3GalNAcOH; the radioactivity was distributed equally between the two peaks. The alkaline borohydride resistant portions of GPIa and GPIb (55% and 12%, respectively) eluting prior to the tetrasaccharide peak on the Bio-Gel P-6 column (Figure 5a,b) were also recovered and examined. Only minor portions (14% and 11%) of these fractions were retained on the WGA–Sepharose 4B column. [^{3}H]Glucosamine accounted for greater than 90% of the radioactivity detectable after total acid hydrolysis, in both cases.

Retreatment of GPIa and GPIb with Pronase did not alter their elution profile on the glyceryl-CPG or Sephadex G-50 columns.

Characterization of the Melanocyte WGA-Binding Glycopeptides. Ion-exchange chromatography on a DEAE-Sephacel column using a 0.01–1.0 M pyridine/acetic acid gradient gave one broad peak which eluted after the WGA-nonbinding (class II) glycopeptides and before the human melanoma class I glycopeptides Ia (Figure 3, bottom). The percent distribution of radioactivity in sialic acid and hexosamines (Table II) resembles that of melanoma Ia glycopeptides. Alkaline borohydride treatment of WGA-binding glycopeptides from cells followed by gel filtration on a Bio-Gel P-6 column indicated that 38% of the radioactivity was released as oligosaccharides. The hexosamine in the alkaline borohydride resistant portion was practically all glucosamine.

Studies on the WGA-Nonbinding (Class II) Glycopeptides. The distribution of radioactivity in sialic acid and hexosamine

Table II: Percent Distribution of ^{3}H Activity in Sialic Acid and Hexosamines in the Glycopeptides Produced by Human Melanocytes in Culture and by Explant Iris Culture[a]

glycopeptides	sialic acid (acid hydrolysis)	$GlcNH_2$	$GalNH_2$
FM6 cell			
class I	31.5	43.8	24.7
class II	20.0*	66.0	14.0
class IIa	5.3	90.8	3.9
class IIb	10.6	82.1	7.3
class IIc	14.3	75.2	10.5
class IId	16.1	71.1	12.8
FM6 spent medium			
class I	34.0	39.5	26.5
class II	20.9*	65.6	13.5
FM6 EGTA supernatant			
class II	21.3	78.7	<1
iris explant cell	16.0	73.1	10.9
iris explant medium	18.6	76.5	4.9

[a] Details are as described in Table I.

Table III: Percent Distribution of ^{3}H Activity in Sialic Acids and Hexosamines in the Melanoma Class II (WGA-Nonbinding) Sialoglycopeptides[a]

glycopeptides	sialic acid: acid hydrolysis	sialic acid: neuraminidase	$GlcNH_2$	$GalNH_2$
cell				
class II	19.6	19.1*	68.3	12.1
GPIIC-2a	15.5	14.0*	79.7	4.8
GPIIC-2c	21.5	20.3*	78.5	0.0
GPIID-2b	23.1	21.7*	76.9	0.0
GPIIE-2b	23.0	20.0*	73.8	3.2
spent medium				
class II	22.9	ND	72.5	4.6
EGTA supernatant				
class II	23.7	ND	63.3	13.0

[a] Details are as described in Table I.

