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Comparison of Glycopeptides from Control and Virus-Transformed Baby Hamster Kidney Fibroblasts†

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ABSTRACT: Glucosamine-labeled glycopeptides from control and virus-transformed BHK fibroblasts were characterized by size, lectin affinity, charge, and composition. As already demonstrated, on the basis of elution position on a column of Sephadex G-50, transformed cells contained a greater proportion of large glycopeptides than did control cells. Transformed cells also contained a larger proportion of glycopeptides which do not bind to Con A–Sepharose. By sequential chromatography on Sephadex G-50, Con A–Sepharose, and DEAE-Sephadex, ~40 individual peaks were partially or completely resolved. If sialic acid was removed from the glycopeptides prior to analysis by ion-exchange chromatography, 95% of the glycopeptides from control cells and 85% of the glycopeptides from transformed cells were no longer bound by DEAE-Sephadex. It was concluded that the DEAE-Sephadex elution properties of the glycopeptides are determined almost entirely by the sialic acid content of the molecules. A comparison of the profiles of control and transformed cell glycopeptides simultaneously eluting from columns of DEAE-Sephadex revealed that the differences between the two cells were largely quantitative; however, the possibility of the existence of qualitative differences as well cannot be excluded. In particular, there was one component present on the surface of transformed cells that was virtually absent in control cells. It was degraded by nitrous acid hydrolysis and heparinase and appeared to be heparan sulfate like material. After fractionation, each isolated glycopeptide population was analyzed for carbohydrate and, in some cases, amino acid content. The apparently larger glycopeptides, group A, the dominant population in transformed cells, were found to contain 3 to 4 mannose residues/glycopeptide when the sugars were normalized to sialic acid content. On the basis of the same criteria, group B glycopeptides contained 4–6 mannose residues/glycopeptide. The carbohydrate and amino acid compositions of the glycopeptides from transformed cells were, with a few exceptions, similar to those from control cells. Some isolated glycopeptides appeared to contain both O-glycosidic and N-glycosidic linkages on the same oligopeptide.

The role of bound carbohydrate in growth control and malignancy has been a subject of great interest in recent years. It has been found that malignant cells in culture are enriched in a population of glycopeptides (group A) (Buck et al., 1970, 1971a,b; Van Beek et al., 1975; Smets et al., 1976). This enrichment has been observed by analysis of limit digest products of Pronase-treated glycoproteins on columns of Sephadex G-50. The degree of increase in the high molecular weight population has been correlated with tumorigenicity (Glick et al., 1973; Smets et al., 1976) and with an active growth state (Buck et al., 1971a,b; Muramatsu et al., 1973). The larger glycopeptides, group A, have an approximate apparent molecular weight range of 4200–5500. The bulk of the glycopeptides from control cells elutes from Sephadex G-50 in regions called groups B and C, which correspond to approximate apparent molecular weight ranges of 3000–4200 and 1500–3000, respectively (Warren et al., 1974).

An increase in group A glycopeptides following transformation has been observed in several species and appears to be independent of the transforming agent (e.g., DNA- or RNA-containing virus, chemical agents, or spontaneous transformation) (Buck et al., 1971a,b; Smets et al., 1976). The increase in the population of group A glycopeptides has been demonstrated in material released from the cell surface by trypsin, as well as in cell pellets and in membranes from each major organelle isolated by cell fractionation (Buck et al., 1974; Keshgegian & Glick, 1973).

A recent study (Glick, 1979) has shown that the cell surface glycopeptides can be separated into seven or more groups by chromatography on DEAE-cellulose.[1] Comparison of the glycopeptides from control and transformed cells by this procedure indicated that the differences between the two cell types were quantitative with the transformed cell type containing an increase in larger glycopeptides. A partial structure for such glycopeptides was proposed (Santer & Glick, 1979; Ogata et al., 1976). In the work presented here, we extended these findings on a more detailed level. We cofractionated cell surface glycopeptides from control and virus-transformed

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[1] Abbreviations used: Con A, concanavalin A; Con A(−), glycopeptides which do not bind to a column of Con A–Sepharose; Con A(+), glycopeptides which bind to a column of Con A–Sepharose; C13, BHK_{21}/C_{13}; B4, C_{13}/B_4; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetate; L-fucose, 6-deoxy-L-galactose; sialic acid, *N*-acetylneuraminic acid; Man and mannose, D-mannose; galactose, D-galactose; glucose, D-glucose; GlcNAc and *N*-acetylglucosamine, 2-acetamido-2-deoxy-D-glucose; *N*-acetylgalactosamine, 2-acetamido-2-deoxy-D-galactose; Asn, asparagine.

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cells on the basis of gel filtration, lectin affinity, and charge properties and have found that the glycopeptides isolated from the cell surface are remarkably heterogeneous, yielding at least 40 fully or partially resolved groups of glycopeptides. Comparative compositional data on the major constituents from both cell types support the premise that most of the differences observed between the glycopeptides from control and transformed cells are quantitative in nature and involve several glycopeptides.

Experimental Procedure

Materials. Radioisotopes purchased from New England Nuclear (Boston, MA) were D-[^{14}C(U)]glucosamine hydrochloride (250 mCi/mmol) and D-[6-^{3}H(N)]glucosamine hydrochloride (20 Ci/mmol). Pronase B grade and *Vibrio cholerae* neuraminidase B grade were purchased from Calbiochem (La Jolla, CA). Hyaluronidase 100 USP was purchased from Worthington Biochemical Co. (Freehold, NJ). Sephadex G-50 (fine), Sephadex G-10 (fine), Sephadex G-25 (coarse), DEAE-Sephadex A-25, and Con A–Sepharose were purchased from Pharmacia, Inc. (Piscataway, NJ). All reagents used in gas–liquid chromatography were Baker Instra Analyzed and were redistilled before use.

