PARTIAL CHARACTERIZATION OF RAT HEPATOMA CELL-SURFACE GLYCOPEPTIDES POSSESSING CONCANAVALIN A RECEPTOR ACTIVITY

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SUMMARY: Papain digestion of Novikoff or AS-30D rat hepatoma cells released glycopeptides from the cell surface. That portion of the glycopeptides accessible to Sephadex G-50 was digested with pronase and the component glycopeptides partially resolved by ion-exchange chromatography. Each tumor type yielded two well resolved sialoglycopeptide fractions which possessed concanavalin A receptor activity. The amino acid and saccharide composition of these low molecular weight (3,100 ± 300 daltons) sialoglycopeptides was determined.

INTRODUCTION: Malignant transformation is accompanied by alterations in the structure, topology and dynamics of plasma membrane glycoproteins (1). Studies using normal and malignant rat liver cells have revealed the existence of qualitative differences in low molecular weight sialoglycopeptides released from the cell surface by proteases (2,3). Low molecular weight sialoglycopeptides partially accessible to Sephadex G-50 are released from the surface of Novikoff or AS-30D rat hepatoma cells by incubation of the intact cells with papain. These glycopeptides possessed Con A (concanavalin A) receptor activity (4,5), whereas similar sialoglycopeptides from rat hepatocytes possessed no detectable Con A receptor activity (2,3). The resolution of the low molecular weight, cell-surface sialoglycopeptides from Novikoff cells, and the partial chemical characterization of the two major low molecular weight sialoglycopeptide fractions derived from Novikoff or AS-30D hepatoma cells are reported herein

MATERIALS AND METHODS:

Rat hepatomas: Two transplantable ascites hepatomas were utilized: Novikoff (6) and AS-30D (7,8). The procedures for tumor transplantation and harvesting of cells has been described (5,7).

Isolation and resolution of glycopeptides: Cell-surface sialoglycopeptides partially accessible to Sephadex G-50 (C-SGP-C) were prepared from Novikoff or

AS-30D cells as described (4,5). AS-30D C-SGP-C was submitted to pronase digestion and subsequent ion-exchange chromatography on DEAE-cellulose, yielding two sialoglycopeptide fractions designated DC2 and DC3 (5). Gel filtration was performed using Sephadex G-25 or G-50 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). DEAE-cellulose (Whatman Microgranular DE 32, 1.0 mequiv/g dry exchanger) was obtained from H. Reeve Angel and Co., Ltd., London. Column effluents were assayed for sialic acid and neutral sugar as described (4). Saccharide concentrations in the column effluents were expressed as μmole/fraction.

Characterization of the glycopeptides: The molecular weights of the purified sialoglycopeptide fractions were determined by gel filtration on Sephadex G-50, as described by Bhatti and Clamp (9). The column (0.4 x 95 cm) was calibrated with maltopentaose (Pierce Chemical Co., Rockford, Ill.) and ovalbumin glycopeptide, prepared as described by Montgomery et al (10). The molecular weight of ovalbumin glycopeptide (2200 ± 100) was determined from the aspartic acid content, assuming one mole per mole of glycopeptide. The amino acid, hexosamine, total neutral sugar and sialic acid content of the glycopeptides was quantitated as described previously (4). Neutral sugars were resolved and quantitated according to Method B[1] described by Walborg et al (11),[1] using hydrolysis with a cation exchange resin as described (4). Lectin receptor activity was determined using the assay of Smith et al (5). Con A, a twice crystallized product dissolved in saturated NaCl (Lot No. 61) was obtained from Miles Laboratories, Kankakee, Ill. This preparation possessed 48,000 HAU per mg, assuming an $E^{1\%}_{280\ nm}$ of 13. WGA (wheat germ agglutinin) was prepared according to the procedure of Nagata and Burger (12). The active fraction from DEAE-cellulose, possessing 15,000 HAU per mg, was utilized.

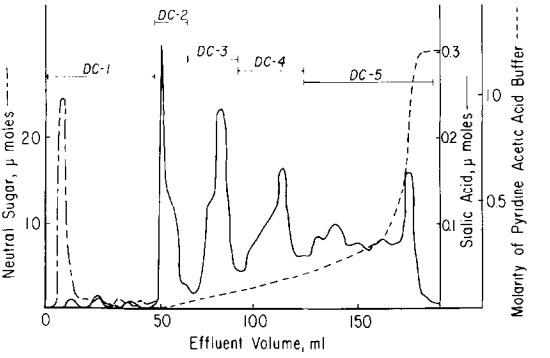

Figure 1: Ion-exchange chromatography of Novikoff P-SGP-C. The sample (25 mg) was dissolved in 2.0 ml of 2 mM pyridine-acetic acid buffer, pH 5.3, and applied to a column (1 x 10 cm) of DEAE-cellulose. Elution was accomplished by sequential addition of the following buffers: a) 20 ml of 2 mM pyridine-acetic acid buffer, pH 5.3; b) a concave gradient of 116 ml of 2 mM pyridine-acetic acid buffer, pH 5.3 vs 26 ml of 1.2 M pyridine-acetic acid buffer, pH 5.3; c) 30 ml of 1.2 M pyridine-acetic acid buffer, pH 5.3. The concentration of each buffer was expressed as the final concentration of pyridine. Neutral sugar analysis was performed only on the first 48 ml of column effluent in order to conserve glycopeptide material. The salt concentration of the gradient was determined conductimetrically.