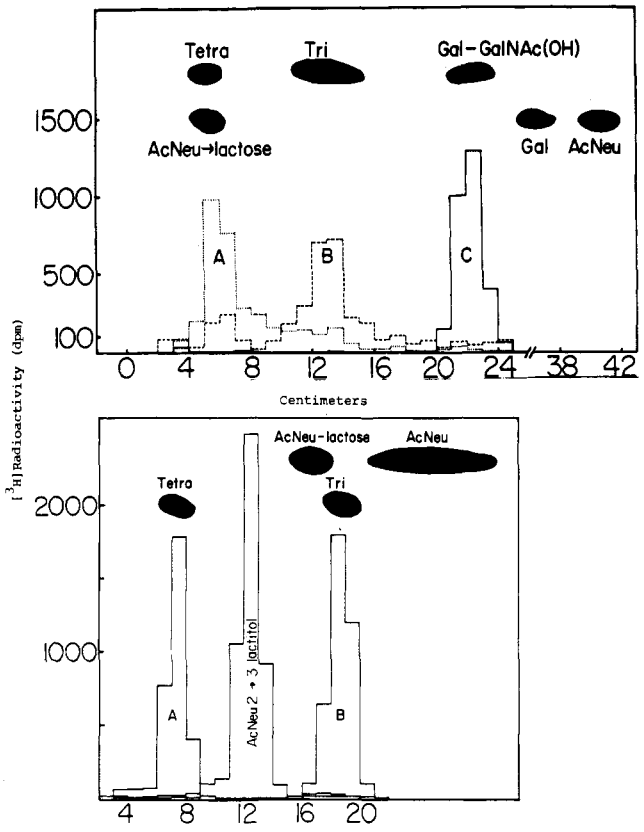


FIGURE 6: Paper chromatography of β-eliminated oligosaccharides from human melanoma glycopeptides Ia (top panel) and Ib (bottom panel). The ^{3}H-labeled oligosaccharides A, B, and C obtained from GPI a (Figure 5, panel a) and A and B from GPIb (Figure 5, panel b) were chromatographed by using solvent systems A and C, respectively. The migration positions of standard sugars detected as described under Methods are indicated. Tetra and Tri refer to the tetra- and trisaccharides described in Figure 5.

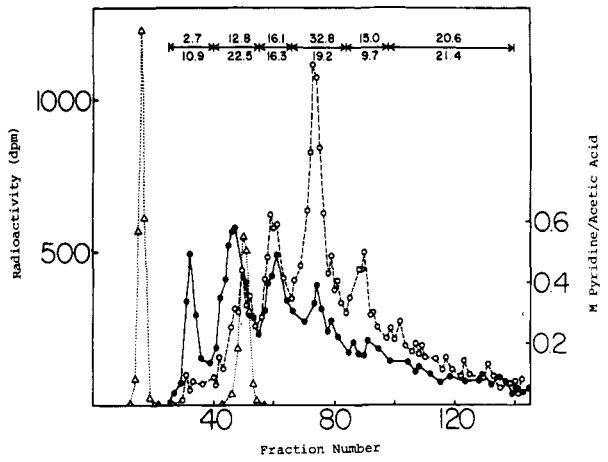


FIGURE 7: Elution profiles of the ^{3}H-labeled glycopeptides from human fetal melanocytes and human melanoma cells on DEAE-Sephacel. The WGA-nonbinding (class II) glycopeptides isolated from [^{3}H]-glucosamine-labeled human fetal melanocytes (●) and human melanoma (○) were separately mixed with [^{14}C]glucose and [^{14}C]AcNeu (▲) and chromatographed on a DEAE-Sephacel column (1.5 × 22 cm). The column was eluted with a linear gradient of 0.01–0.5 M pyridine/acetic acid, pH 5.0 (300 mL), at 15 mL/h. Fractions of 2.1 mL were collected and 1-mL aliquots analyzed for radioactivity. The results are presented by superimposing the elution profiles. The present of radioactivity of the melanoma and melanocyte glycopeptides eluting in the area indicated by the horizontal arrows are given above and below the arrows, respectively.

in the class II glycopeptides is given in Tables II and III.

The elution profiles of the WGA-nonbinding glycopeptides obtained from melanoma cells and melanocytes on an ion-exchange column is illustrated in Figure 7. There is a higher proportion of the less anionic glycopeptides in the melanocyte-derived glycoconjugates. For example, 49.7% of the ^{3}H-labeled glycopeptides from melanocytes elutes in fractions 25–66 as opposed to only 31.6% in the case of the melanoma-derived glycopeptides. Gel filtration on glyceryl-CPG, Sephadex G-50, and Bio-Gel P-10 indicated that these glycopeptides are heterogeneous and of lower molecular weight when compared to the class I glycopeptides. The melanoma cells, however, contained a higher proportion of the larger glycopeptides than did the melanocytes (cf. Figure 1).