Preparation of Glycopeptides. Baby hamster kidney fibroblasts (BHK_{21}/C_{13}) and a cell line derived from transformation of the same clone with the Bryan high-titer strain of Rous sarcoma virus (C_{13}/B_4) were cultured in plastic roller bottles under identical conditions as described previously (Buck et al., 1970, 1974). The medium was replaced after 24 h with fresh medium containing 20 μCi of D-[^{3}H]- or D-[^{14}C]-glucosamine. One cell line received one isotope and the other received the other isotope of the same carbohydrate precursor. Fresh medium was added to the radioactive medium after 24 h (day 3). The cells, which have essentially the same doubling time, were harvested on day 4 while still in log phase of growth. The medium was decanted, and the cells were washed and detached from the plastic by incubation with trypsin as previously described (Buck et al., 1970, 1974). Viability of the cells after this step was always greater than 90% by the trypan blue exclusion test. The suspension was removed from the bottles and centrifuged. The supernatant containing the trypsin-released material was lyophilized. The trypsinates were resuspended in a minimal volume of buffer (0.1 M Tris and 0.0015 M $CaCl_2$, pH 7.8) with predigested Pronase (1 mg/mL) and 1% v/v toluene. The samples were then incubated at 37 °C. Fresh Pronase (1 mg/mL) and toluene were added daily for 5 days. The Pronase-treated material was extensively dialyzed (dialyzer tubing, molecular weight cutoff 3500; Arthur H. Thomas Co., Philadelphia, PA) against distilled water. Although the dialysis tubing should be permeable to molecules with a molecular weight of less than 3500, group C (with a molecular weight of ~1500–3000) was not lost during dialysis. The dialyzed material was lyophilized, and the samples were redissolved in a small volume of distilled water. At this point, the material from the two cell lines (control and transformed) had been treated identically but separately. For analytical purposes, portions from each were now mixed together and all further steps were carried out with the mixture.

Chromatography on Sephadex G-50. The material was diluted with elution buffer (0.01 M ammonium acetate in 20% ethanol) to a total volume of 5 mL, and 0.05 mL of a 10% solution of phenol red was added. The material was chromatographed on a column of Sephadex G-50 (118 × 3 cm) with an included volume of 440 mL (Buck et al., 1970). Fractions of 2.2 mL were collected, and an aliquot was assayed for radioactivity. All samples were counted in 4 mL of Formula 963A scintillation fluid (New England Nuclear, Boston, MA). Selected fractions were pooled and lyophilized.

Analysis of Glycopeptides by Con A–Sepharose. A column of Con A–Sepharose (10 × 1 cm) was prepared by preadsorption of nonspecific binding sites with bovine serum albumin (5 mg/mL) in 10 mL of elution buffer (0.03 M Tris–acetate, 0.001 M $CaCl_2$, 0.001 M $MgCl_2$, 0.001 M $MnCl_2$, 0.03% NaN_3, and 0.8% NaCl, titrated with acetic acid to pH 7.0). The column was eluted with 50 mL of Con A elution buffer and 40 mg/mL α-methyl D-mannoside followed by 200 mL of Con A elution buffer. Three-milliliter fractions were collected. Bound and unbound fractions were each pooled, dialyzed against three changes (2 L each) of distilled water, and then lyophilized. When these fractions were again placed on columns of Con A, they passed through or were bound as expected.

Ion-Exchange Column Chromatography. A DEAE-Sephadex A-25 column (10 × 1 cm) overlaid with Sephadex G-25 (coarse) (1 × 1 cm) was prepared by washing the column with 250 mL of 0.01 M pyridine acetate, pH 4.5.

The sample was loaded onto the prewashed column and eluted with 10 mL of 0.01 M pyridine acetate (buffer I), pH 4.5, followed by a linear gradient of 0–0.1 M sodium borate prepared from 50 mL of buffer I and 50 mL of buffer II (0.1 M sodium borate in 0.01 M pyridine, buffered with glacial acetic acid to pH 4.5). The flow rate was adjusted to 0.5 mL/min, and fractions of 0.5 mL were collected. A second gradient was then employed. The column was washed with 10 mL of buffer II, followed by a linear gradient of 0–1.0 M sodium acetate prepared from 25 mL of buffer II and 25 mL of buffer III (1.0 M sodium acetate and 0.1 M sodium borate in 0.01 M pyridine, buffered with glacial acetic acid to pH 4.5). The second gradient was followed by a wash with 5 mL of buffer III. A final wash with 10 mL of buffer IV (1.5 M sodium acetate in 0.01 M pyridine acetate, pH 4.5) was used to elute the remaining material. At the start of the second gradient, the flow rate was adjusted to 0.5 mL/min and a fraction volume of 0.5 mL was collected. Ionic strength was determined by refractive index measured on a Bausch and Lomb refractometer. Fractions corresponding to resolved peaks on the chromatograms were pooled and lyophilized. Recovery of material from the column was 95 ± 5% of the applied radioactivity.

Analysis of Glycopeptides by Gas–Liquid Chromatography. The above isolation and fractionation procedures were repeated separately for BHK_{21}/C_{13} and C_{13}/B_4 cells in order to prepare glycopeptides for compositional analysis.

Following DEAE-Sephadex chromatography, the purified glycopeptides were desalted by sieving through a column of Bio-Gel P2 (15 × 2.5 cm) (Bio-Rad Laboratories, Richmond, CA) overlaid with Sephadex G-10 (fine) (5 × 2.5 cm) for removal of borate ions and Sephadex G-25 (coarse) (1 × 2.5 cm). The column was eluted with distilled water, and fractions of 1 mL were collected and assayed for radioactivity and refractive index. The desalted samples were lyophilized and redissolved in a small volume, and the glycopeptides were converted to alditol acetate derivatives by a modification of the procedure of Lenhardt & Winzler (1968). Samples (~100–200 nmol of total sugar) were hydrolyzed for 40 h at 100 °C in 0.04 M HCl in the presence of 20% v/v Dowex 50 X-4. A hydrolysis curve for each monosaccharide was performed under the same conditions for determination of the percentage of recovery of each sugar after incubation (duration of incubation varied from 0 to 48 h). After hydrolysis, 10 μg of