[1] The buffer flow rate was 20 ml/hr, not 60 ml/hr, as incorrectly reported in reference 11.

RESULTS:

Resolution of low molecular weight sialoglycopeptide fractions from the surface of Novikoff cells: The pronase digest of Novikoff C-SGP-C was submitted to gel filtration on Sephadex G-25 (5). The sialoglycopeptide fraction excluded from Sephadex G-25 (P-SGP-C) was submitted to chromatography on DEAE-cellulose as described previously for AS-30D P-SGP-C (5). See Figure 1. The fractions resolved by ion-exchange chromatography were pooled and lyophilized repeatedly to remove salt. The relative weight recoveries of the glycopeptide fractions are shown in Table 1.

Lectin receptor activity of cell-surface glycopeptide fractions: The Con A

Table 1: Con A Receptor Activity of Cell-Surface Glycopeptide Fractions

Fraction	Yield mg per 100 mg of C-SGP[a]	Specific Receptor Activity[b] HAIU/mg	Total Receptor Activity HAIU/Fraction
Novikoff			
C-SGP-C	28	710	20,000
P-SGP-C	18	800	15,000
DC1	3.8	800	3,000
DC2	2.2	2,700	5,900
DC3	2.7	980	2,600
DC4	2.5	490	1,200
DC5	7.3	220	1,600
AS-30D[c]			
DC2	5	1,100	5,500
DC3	8	490	3,900

[a]Nondialyzable glycopeptides cleaved from the surface of hepatoma cells by papain (4,5).

[b]Lectin receptor activities were measured by hemagglutination inhibition assay (5). One hemagglutination inhibitory unit (HAIU) is defined as the minimum amount of inhibitor required to inhibit completely 3 hemagglutination units of lectin. Any differences from previously reported data reflect variations in the specific activities of different batches of glycopeptides and selection of more recent assays in which more inhibitors have been directly compared in a single assay, using freshly prepared inhibitors and the same lectin solutions. Ovalbumin glycopeptide, prepared as described by Montgomery et al (10) possessed 2,800 Con A-HAIU/mg.

[c]See reference 5.

receptor activities of the low molecular weight glycopeptide fractions from the surface of Novikoff and AS-30D cells are shown in Table 1. The major portion (75%) of the Con A receptor activity of Novikoff C-SGP-C was recovered in P-SGP-C. All of the Con A receptor activity of Novikoff P-SGP-C was recovered in the components resolved on DEAE-cellulose, with sialoglycopeptide fractions DC2 and DC3 accounting for 56% of the Con A receptor activity of P-SGP-C. The glycopeptide fractions shown in Table 1 exhibited no detectable WGA receptor activity, i.e. <70 WGA-HAIU/mg. In the same assay ovomucoid (Worthington Biochemical Co., Freehold, N.J.) possessed 890 WGA-HAIU/mg.

Purity of glycopeptide fractions DC2 and DC3: DC2 and DC3 from Novikoff or AS-30D cells were submitted to rechromatography on DEAE-cellulose. Each fraction eluted as a single symmetrical peak within a restricted portion of the gradient: Novikoff or AS-30D DC2 at 0.01 to 0.05 M pyridine acetate and Novikoff or AS-30D DC3 at 0.05 to 0.10 M pyridine acetate. The glycopeptides eluted as single symmetrical peaks upon gel filtration on a calibrated column of Sephadex G-50, indicating the following molecular weights: Novikoff DC2, 2880 ± 250, Novikoff DC3, 3200 ± 250; AS-30D DC2, 3120 ± 250 and AS-30D DC3, 3390 ± 250 daltons.

Compositional analysis of DC2 and DC3: The amino acid and saccharide composition of these glycopeptides are shown in Table 2. In each case aspartic acid, threonine and serine accounted for 52 - 58% of the weight of the peptide moiety with only aspartic acid present in molar quantities. The following molecular weights were estimated from the aspartic acid contents, assuming one mole of aspartic acid per mole of glycopeptide: Novikoff DC2, 2840 ± 20; Novikoff DC3, 3080 ± 50; AS-30D DC2, 2850 ± 30; and AS-30D DC3, 3050 ± 190 daltons. The total neutral sugar contents of Novikoff DC2, Novikoff DC3, AS-30D DC2 and AS-30D DC3 were 2.37, 2.08, 2.59, and 2.28 μmole neutral sugar/mg glycopeptide, respectively. This indicates that the conditions of hydrolysis for the liberation of neutral sugars were 70 - 90% effective.