The size of the class II glycopeptides was reduced on desialylation (about 80% of radioactivity was now dialyzable), and the asialoglycopeptides were still highly heterogeneous. It was therefore apparent that the heterogeneity of the class II glycopeptides is not solely due to differences in the degree of sialylation, even though this may be a factor. This was further illustrated by chromatography of the untreated and desialylated glycopeptides on a DEAE-Sepharose column (Figure 3, top).

Characterization of the Melanoma Class II Glycopeptides. The melanoma sialo- and asialoglycopeptides on treatment with endo-α-*N*-acetylgalactosaminidase or with endo-β-galactosidase released insignificant quantities of radioactivity. The results of alkaline borohydride treatment of the class II glycopeptides from melanoma cells followed by gel filtration is illustrated in Figure 5c. About 5% and 4% of the radioactivities were in the released tetra- and trisaccharides, respectively.

Attempts to fractionate the class II glycopeptides on WGA–, Con A–, RCA_{120}–, and RCA_{60}–Sepharose columns were unsuccessful since these glycopeptides were not retained on these columns. The asialoglycopeptides also did not interact with either WGA– or Con A–Sepharose but were partially (77% and 24%) retained on RCA_{120}– and RCA_{60}–Sepharose columns. On a RCA_{120}–Sepharose column, class II asialoglycopeptides separated into three categories: not retained (excluded from the column), 12%; retarded but eluted with buffer, 76%; retained and eluted with 0.1 M lactose, 12%.

Fractionation of the Melanoma Class II Glycopeptides (Refer to Scheme I). The class II glycopeptides from melanoma cells were fractionated on a DEAE-Sepharose column into six fractions (GPIIA–F) by using a 0.01–1.0 M pyridine/acetic acid, pH 5.0, gradient. The three major fractions (GPIIC, GPIID, and GPIIE) were separately rechromatographed on DEAE-Sepharose by using a 0–0.5 M pyridine/acetic acid gradient, and the material eluting in sharp peak areas GPIIC-2, GPIID-2, and GPIIE-2 was recovered (Figure 8) and chromatographed on a column (0.9 × 140 cm) of Sephadex G-50 (fine). Fraction GPIIC-2 yielded two peaks and fractions GPIID-2 and GPIIE-2 one major peak each. The material in the major peaks in each case was rechromatographed on the Sephadex G-50 column, recovered, and designated GPIIC-2a, GPIIC-2c, GPIID-2b, and GPIIE-2b. These glycopeptides represented 4.0%, 5.4%, 10.5%, and 3.3% of the total radioactivity in the glycopeptides (cetylpyridinium chloride supernatant).

Partial Characterization of GPIIC-2a, GPIIC-2c, GPIID-2b, and GPIIE-2b. The distribution of ^{3}H counts in sialic acid and hexosamine in these glycopeptides is given in Table III. All four glycopeptides had a lower proportion of radioactivity in sialic acid and a much higher proportion in glucosamine as compared to GPIa and GPIb. This indicated that these may contain N-glycosidic linkages between GlcNAc and asparagine.