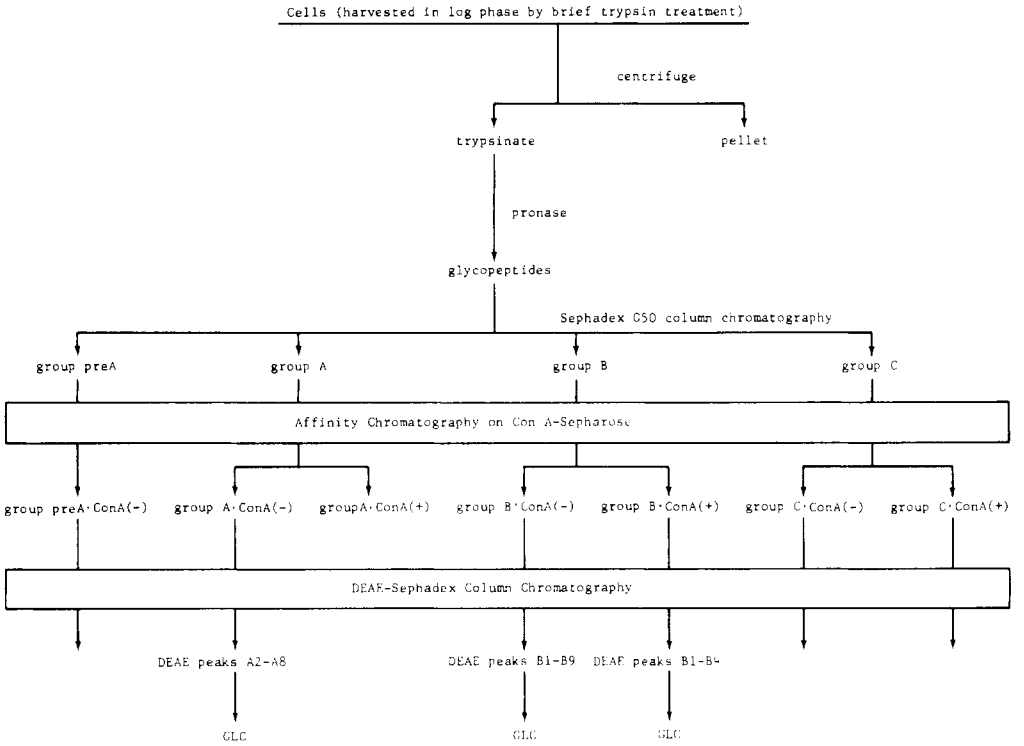

FIGURE 1: Procedures for preparation and analysis of glycopeptides.

2-deoxyglucose was added to each tube as an internal standard. The samples were reduced with 1 mg of sodium borohydride for 45 min at room temperature. Excess sodium borohydride was destroyed by the addition of glacial acetic acid. The borate was removed as methyl borate by the addition of methanol–HCl (1000:1 v/v) and lyophilization. The reduced sugars were acetylated with acetic anhydride in pyridine (Lenhardt & Winzler, 1968). Samples were analyzed on a Hewlett-Packard 5830 gas chromatography instrument. Glass columns (6 ft × 2 mm i.d.) were packed with 3% ECNSS-M on Chromosorb Q (100–120 mesh) (Applied Science Laboratories, Inc., State College, PA). Resolution of the monosaccharde derivatives was obtained by temperature programming from 175 to 210 °C for neutral sugars and by isothermal programming at 230 °C for amino sugars. Response factors for the individual monosaccharides were calculated from solutions of standard alditol acetates (obtained in derivatized form from Supelco, Inc., Bellefonte, PA) compared to a known concentration of 2-deoxyglucose pentaacetate and from the recovery percentages of monosaccharides obtained from the hydrolysis curve after incubation for 40 and 0 h and then converted to alditol acetates.

Removal of Sialic Acid from Glycopeptides. (*1*) *Neuraminidase Treatment.* Glycopeptides were incubated for 3 h at 37 °C in a solution of sodium acetate (0.1 M) and $CaCl_2$ (0.003 M), pH 5.2, in the presence of *V. cholerae* neuraminidase (15 units) in a reaction volume of 0.2 mL. The reaction was stopped by boiling the reaction mixture for 4 min.

(*2*) *Acid Hydrolysis.* Glycopeptides were incubated for 1 h at 80 °C in 0.2 N H_2SO_4 in a reaction volume of 0.2 mL. Samples were neutralized after the reaction period, and sialic acid was determined by the thiobarbituric acid assay (Warren, 1959).

(*3*) *Hyaluronidase Treatment.* Glycopeptides were incubated with 100 μg of hyaluronidase in NaH_2PO_4 (0.1 M) and NaCl (0.15 M), pH 5.3, at 37 °C for 18 h in a reaction volume of 0.2 mL. The reaction was stopped by boiling the reaction mixture for 5 min.

(*4*) *Treatment with Nitrous Acid.* Samples were treated with nitrous acid as described by Hart (1976). The reaction mixtures were neutralized after 2 h with NaOH, and the hydrolysis products were assayed by Sephadex G-50 column chromatography.

(*5*) *Treatment with Heparinase.* Heparinase was a generous gift from Dr. Eugene A. Davidson. Samples were incubated at 30 °C for 24 h with 250 μg of heparinase in 0.1 M sodium acetate and 1 mM $CaCl_2$, pH 7.0, in a reaction volume of 0.2 mL. The reaction mixture was assayed by Sephadex G-50 column chromatography.

(*6*) *Alkaline Treatment.* For determination of the presence of O-glycosidically linked material, several glycopeptides were incubated under reducing alkaline conditions, as described by Arima et al. (1972). After 48 h, the reaction mixtures were neutralized and assayed by Sephadex G-50 column chromatography.

Results

BHK_{21}/C_{13} and C_{13}/B_4 cells were cultured for 48 h in the presence of D-[^{14}C]- or D-[^{3}H]glucosamine, respectively. The cells were briefly treated with trypsin for removal of the surface glycopeptides and the released material (~34% of the total nondialyzable radioactivity). The preparation and analysis of the glycopeptides are summarized in a flow diagram (Figure 1).

