CHARACTERISTICS OF A MUCIN-TYPE SIALOGLYCOPEPTIDE PRODUCED BY B16 MOUSE MELANOMA CELLS

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SUMMARY: The glycopeptides produced by B16 mouse melanoma cells grown in the presence of [^{3}H]glucosamine were isolated and fractionated into two classes (I and II) with cetyl pyridinium chloride. The class I glycopeptides were of higher molecular weight and of higher negative charge (sialic acid content) than those in class II. Class I glycopeptides contained N-acetyl neuraminic acid, galactose and N-acetylgalactosamine and on treatment with alkaline-borohydride were degraded to apparently tri- and tetrasaccharides. The presence of this mucin-type glycoprotein on the cell surface was detected by mild trypsinization of intact cells.

Examination of proteins and glycoproteins of control and transformed cells by gel filtration or by gel electrophoresis has shown numerous differences of a quantitative nature. These include a sialoglycopeptide derived from membranes (1,2) and a high molecular weight surface protein that disappears on transformation (2-4).

While studying the polysaccharides produced by B16 mouse melanoma cells it was discovered that cetyl pyridinium chloride (CPC)[1] was capable of precipitating a sialoglycopeptide from the pronase digested cells and from spent media in which these cells were grown. Markedly reduced amounts of this sialoglycoprotein were produced by a control population of mouse iris melanocytes (5).

This study describes the partial characterization of this sialoglycopeptide. Its properties are compared with those of the glycopeptides which are not precipitated by CPC and remain in the supernatant.

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[1] Abbreviations used: CPC, cetyl pyridinium chloride; TPCK-trypsin, L-(1-tosylamido-2-phenyl)-ethyl chloromethylketone treated trypsin; GalNAc, N-acetyl galactosamine.

MATERIALS AND METHODS

Bl6 mouse melanoma cells were grown and the complex saccharides labeled with [^{3}H]glucosamine and $Na_2{}^{35}SO_4$ as described previously (5,6).

The glycopeptides were isolated from labeled cells as well as the spent media by exhaustive digestion with pronase and fractionation of the solubilized components with CPC (6). For isolation of cell surface complex saccharides, cells were cultured for 48 hours prior to harvest in medium containing 1/3 the usual amount of glucose and 0.5 μCi ml^{-1} of D-[^{14}C]glucosamine (New England Nuclear, 51.5 mCi $mmole^{-1}$). The cells were subjected to short-term successive incubations with TPCK-trypsin (Worthington Biochemical Corp.) according to Codington *et al* (7) and the resulting trypsinates digested with pronase to yield cell surface glycopeptides.

Hexosamine determinations on isotopically labeled glycopeptides were carried out on acid hydrolysates (4 N HCl, 8 hr, 100°C) on a Beckman 120C amino acid analyzer employing a stream-splitting attachment. Unlabeled and [^{14}C]-labeled glycine, glucosamine and galactosamine were used as markers. To identify hexosaminitols, the citrate-borate buffer system as described by Bella and Kim (8) was used. Labeled sialic acid in glycopeptides was determined either by acid hydrolysis (0.1 N H_2SO_4, 80°C, 1 hr) or neuraminidase treatment followed by separation of sialic acid from asialoglycopeptide on a BioGel P2 200-400 mesh column (0.9 x 70 cm) by elution with 0.1 M pyridine acetate, pH 5.0. Radioactivity in column fractions was determined as described previously (6). To locate radioactivity on paper chromatograms, 1 cm paper strips were extracted with 1 ml water in counting vials prior to addition of scintillation liquid and counting. Paper chromatography was done on Whatman No. 1 paper using the following solvents: A, N-butyl acetate:glacial acetic acid:water (3:2:1); B, N-butyl alcohol:pyridine:water (6:4:3); C, ethylacetate:pyridine:water (9:3:2). Marker sugars were detected by a silver nitrate stain.