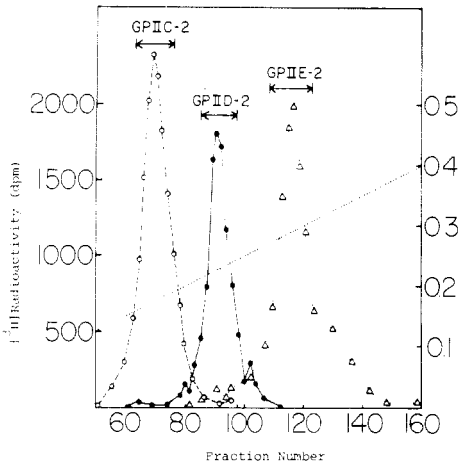


FIGURE 8: Fractionation of ^{3}H-labeled class II glycopeptides from human melanoma cells on DEAE-Sepharose. The human melanoma class II glycopeptides isolated from [^{3}H]glucosamine-labeled cells were chromatographed on a DEAE-Sepharose column (1.5 × 25 cm). The column was eluted with a linear gradient of 0.01–1.0 M pyridine/acetic acid, pH 5.0. The total volume of the gradient was 400 mL, and the rate of elution was about 20 mL/h. Fractions of 2 mL were collected, and aliquots of 25 μL were analyzed for radioactivity. The glycopeptides eluting in fractions 42–51, 52–62, and 63–74 were recovered by lyophilization and designated GPIIC, GPIID, and GPIIE. They were then individually rechromatographed on the same DEAE-Sepharose column by using a 0–0.5 M pyridine/acetic acid gradient. In each case, the material eluting in the area indicated by horizontal arrows (GPIIC-2, GPIID-2, and GPIIE-2) was recovered by lyophilization.

Asialo-GPIIC-2c and -GPIID-2b, prepared by treatment with *V. cholerae* neuraminidase followed by chromatography on Bio-Gel P-2, interacted specifically with RCA_{120}–Sepharose but not with Con A–Sepharose. Treatment of the asialo-GPIIC-2c and -GPIID-2b with *D. pneumoniae* β-galactosidase and β-*N*-acetylhexosaminidase released a product containing 45% and 55% of the radioactivity, respectively. This product eluting in the same position as GlcNAc on a Bio-Gel P-2 column was isolated and desalted by passage through Dowex AG50 (H+) and Dowex AG1 (formate) columns. On paper chromatography using solvent system B, it was indistinguishable from standard GlcNAc. In control experiments, treatment of the asialoglycopeptides with either enzyme alone failed to release any labeled products.

The portion of asialo-GPIIC-2c undigested by the exoglycosidases eluting at the void volume of the Bio-Gel P-2 column was recovered by lyophilization. It was not retained on a Con A–Sepharose column and was resistant to degradation by endo-*N*-acetylglucosaminidase H. However, on treatment with endo-*N*-acetylglucosaminidase D followed by chromatography on a Bio-Gel P-4 column, 32% of the radioactivity eluted in the position of reference GlcNAc→Asn. Hydrazinolysis of GPIIC-2c and GPIID-2b followed by gel filtration of a Bio-Gel P-6 column gave included peaks, indicating that these glycopeptides had oligosaccharides linked N-glycosidically to the peptide.

Fractionation of the Melanocyte Class II Glycopeptides. The cell-associated class II sialoglycopeptides were preparatively fractionated on the DEAE-Sepharose column into four fractions (a–d). The distribution of radioactivity in the peaks marked a, b, c, and d in Figure 3 (bottom) was 23%, 33%, 23%, and 21%. The fractions were rechromagraphed on a DEAE-Sephacel column using 0–0.5 M pyridine/acetic acid gradient, and the material eluting in sharp peaks was recovered and designated class IIa, class IIb, and class IIc, and class IId. The distribution of ^{3}H activity in sialic acid and the significant quantities of galactosamine in these glycopeptides compared to the human melanoma class II glycopeptides (Table III) is of interest. The results suggest that the major portion of the carbohydrate in these glycopeptides is of the N-glycosidically linked type. Insufficient material was available for further purification and characterization of these four subfractions.

Characteristics of Glycopeptides from Human Embryonic Iris. Chromatography on Sephadex G-50 of the glycopeptides from both tissue and medium gave patterns very similar to that of the asialoglycopeptides isolated from cultured melanocytes. On gel filtration on a calibrated CPG column, the iris glycopeptides eluted as a broad peak, with a maximum two fractions before glucuronic acid. The elution pattern of the iris glycopeptides on a DEAE-Sepharose column was unusual and markedly different from those of the melanocyte and melanoma glycopeptides. A significant portion (27%) of the iris glycopeptides eluted in a sharp peak very early; these are probably uncharged (nonsialylated) or low sialic acid glycopeptides. The overall reduction in sialylation in the iris glycopeptides is also reflected by the low percent of radioactivity in the sialic acid, 16.0% and 18.6;, for the tissue and medium derived glycopeptides, respectively. Alkaline borohydride treatment of these iris medium glycopeptides followed by chromatography on Bio-Gel P-6 showed that about 12% of the radioactivity was released as tetra- and trisaccharides. This is in agreement with the presence of 11% and 5% [^{3}H]-galactosamine in the iris tissue and medium glycopeptide, respectively. The elution pattern of the elimination products on the Bio-Gel P-6 column was very similar to that obtained for melanocyte glycopeptides after the same treatment. These O-glycosidically linked oligosaccharides are apparently not present as clusters and thus fail to bind to the WGA–Sepharose.