Chromatography of Glycopeptides on Sephadex G-50. The combined Pronase-digested glycopeptides containing equivalent amounts of ^{14}C and ^{3}H were fractionated on the basis of size by chromatography on a column of Sephadex G-50 and the eluting material was divided into four size ranges, designated groups pre-A, A, B, and C (Figure 2a). The glycopeptides from the transformed cells (C_{13}/B_4) were significantly enriched in the high molecular weight fractions (groups pre-A and A) relative to those from the control cells (BHK_{21}/C_{13}). The fractions in each size range were pooled and rechromatographed separately on Sephadex G-50 in order to minimize overlap between the size ranges (Figure 2b). The material

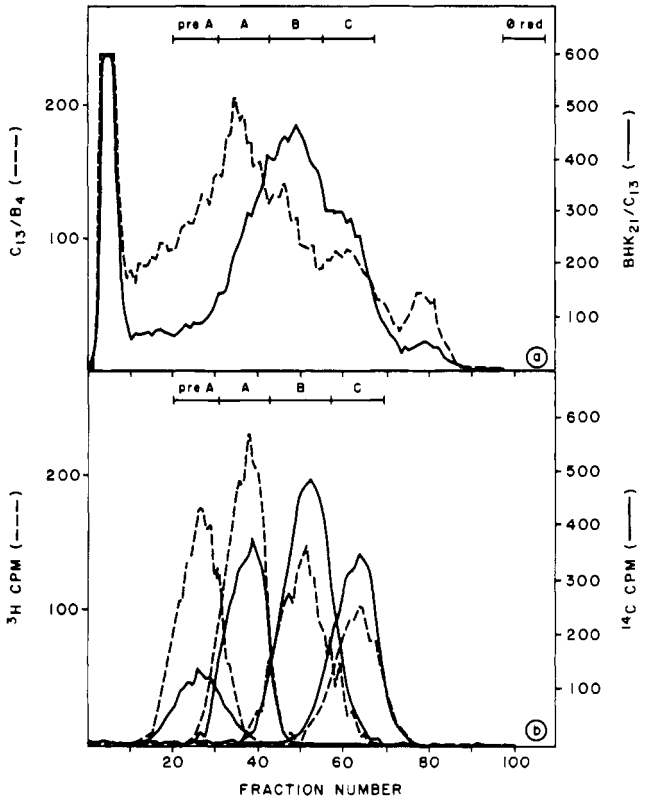

FIGURE 2: Sephadex G-50 chromatography of glucosamine-labeled glycopeptides from control (BHK_{21}/C_{13}) and transformed (C_{13}/B_4) hamster cells. (a) Cells were grown in the presence of either D-[^{14}C]- or D-[^{3}H]glucosamine. The Pronase-digested glycopeptides from each cell line were combined and subjected to chromatography on a column of Sephadex G-50 as described under Experimental Procedure. (—) Control cells labeled with [^{14}C]glucosamine; (---) transformed cells labeled with [^{3}H]glucosamine; ϕR = phenol red. (b) The fractions indicated in (a) were pooled, and each group was subjected to rechromatography on the same column of Sephadex G-50. The four elution patterns, i.e., groups pre-A, A, B, and C, were superimposed on one graph on the basis of their elution positions relative to added phenol red. The bars above the rechromatographed fractions indicate the regions pooled for further analysis.

from the pooled regions, groups pre-A, A, B, and C, was eluted in a restricted volume, and the positions of elution were found to correspond to the locations of the originally pooled areas. The bar above each of the peaks (Figure 2b) indicates the fractions which were pooled for further analysis.

Affinity Chromatography of Glycopeptides on Con A–Sepharose. Each population of glycopeptides obtained from Sephadex G-50 fractionation was analyzed for its ability to bind to Con A on a column of Con A–Sepharose (see Experimental Procedure for details). The results are seen in Table I. The larger glycopeptides, groups pre-A and A, showed little or no ability to bind to Con A–Sepharose and therefore passed through the column. These glycopeptides were referred to as Con A(−). The populations of smaller glycopeptides, groups B and C, appeared to be a mixture of glycopeptides with or without the ability to bind to Con A. Those glycopeptides which bound to Con A, referred to as Con A(+), were eluted from the column with α-methyl D-mannoside. Within each size range, particularly groups B and C, a smaller percentage of the glycopeptides of the transformed cells (C_{13}/B_4) bound to Con A (Table I).

Analysis of Glycopeptides by Ion-Exchange Column Chromatography. Fractionation yielded six populations of glycopeptides: group pre-A·Con A(−), group A·Con A(−), group B·Con A(−), group C·Con A(−), group B·Con A(+), and group C·Con A(+). Each of these groups was further

Table I: Distribution of Con A Binding and Nonbinding Glucosamine-Labeled Glycopeptides in Control and Transformed Hamster Cells[a]

	distribution of binding (%)									
	total[b,c]		group pre-A		group A		group B		group C	
	C13	B4	C13	B4	C13	B4	C13	B4	C13	B4
Con A(−)[d]	61	86	93	98	91	97	50	68	45	60
Con A(+)[e]	39	14	7	2	9	3	50	32	55	40

[a] The percentages were calculated on the basis of 100% recovery of the radioactivity. [b] Combined glycopeptides from all four groups (pre-A + A + B + C). [c] C13 = BHK_{21}/C_{13}; B4 = C_{13}/B_4. [d] Con A(−) indicates material which did not bind to Con A–Sepharose. [e] Con A(+) indicates material which bound to Con A–Sepharose and was eluted with α-methyl D-mannoside.