Vibrio cholerae neuraminidase (Calbiochem) digestion was done in 0.1 M Na acetate buffer, pH 5.6 containing 0.001 M $CaCl_2$ with 188 milliunits of enzyme in a total digest volume of 200 μl; incubation was performed at 37°C. Influenza virus neuraminidase (Calbiochem) digestion was carried out in 0.05 M Tris-maleate buffer, pH 6.5 containing 0.001 M $CaCl_2$ with 174 milliunits of the enzyme in a volume of 200 μl. Incubation with β-galactosidase from Jack bean (9) was performed in 0.05 M citrate-buffer, pH 4.0 in a volume of 50 μl using 0.1 units enzyme for 24 hr at 37°C. Digestions with β-N-acetyl hexosaminidase and with α-N-acetyl galactosaminidase both from *Charonia lampus* (Miles Laboratories) were also done under the above conditions using 0.1 unit or 10 milliunit of the enzymes respectively. The released labeled sugars were estimated by chromatography of the digest on BioGel P2 columns as described above.

RESULTS AND DISCUSSION

The labeled glycopeptides synthesized by the mouse melanoma and present in the pronase digest of the cells or media were fractionated into two classes on the basis of their interaction with CPC. The Class I glycopeptides were selectively eluted from the CPC precipitate with 0.2 M NaCl, whereas the Class II glycopeptides which remain in the CPC supernate were isolated after precipitation of CPC with potassium thiocyanate and extensive dialysis. The Class I glycopeptides contained 4-5% of the incorporated tritium label and those in Class II about 90%; the balance of the tritium

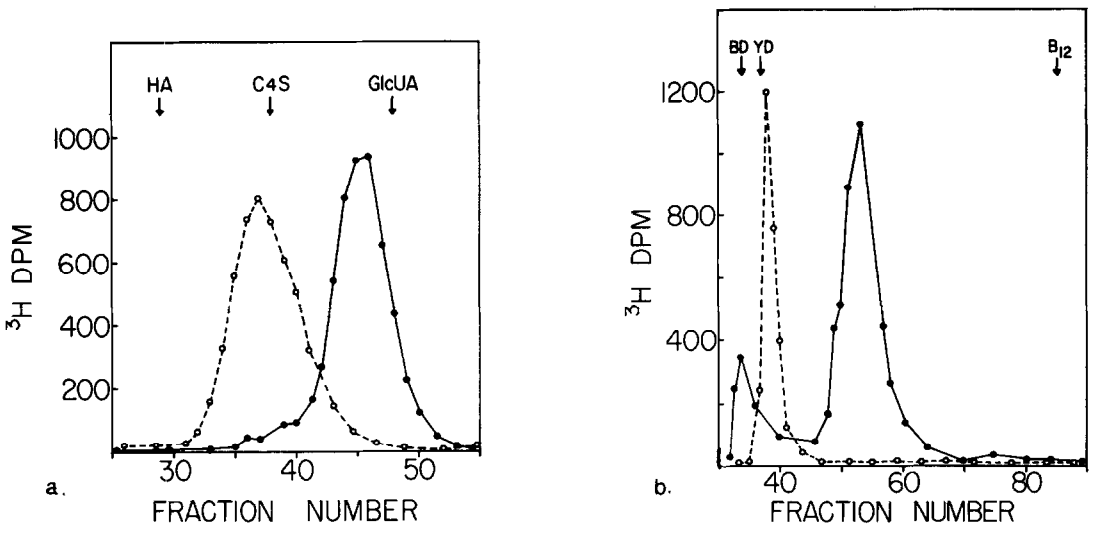

Fig. 1: Chromatography of mouse melanoma glycopeptides (a) on CPG 10-240 glass bead column (0.9 x 90 cm). Elution was with 0.5 M $CaCl_2$ at 30 ml per hr. The peak elution positions of marker saccharides (HA; vitreous humor hyaluronic acid; C4S porcine rib cartilage chondroitin-4-sulfate and GlCUA, glucuronic acid) determined by the oricinol reaction are indicated by arrows. (b) On Sephadex G-50 column (0.9 x 140 cm) eluted with 0.1 M pyridine acetate pH 5.0. The peak elution positions of markers BD, blue dextran 2000; YD, yellow dextran and Bl2, vitamin B_{12} are indicated by arrows. One (1) ml fractions were collected and aliquots analyzed for radioactivity. Class I (---O---O---) and Class II (—●—●—) glycopeptides were run in separate experiments and the elution patterns superimposed.

label and virtually all of the non-dialyzable ^{35}S label were in mucopolysaccharides.