Discussion

The WGA-binding glycopeptide, GPIb, had a mobility on a glyceryl-CPG column identical with that of chondroitin 4-sulfate from cartilage and of mouse melanoma class I glycopeptides (Bhavanandan & Davidson, 1976), indicating an apparent molecular weight in the range of 12000–15000, whereas glycopeptide GPIa had an apparent molecular weight of ~8000–10000. The reason for the anomalous behavior of GPIa on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Figure 4) is not entirely clear but is probably dependent on higher carbohydrate content of GPIa (expected to be greater than 90%) compared to that of glycophorin (55%). The high sialic acid content is likely to be the primary determinant of the ion-exchange chromatographic behavior of class I glycopeptides Ia and Ib (Figure 3, top) even though factors such as amino acid composition and size are likely to contribute.

Chromatographic and enzymatic data suggest the presence of the sequence Gal1→3[^{3}H]GalNAc in asialo-GPIa and -GPIb. GPIb has a large portion of tetra- (51%) and trisaccharides (31%) linked O-glycosidically in clusters to serine or threonine, as suggested by affinity chromatography on WGA–Sepharose (Bhavanandan & Katlic, 1979a) and resistance to degradation by Pronase. Thus GPIb has a structure very similar to that suggested for the mucin-type (class I) sialoglycopeptides obtained from mouse melanoma (Bhavanandan & Davidson, 1976; Bhavanandan et al., 1977). However, it differs from the mouse melanoma glycopeptide in containing a small proportion of N-glycosidically linked glucosamine-containing oligosaccharides and in not being precipitated by cetylpyridinium chloride. Since neither glycophorin nor glycopeptides derived from it is precipitated to any extent by this cationic detergent, the class I mouse me-

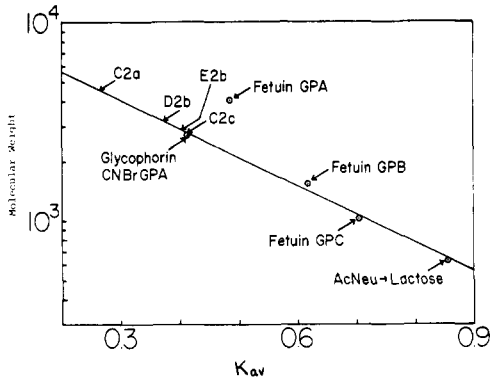


FIGURE 9: Molecular weight estimation of human melanoma glycopeptides IIC-2a, IIC-2c, IID-2b, and IIE-2b. The purified ^{3}H-labeled human melanoma glycopeptides IIC-2a, IIC-2c, IID-2b, and IIE-2b were individually mixed with ^{14}C-labeled fetuin and [^{14}C]-glucose and chromatographed on the calibrated Sephadex G-50 column. The K_{av} values are plotted against log molecular weight.

lanoma glycopeptides may contain other anionic groups (sulfate or phosphate) or a closer spacing of the sialic acid residues and therefore a higher charge density. Further work is necessary to resolve this discrepancy.

The higher proportion of glucosamine, the difference in the ratio of O-glycosidically linked oligosaccharides, and the inability to convert GPIa to GPIb by treatment with Pronase excludes the possibility that GPIa is a precursor of GPIb, although both bind to WGA–Sepharose.