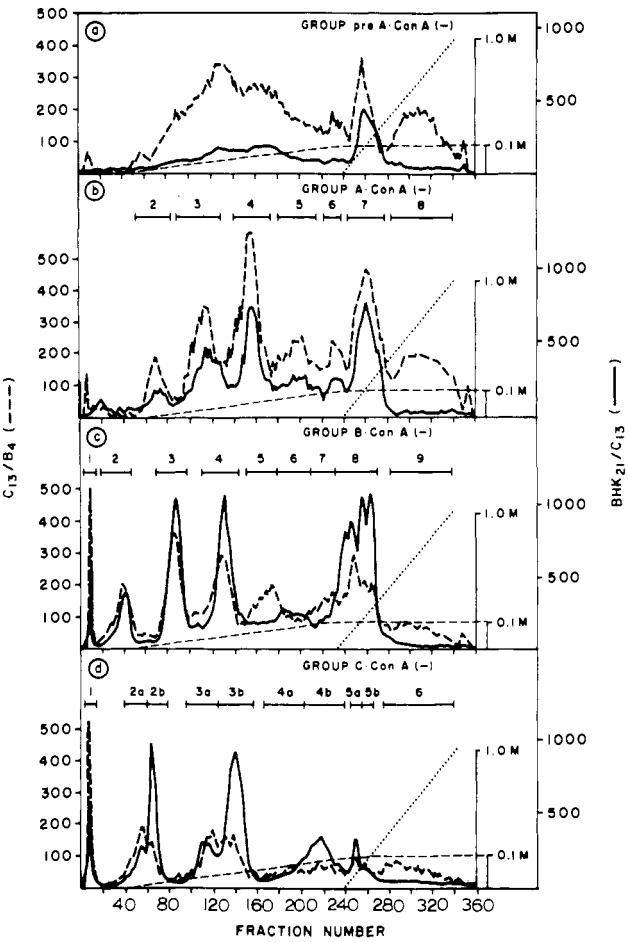

FIGURE 3: DEAE-Sephadex chromatography of glucosamine-labeled glycopeptides which do not bind to Con A. The glycopeptides were subdivided on the basis of Sephadex G-50 elution position (Figure 2) and lack of binding affinity for Con A into groups pre-A·Con A(−), A·Con A(−), B·Con A(−), and C·Con A(−). Each group was subjected to ion-exchange chromatography on DEAE-Sephadex A-25. The glycopeptides were eluted first with a linear salt gradient from 0 to 0.1 M sodium borate in 0.01 M pyridine acetate, pH 4.5, and then with a second linear salt gradient from 0 to 1.0 M sodium acetate in 0.1 M sodium borate and 0.01 M pyridine acetate, pH 4.5. This was followed by a final wash with 1.5 M sodium acetate. (—) Glycopeptides from ^{14}C-labeled control cells; (– – –) glycopeptides from ^{3}H-labeled transformed cells; (- - -) linear salt gradient based on the molarity of sodium borate; (···) linear salt gradient based on the molarity of sodium acetate.

subdivided on the basis of charge by ion-exchange chromatography on DEAE-Sephadex (Figures 3 and 4). It is apparent from the data obtained that the cell is capable of generating a great diversity of glycopeptides; ~40 different

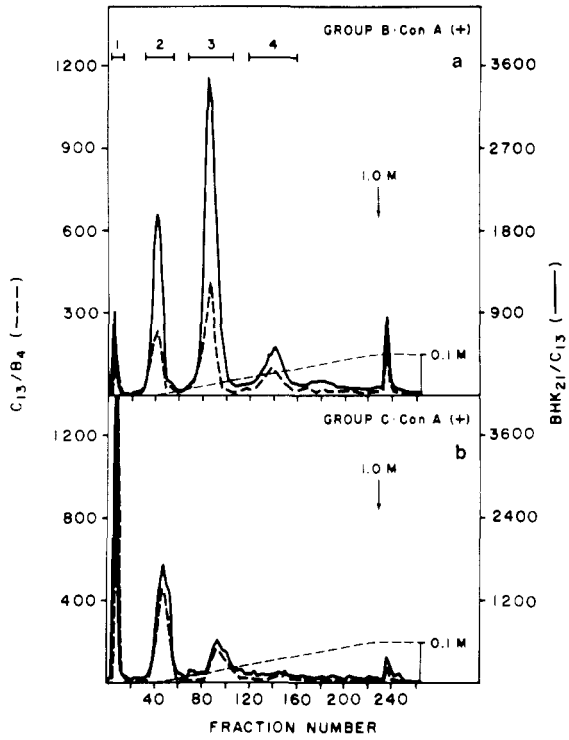

FIGURE 4: DEAE-Sephadex chromatography of glucosamine-labeled glycopeptides which bind to Con A. See the legend to Figure 3.

components were fully or partially resolved from the four size groups. The number of components and the ionic strength required for elution of each component within a group were entirely reproducible.

The DEAE-Sephadex elution profiles indicate substantial quantitative changes in nearly all of the components. Group pre-A·Con A(−) was poorly resolved on DEAE-Sephadex (Figure 3a). When a restricted region (i.e., tubes 105–115) was pooled, dialyzed, and rechromatographed on DEAE-Sephadex, the material was eluted as a single narrow peak at precisely the same ionic strength, indicating that the poor resolution observed with the glycopeptides in group pre-A is due to a high degree of charge heterogeneity. The lack of resolution of these glycopeptides may conceal qualitative differences.

Compared with group pre-A, the glycopeptides in group A·Con A(−) (Figure 3b) appeared to contain less charge heterogeneity. The transformed cell contained a greater amount of each of the components in group A·Con A(−); in particular, a striking quantitative difference was observed in the amount of material eluting at high ionic strength (tubes 280–340). This material (peak no. A8) was further examined and appears to be heparan sulfate (see below).

Group B·Con A(−) (Figure 3c) and group C·Con A(−) (Figure 3d) were enriched in glycopeptides which eluted at low ionic strength relative to the components in groups pre-A and A. Several of the components from group B·Con A(−) which show quantitative differences between the two cell types (peaks no. B7, B8, and B9) were examined further (see below).

Glycopeptides which can bind to Con A–Sepharose were found primarily in low molecular weight ranges, in agreement with the results of Ogata et al. (1976). All of the components of group B·Con A(+) were substantially decreased in the transformed cell type relative to the control cell type. Slight reductions were also consistently observed in the transformed cell components of group C·Con A(+). Resolution of the individual components in the Con A(+) category was remarkably complete and periodic, suggesting that the glycopeptides may be separated on the basis of 0, 1, 2, and 3 residues of sialic acid (or some other negatively charged species) per glycopeptide.

Removal of sialic acid had no effect on the ability of any of the glycopeptides to bind to Con A–Sepharose; this result is in agreement with the findings of Ogata et al. (1976). However, sialic acid appeared to be the dominant factor in determining the elution properties of the glycopeptides on DEAE-Sephadex. When sialic acid was removed from the glycopeptides either by neuraminidase treatment or by mild acid hydrolysis, 95% of the glycopeptides derived from BHK_{21}/C_{13} cells were no longer retained by DEAE-Sephadex. Similarly, 85% of the glycopeptides from transformed C_{13}/B_4 cells were no longer retained by DEAE-Sephadex after removal of sialic acid. The difference in the percentages of retained, desialylated material from the two cell lines was largely due to the presence in transformed cells of an increased amount of material requiring high ionic strength for elution from DEAE-Sephadex (Figure 3). After neuraminidase or mild acid treatment, this component eluted from DEAE-Sephadex at the same ionic strength as did DEAE peak no. A8 (tubes 280–340, Figure 3b) from untreated material. No sialic acid was released.