The Class I glycopeptides were of higher molecular weight (Fig. 1) and higher negative charge (Fig. 2) than those in Class II. The former appeared relatively homogenous on the basis of size and charge whereas the latter was obviously a complex mixture. The tritium label in these glycopeptides was distributed between sialic acid and hexosamines. Class I glycopeptides had about 38% of their label in sialic acid, the balance being in galactosamine. The label in Class II glycopeptides was distributed between sialic acid (25%), glucosamine (66%) and galactosamine (9%). The sialic acid of glycopeptide I was identified as [^{3}H]-N-acetyl neuraminic acid by co-chromatography with standard on a BioGel P2 column, by paper chromatography in

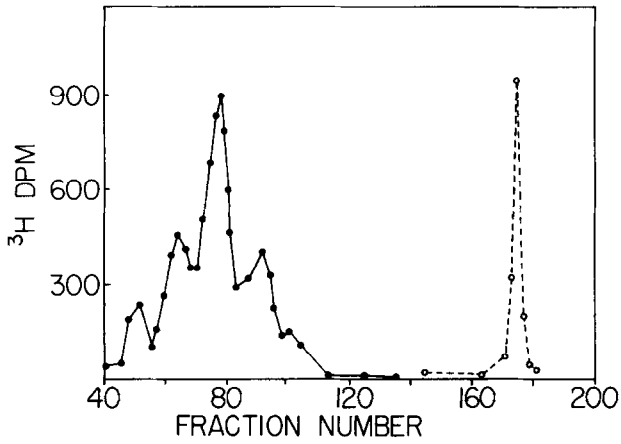

Fig. 2: Chromatography of Class I and II glycopeptides from mouse melanoma cells on DEAE cellulose column (0.9 x 70 cm). Elution was with a linear gradient of 0.01 M to 0.5 M pyridine acetate buffer, pH 5.1 (fractions 1-140), followed by 1 M pyridine acetate of same pH. Rate of flow was 30 ml per hr. Fractions of 2.5 ml were collected and aliquots analyzed for radioactivity. Class I (---O---O---) and Class II (—●—●—) were analyzed on separate runs and the results are presented by superimposing the elution patterns.

solvent A and by paper electrophoresis (3 MM paper, pyridine acetate buffer, pH 3.5, 10V/cm, 3 hr). Further evidence that the hexosamine of glycopeptide I is galactosamine was obtained by ninhydrin oxidation (10) followed by identification of [^{3}H]-lyxose as the only labeled component by paper chromatography (solvent C). [^{3}H]-galactose was the only neutral sugar detected by paper chromatography of a total acid hydrolysate of Class I glycopeptides isolated from cells grown in the presence of [^{3}H]glucose (V. P. Bhavanandan, J. Umemoto, J. Banks and E. A. Davidson, unpublished results).

Vibrio cholerae neuraminidase digestion of Class I glycopeptides released virtually 100% of the labeled sialic acid in 24 hr, indicating that all the sialic acid was present as terminal residues. However, influenza virus neuraminidase released only about 50% of the sialic acid in 24 hr, suggesting that sialic acid may be involved in more than one type of linkage. It has been reported that whereas *Vibrio cholerae* neuraminidase acts equally on both 2→3 and 2→6 linked sialic acid, influenza virus

enzyme acts preferentially on 2→3 and only weakly on 2→6 linkage (11).