Table 2: Peptide and Saccharide Composition of Cell Surface Glycopeptide Fractions from Novikoff and AS-30D Hepatoma Cells

	Amino acid and saccharide composition mole/mole glycopeptide[a]			
	Novikoff		AS-30D	
Component	DC2	DC3	DC2	DC3
Peptide				
Aspartic acid[b]	1.00	1.03	1.04	0.98
Threonine	0.45	0.47	0.42	0.66
Serine	0.52	0.50	0.42	0.45
Glutamic acid	0.22	0.38	0.24	0.36
Proline	0.40	0.31	0.29	0.26
Glycine	0.23	0.25	0.22	0.27
Alanine	0.22	0.21	0.24	0.26
Other amino acids	0.35	0.35	0.40	0.63
Carbohydrate				
Sialic acid	0.98	1.23	0.97	0.94
Glucosamine	3.20	3.57	4.49	3.74
Galactosamine	0.20	0.27	0.31	0.43
Mannose	3.58	2.92	2.31	1.85
Fucose	0.74	0.60	0.88	0.55
Galactose	2.03	2.54	2.38	2.43
Glucose	0.43	0.47	N.D.[c]	N.D.[c]

[a]Calculated using average of the molecular weights determined by gel filtration and aspartic acid content.

[b]The aspartic acid content of Novikoff DC2, Novikoff DC3, AS-30 DC2 and AS-30D DC3 was 0.35, 0.33, 0.35, and 0.33 μmole per mg glycopeptide, respectively. Amino acids and saccharides accounted for 84.6, 82.0, 83.3 and 76.6% of the weight of Novikoff DC2, Novikoff DC3, AS-30D DC2 and AS-30D DC3, respectively.

[c]Not detected.

DISCUSSION: The low molecular weight glycopeptides released from the surface of Novikoff or AS-30D cells were submitted to digestion with pronase to minimize variations in their peptide chain length and partially resolved into their components by ion-exchange chromatography (Fig. 1). DC1, a fraction which was not bound to the ion-exchanger at low ionic strength, was rich in neutral sugar, but essentially devoid of sialic acid. Since neutral sugar-containing components devoid of sialic acid are liberated from hepatoma cells incubated in the absence of papain (7), this may not represent material of cell-surface

origin. The sialoglycopeptides were resolved into four fractions: DC2, DC3, DC4, and DC5. The two sialoglycopeptide fractions eluted at low ionic strength were well resolved and therefore were subjected to further examination.

DC2 and DC3 from Novikoff or AS-30D cells behaved as homogeneous components upon rechromatography on DEAE-cellulose or upon gel filtration on Sephadex G-50, indicating relatively high purity with regard to ionic charge and molecular size. The amino acid and saccharide composition of the glycopeptides (Table 2), however, provided conclusive evidence that the sialoglycopeptide fractions were not homogeneous. Submolar quantities of threonine and serine indicate considerable heterogeneity in the peptide moiety. This is not unexpected since it is extremely difficult to degrade the peptide moiety of sialoglycopeptides to asparaginyl oligosaccharide using proteases (13). Aspartic acid was the only amino acid present in molar quantities; strong presumptive evidence for the existence of an N-glycosylamine type linkage between asparagine and glucosamine residues. The saccharide analysis provides evidence for microheterogeneity within the oligosaccharide moieties (14), since nonunitary molar quantities of hexosamines and neutral sugars were present. Submolar quantities of galactosamine were also present. The submolar quantities of glucose present in Novikoff DC2 and DC3 may represent contamination of the sample by glucose-containing components present in the DEAE-cellulose. Each of the four sialoglycopeptide fractions contained one mole of sialic acid per mole of glycopeptide, consequently the resolution of the glycopeptides on DEAE-cellulose cannot be explained simply on the basis of charge differences due to sialic acid content. A similar situation was found for sialoglycopeptides obtained by pronase digestion of human ceruloplasmin (15).

The lectin specificity of DC2 and DC3 from Novikoff and AS-30D cells makes these sialoglycopeptide fractions candidates for studies to elucidate the minimum structural requirements for Con A binding. The glycopeptides possessed Con A receptor activity, but no detectable WGA receptor activity. Novikoff C-SGP-C possessed no detectable Ricinus communis agglutinin I or II or soybean agglutinin

receptor activity (16). The compositional data reported herein provides an analytical foundation for future structural investigations.

Malignant transformation is accompanied by alterations of low molecular weight, fucose-containing, cell-surface sialoglycopeptides (17,18). It has been proposed that this alteration results from the increased sialylation of glycopeptides derived from transformed cells (19). Warren et al (19) have demonstrated the presence of a tumor-associated sialyltransferase which transfers sialic acid to desialylated glycopeptides derived from transformed cells. The fucose-containing sialoglycopeptides which serve as glycosyl acceptors for the tumor-associated sialyltransferase are comparable to the C-SGP-C fraction described herein; a conclusion based on similar methods of isolation, their compositional analysis (20) and their behavior on Sephadex G-50 gels. Furthermore Warren et al (20) demonstrated that desialylated Novikoff C-SGP-C, DC2 and DC3 possessed good acceptor activity for the tumor-associated sialyltransferase. Further structural investigations utilizing sialoglycopeptide components of C-SGP-C may aid in elucidating the structural basis for the acceptor specificity of this tumor-associated sialyltransferase.

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