Amino acid analyses of many of the glycopeptides did not indicate the presence of significant amounts of basic amino acids. This finding combined with the behavior of desialylated material on DEAE-Sephadex indicated that the ion-exchange properties of the glycopeptides are determined by the sialic acid content of the molecule. It should be noted that there are no amino acid data presented for group A·Con A(−) and group B·Con (A−) glycopeptides. A control preparation containing similar amounts of Pronase used to digest the trypsin-released material was subjected to chromatography on Sephadex G-50, and the fractions corresponding to groups A and B were analyzed for the presence of carbohydrate and protein. No carbohydrate was detected; however, a significant amount of protein, probably resulting from autodigestion of Pronase, was found. Thus, amino acid analysis on Pronase-treated glycopeptides which have not been purified by adsorption to a column of Con A–Sepharose would be misleading.

Carbohydrate Composition. The major fractions from groups A·Con A(−), B·Con A(−), and B·Con A(+) were pooled, as indicated by the bars in parts b and c of Figure 3 and Figure 4a, respectively, desalted, and analyzed for carbohydrate composition. The compositional data are presented in molar ratios based on normalization of the sialic content of the glycopeptides as discussed in the previous section. The only form of sialic acid present is *N*-acetylneuraminic acid as shown by paper chromatography (P. Broquet). This material, when free, produced a chromophore with a maximum absorption of light at 549 nm in the thiobarbituric acid assay. It eluted with authentic *N*-acetylneuraminic acid from columns of Sephadex G-50 and DEAE-Sephadex.

Carbohydrate Composition of Group A·Con A(−) Glycopeptides. The carbohydrate compositions of the glycopeptides from group A·Con A(−) resolved on DEAE-Sephadex are presented in Table II. When equivalent fractions from BHK_{21}/C_{13} (C13) and C_{13}/B_4 (B4) were compared (refer to Figure 3b), it was found that for most of the fractions, i.e., for DEAE peaks no. A2, A3, A5, A6, and A7, the glycopeptides from transformed (C_{13}/B_4) cells contained an additional 1–2 mol of *N*-acetylglucosamine compared to the control (BHK_{21}/C_{13}) cell glycopeptides. Furthermore, in many of these fractions from transformed cells, i.e., in DEAE peaks

Table II: Carbohydrate Composition of DEAE-Fractionated Glycopeptides from GroupA·Con A(−)[a]

	comparison of molar ratios based on sialic acid											
	DEAE peak no. 2		DEAE peak no. 3		DEAE peak no. 4		DEAE peak no. 5		DEAE peak no. 6		DEAE peak no. 7	
	C13	B4	C13	B4	C13	B4	C13	B4	C13	B4	C13	B4
sialic acid	2.0	2.0	3.0	3.0	4.0	4.0	4.0	4.0	5.0	5.0	6.0	6.0
mannose	3.49	3.57	3.73	3.53	3.99	3.32	3.02	3.22	3.52	3.68	1.73	2.80
galactose	5.58	6.67	5.40	6.02	5.67	5.52	4.71	5.38	5.64	5.76	6.15	8.32
N-acetylglucosamine	5.82	8.56	6.44	7.53	6.99	6.95	5.20	5.85	5.72	6.58	3.14	4.90
N-acetylgalactosamine	0.27	0.68	0.31	0.34	0.15	0.31	0.36	0.32	0.88	0.66	2.37	2.22
fucose	1.23	1.71	1.26	1.41	1.38	1.18	1.06	0.96	0.93	0.97	0.58	1.01
approx M_r of subunit	3800	4700	4200	4600	4800	4600	4000	4300	4800	5000	4600	5600

[a] The carbohydrate compositions represent the average of two to four determinations. The variability for the individual sugars was as follows: sialic acid, ±1–4%; mannose, ±2–7%; galactose, ±1–4%; *N*-acetylglucosamine, ±2–8%; fucose, ±4–7%. C13 = BHK_{21}/C_{13}; B4 = C_{13}/B_4.

Table III: Carbohydrate Composition of DEAE-Fractionated Glycopeptides from Group B·Con A(−)[a]

	comparison of molar ratios based on sialic acid													
	DEAE peak no. 2		DEAE peak no. 3		DEAE peak no. 4		DEAE peak no. 5		DEAE peak no. 6		DEAE peak no. 7		DEAE peak no. 8	
	C13	B4	C13	B4	C13	B4	C13	B4	C13	B4	C13	B4	C13	B4
sialic acid	1.0	1.0	2.0	2.0	3.0	3.0	3.0	3.0	4.0	4.0	5.0	5.0	6.0	6.0
mannose	5.45	5.45	4.96	5.94	4.29	4.11	3.90	2.88	3.49	2.64	2.23	1.87	0.89	1.05
galactose	3.22	3.04	4.50	4.57	4.96	4.67	5.69	4.19	6.27	5.10	7.55	6.58	6.40	7.59
N-acetylglucosamine	9.15	7.98	9.17	8.75	5.56	6.52	6.61	4.94	4.86	4.71	4.39	4.08	1.41	1.66
N-acetylgalactosamine					0.41	0.62	0.97	0.72	1.51	1.51	2.76	2.68	4.33	4.21
fucose	2.24	2.22	2.08	2.46	1.56	1.23	1.48	0.96	0.97	0.84	1.78	1.56	0.38	0.55
approx M_r of subunit	4300	4000	4700	4900	4100	4200	4500	3700	4600	4200	5200	4900	4800	4800

[a] See footnote *a* of Table II.

no. A2, A3, A5, and A7, there was a higher galactose content. The mannose content of group A·Con A(−) glycopeptides was 3 to 4 residues/mol, and it was similar in the two cell lines. Significant amounts of *N*-acetylgalactosamine were found in the components requiring higher ionic strength for elution from DEAE-Sephadex (DEAE peaks no. A6 and A7); this indicated the possible presence of O-glycosidically linked glycopeptides.