Incubation of the asialoglycopeptide (prepared by *Vibrio cholerae* neuraminidase treatment) with *Charonia lampus* β-hexosaminidase, *Charonia lampus* α-N-acetyl galactosaminidase or Jack bean β-galactosidase followed by β-hexosaminidase failed to release any labeled sugar. However, when the asialoglycopeptide was treated with Jack bean β-galactosidase followed by α-N-acetyl galactosaminidase, a component with 27% of the label was released and was identified as N-acetyl galactosamine by paper chromatography in solvents A and B. This suggests the presence of the sequence $Gal \xrightarrow{\beta} [^3H]GalNAc \xrightarrow{\alpha}$ in the asialoglycopeptide.

In order to examine the nature of the linkage between carbohydrate and peptide, the Class I glycopeptides were treated under nitrogen with 0.3 M $NaBH_4$ - 0.1 N NaOH for 90 hr at 37°C in sealed tubes in the dark. The excess borohydride was destroyed by careful addition of 1 N acetic acid and the neutralized solution chromatographed on controlled pore glass bead column. The radioactive material now migrated in the low molecular weight region with the peak elution position at fraction 48 (Fig. 1a). On BioGel P2 and P4 columns, about 90% of the radioactive material was included whereas the untreated sample was entirely excluded. Paper chromatography of the alkali degraded product gave two spots, the major one having $R_{Lactose}$ 0.34 and 0.09 and the minor one 0.79 and 0.56 in solvent systems A and B respectively. On hydrolysis of the β-elimination products, only [^{3}H]galactosaminitol was detectable with virtually no [^{3}H]galactosamine indicating that only one galactosamine per prosthetic group was present and that this was involved in an alkali-labile O-glycosidic linkage apparently to serine and/or threonine.

The class I glycopeptide is rich in sialic acid and galactosamine, free of glucosamine and of higher molecular weight than the other glycopeptides produced by melanoma cells. It is distinct from the peak A glycopeptides described by Warren and co-workers (1,12) since these glycopeptides have been shown to have the serum-type N-glycosidic linkage (13). Even though an

accurate estimation of the size of the prosthetic group has not been made, its mobility on BioGel columns and paper chromatograms indicate a mixture of tetra- and trisaccharides. The enzymatic digestion data are consistent with the sequence Gal$\xrightarrow{\beta}$ GalNAc $\xrightarrow{\alpha}$ Ser/Thr. To this core, one or two sialic acid residues are probably attached, forming tri- or tetrasaccharide units. In the case of the later, the two sialic acid residues are most likely attached by two types of linkages (e.g. 2→3 and 2→6). The glycopeptide appears to be of molecular weight 10-15,000 on the basis of its behavior on controlled pore glass and Sephadex G-50 columns in comparison with standard anionic saccharides. Thus it is conceivable that several oligosaccharides are present in a cluster attached to the peptide core resulting in a molecule resistant to further proteolytic digestion.

When the mouse melanoma ^{3}H-glycopeptides isolated from the media were co-chromatographed together with the [^{14}C]-glucosamine labeled components isolated from the surface of intact mouse melanoma cells, strikingly similar profiles were obtained both on molecular sieve (CPG; Sephadex G-50) as well as ion exchange (DEAE cellulose) columns (not illustrated). This suggests that the Class I and II glycopeptides are located at least partly at the cell surface. These components may be anchored in the membrane or they may reside only temporarily in the glycocalyx (cell coat) in the process of being shed into the medium.

The production of this glycoprotein by melanoma cells but not by control melanocytes (5) is of extreme interest. Mucin-type glycoproteins have been isolated from the membranes of mammalian erythrocytes (14,15) and of cancerous cells grown in ascites form (16-18), whereas solid tissue controls (19) did not make comparable glycopeptides. The production of this class of glycoproteins by substratum grown mouse melanoma as well as human melanoma cells (20; V. P. Bhavanandan, J. Banks and E. A. Davidson, unpublished results) seems to indicate that this may be a more general characteristic of cancer cells.

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