The four substantially homogeneous WGA-nonbinding (class II) glycopeptides from melanoma cells have apparent molecular weights of 7000, 3700, 4500, and 3800 based on their mobilities on a Sephadex G-50 column (Figure 9). The anomalous behavior of sialoglycopeptides (Alhadeff, 1978; Santer & Glick, 1979) in gel filtration precludes a more accurate determination of their molecular weights by this technique. Glycopeptides IIC-2c and IID-2b were shown to have Gal→GlcNAc sequences penultimate to sialic acid and *N,N'*-diacetylchitobiosyl linkages to asparagine residues, probably with fucose substitution at the internal GlcNAc residue (Muramatsu et al., 1978). The various class II glycopeptides may differ from each other in monosaccharide composition and the number and completeness of the branches, i.e., bi-, tri-, and tetraantennary structures; differences in the core region are also possible.

N-Glycosidically linked simple (oligomannosyl) type oligosaccharides were not detected in these studies, probably due to the isolation procedure which involved extensive dialysis. In fact, the gel filtration profile on Sephadex G-50 of the total Pronase digest of the cells before and after exhaustive dialysis suggests the loss of low molecular weight fragments. Other glycopeptides which would have been lost by dialysis are those having nonclustered O-glycosidically linked oligosaccharides as are present in fetuin. The failure of endo-β-galactosidase from *E. Freundii* to degrade GPIa, GPIb, or class II sialo- or asialoglycopeptides indicates the absence of repeating Gal→GlcNAc structures in these fractions (Krusius et al., 1978; Li et al., 1980). The sialic acid and hexosamine compositions of the partially purified melanocyte class II glycopeptides and of iris glycopeptides (Table II) show that the major portion of the carbohydrate in these glycopeptides are N-glycosidically linked *N*-acetyllactosaminyl type, although they differ from the analogous melanoma glycopeptides in having significant quantities of galactosamine.

An increase in the molecular size of N-glycosidic complex glycopeptides from malignant cells (Warren et al., 1978) was also observed in this study. The increased production of these higher molecular weight glycopeptides (referred to as group A by Warren et al., 1978) has been correlated with the active growth state and tumorigenicity of cancer cells (Buck et al., 1971; Glick et al., 1973; Muramatsu et al., 1973; Van Beek et al., 1977). The purification and partial structure of one of these larger glycopeptides isolated from Rous sarcoma virus transformed baby hamster kidney cells were recently reported (Glick, 1979; Santer & Glick, 1979).

Another difference noted in our studies was a striking increase in the WGA-binding glycopeptides in the melanoma culture compared to the melanocyte culture (Figures 1 and 2). Similar increases in the production of WGA-binding glycopeptides by B16 mouse melanoma cells (Satoh et al., 1974; Bhavanandan & Davidson, 1976; Bhavanandan et al., 1977) and by two mammary cancer cells (Chandrasekaran & Davidson, 1979) compared to their normal counterparts have also been noted. The relevance of these alterations to malignancy is not clear, but both alterations noted indicate a higher degree of glycosylation (specifically sialylation) in cancer cells. This is in agreement with the overall increase in the negative charge of the cancer cell as detected by whole cell electrophoresis (Abercrombie & Ambrose, 1962) and with the increase in sialyltransferase activities of cancer cells (Warren et al., 1972; Bosmann & Hall, 1974; Bernacki & Kim, 1977). Further, studies with malignant cells in vivo have consistently shown that plasma membrane sialic acid is elevated relative to control cells (Mabry & Carubelli, 1972; Bryant et al., 1974; Bosmann et al., 1974; Dristrian et al., 1977). This increased cell surface sialic acid has been implicated in reducing the immunogenicity of neoplastic cells by masking antigenic determinants.

Since the melanocytes were of embryonic origin, the differences noted in our studies may not be entirely related to the malignant state of the melanoma cells. It is also difficult to draw conclusions regarding the changes in the individual glycoconjugates based solely on the results of these studies. Recently, we succeeded in purifying and partially characterizing a mucin-type glycoprotein from HM7 human melanoma cells (Umemoto et al., 1981).

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