Carbohydrate Composition of Group B·Con A(−) Glycopeptides. The carbohydrate compositions of the glycopeptides from group B·Con A(−) fractionated on DEAE-Sephadex (refer to Figure 3c) are presented in Table III. Calculation of the data in this way resulted in molecular weights which were consistent within group B but slightly higher than the previously estimated (Warren et al., 1974) size range of group B glycopeptides. The mannose content in the peaks eluted at low ionic strength (DEAE peaks no. B2, B3, and B4) was calculated by this method to be four to six residues of mannose per glycopeptide unit. In the fractions which eluted at a high ionic strength, i.e., DEAE peaks no. B5, B6, and B7, several observations are significant. The ratio of *N*-acetylgalactosamine to sialic acid was the same in the material from both types of cells. As the amount of *N*-acetylgalactosamine increased, so did the amount of galactose; however, there was an additional galactose residue in the glycopeptides from control cells relative to the corresponding material from transformed cells. The relative amounts of mannose in each pair of peaks decreased as the amounts of sialic acid, *N*-acetylgalactosamine, and galactose increased. These results suggest that, for group B·Con A(−) glycopeptides, the material requiring high ionic strength for elution contained a greater proportion of O-glycosidically linked material than of asparagine-linked (N-glycosidic) material. DEAE peak no. B8 contained very little *N*-acetylglucosamine, mannose, or fucose and therefore probably contained little asparagine-linked material. The composition of the material of this peak was similar in the two cell types (BHK_{21}/C_{13} and C_{13}/B_4) except for an additional galactose residue in the material from transformed cells.

In the DEAE-Sephadex fractions which eluted at low ionic strength, a comparison of groups A and B indicated that the ratios of sialic acid to mannose were quite different in the two groups. A comparison based on sialic acid content indicated that group B·Con A(−) (DEAE peaks no. B3 and B4, Table III) may contain one to two additional mannose residues per glycopeptide relative to group A·Con A(−) (DEAE peaks no. A2 and A3, Table II). A mannose content of 3 residues/glycopeptide has been found in many glycoproteins (Etchison et al., 1977; Reading et al., 1978; Kornfeld & Kornfeld, 1976; Kornfeld et al., 1971; Spik et al., 1975; Toyashima et al., 1972).

Composition of Group B·Con A(+) Glycopeptides. The glycopeptides from group B·Con A(+) (Figure 4a) were analyzed for carbohydrate and amino acid composition. The compositions of the glycopeptides from transformed and control cells were very similar (Table IV). The subunit sizes were close to the molecular weights predicted by gel filtration for group B glycopeptides when sialic acid was normalized to 1, 2, or 3 residues/glycopeptide. Calculations indicated that the glycopeptides probably contain four to six mannose residues. The ratios of galactose to mannose and of *N*-acetylglucosamine to mannose were much lower in the group B·Con A(+) material than in the corresponding DEAE peaks from group B·Con A(−). The compositions of all amino acids, with the exception of proline, were very similar in the DEAE peaks of material from transformed cells and control cells. However, when the carbohydrate data for DEAE peak no. 1, which did not contain sialic acid, were calculated on the basis of an aspartic acid (asparagine) content of 1.0, a higher mannose content was found in DEAE peak no. B1 from BHK_{21}/C_{13} cells than in DEAE peak no. B1 from C_{13}/B_4 cells; the other

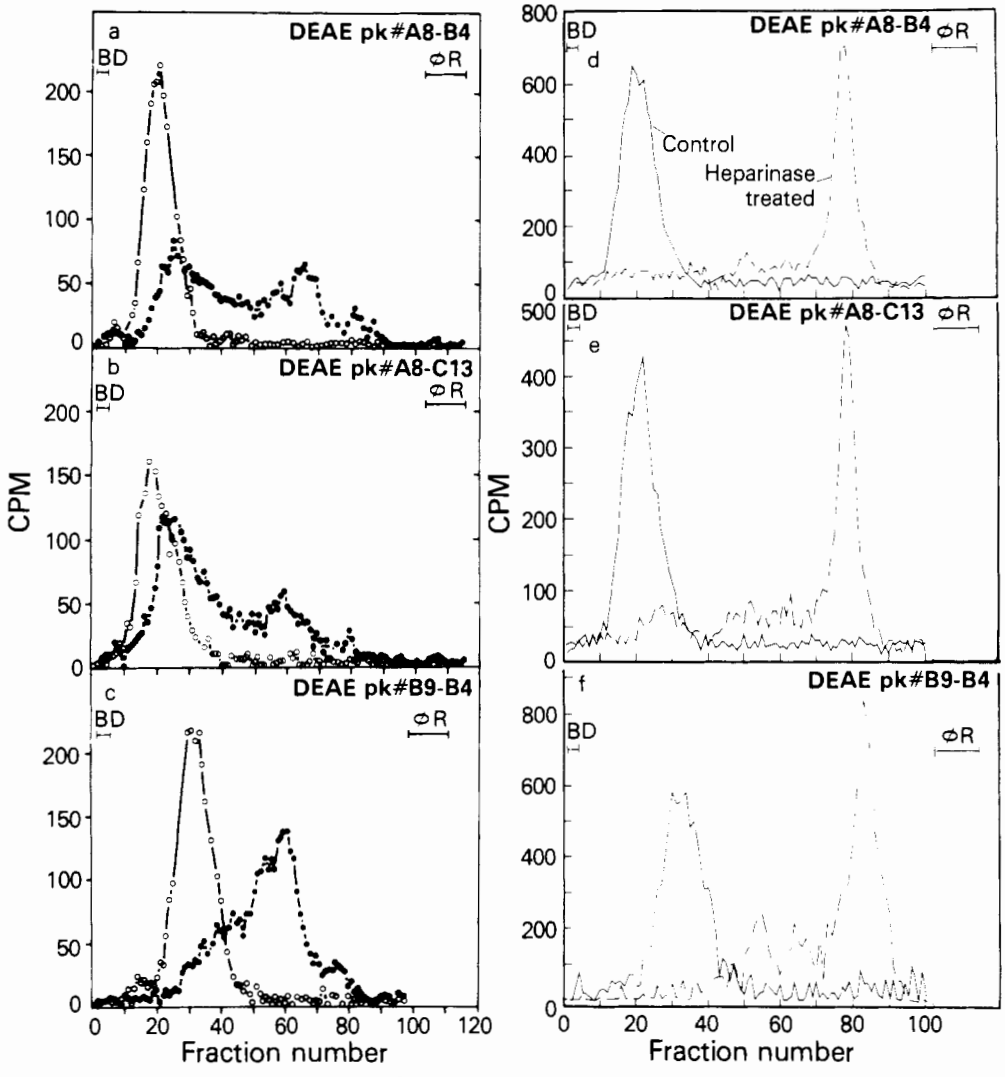

FIGURE 5: Sephadex G-50 chromatography of the products of nitrous acid hydrolysis or heparinase treatment of neuraminidase-resistant material. The material which required a high salt concentration for elution from DEAE-Sephadex was digested with nitrous acid (a–c) or heparinase (d–f) as described under Experimental Procedure. The products were analyzed by Sephadex G-50 column chromatography. The digested material and control material were run separately on the same column and their elution profiles superimposed on the basis of the positions of the marker dyes Blue Dextran (BD) and phenol red (ϕR). (a) DEAE peak no. A8 (Figure 3b) from transformed cells (C_{13}/B_4), (b) DEAE peak no. A8 (Figure 3b) from control cells (BHK_{21}/C_{13}), and (c) DEAE peak no. B9 (Figure 3c) from transformed cells (C_{13}/B_4) (○) before hydrolysis and (●) after hydrolysis. (d) DEAE peak no. A8 (Figure 3b) from transformed cells (C_{13}/B_4), (e) DEAE peak no. A8 (Figure 3b) from control cells (BHK_{21}/C_{13}), and (f) DEAE peak no. B9 (Figure 3c) from transformed cells (C_{13}/B_4) (—) before enzyme treatment and (– · –) after enzyme treatment.

sugars remained approximately equivalent in amount in the two cell lines.

Characterization of Material Requiring High Ionic Strength for Elution from DEAE-Sephadex. Some of the peaks fractionated by DEAE-Sephadex column chromatography were further examined.

Neuraminidase-Resistant Material. DEAE peak no. A8 from group A·Con A(–) (Figure 3b) was found to be present in a much greater quantity in transformed than in normal cells. Elution of this material from DEAE-Sephadex was unchanged by neuraminidase treatment but it eluted at a slightly lower ionic strength after mild acid hydrolysis. Because of the high ionic strength required for elution from DEAE-Sephadex (Figure 3b), it was thought that this glucosamine-labeled material might be a glycosaminoglycan. Radioactive fucose was not incorporated into the material (unpublished observations), and no fucose was found upon analysis of the material for carbohydrate.

When this material was incubated with hyaluronidase (containing chondroitinase A and C activity) and assayed by Sephadex G-50 column chromatography, no change was observed. The material was subjected to nitrous acid hydrolysis in order to determine whether heparin or heparan sulfate was present. This procedure is specific for the hydrolysis of compounds such as heparan sulfate and heparin, containing *N*-sulfate groups (Conrad & Hart, 1975). Considerable degradation was seen following nitrous acid treatment (parts a–c of Figure 5) of the neuraminidase-resistant components (DEAE peaks no. A8 control, A8 transformed, and B9 transformed, Figure 3). Incubation of all three components with heparinase resulted in complete digestion of each of the components (parts d–f of Figure 5). It would appear from the results presented in Figure 5 that the material which eluted from DEAE-Sephadex at high ionic strength was composed largely of heparan sulfate like material. In contrast to our results, Chiarugi et al. (1974) found a decrease in sulfated glycosylaminoglycans at the surface of Rous virus and polyoma transformed BHK cells compared to control cells. At the present time, we have no explanation for the difference in our findings. Some experimental details differ; e.g., Chiarugi et

Table IV: Composition of DEAE-Fractionated Glycopeptides from Group B·Con A(+)[a]

	comparison of molar ratios based on sialic acid							
	DEAE peak no. 1		DEAE peak no. 2		DEAE peak no. 3		DEAE peak no. 4	
	C13	B4	C13	B4	C13	B4	C13	B4
sialic acid	ND	ND	1.0	1.0	2.0	2.0	3.0	3.0
mannose	8.02	5.57	4.16	4.64	4.69	4.53	6.12	5.36
galactose	1.36	1.03	2.08	2.15	2.99	2.87	4.02	3.36
N-acetylglucosamine	3.70	4.01	4.36	4.02	5.07	5.12	6.69	5.86
fucose	1.09	1.18	1.23	1.33	1.42	1.27	2.10	1.57
glucose	1.25	0.92	0.20	0.14	0.12	0.21	0.26	0.23
approx M_r	2920	2447	2633	2664	3364	3314	4604	4067
aspartic acid	1.0[b]	1.0[b]	0.95	1.15	1.29	1.21	1.46	1.50
threonine	0.73	0.71	0.33	0.42	0.91	0.45	0.32	0.47
serine	1.61	1.17	0.34	0.40	0.90	0.51	0.38	0.73
glutamic acid	0.23	0.09	+	+	0.29	0.21	0.44	0.83
proline	0.63	3.22	–	–	0.91	0.10	0.17	–
glycine	1.16	0.96	0.36	0.31	0.59	0.36	0.19	0.32
alanine	0.58	0.32	0.25	0.15	0.55	0.18	0.12	0.14
valine	0.32	0.13	+	0.14	0.10	0.16	–	–
leucine	0.33	+	+	+	+	+	0.12	+
lysine			–	–	–	–	–	–
arginine			–	–	–	–	–	–
histidine			–	–	–	–	–	–

[a] See footnote *a* of Table II. (+) indicates the presence of 0.10 nmol of amino acid, and (–) indicates that none was detectable. ND = not determined. [b] Aspartic acid was normalized to 1.0.

al. harvested their cells at confluency while our cells were processed in full log phase of growth.

Characterization of Material Containing N-Acetylgalactosamine. The presence of *N*-acetylgalactosamine in DEAE peaks no. A7, B6, B7, and B8 indicates that the material probably contained O-glycosidically linked carbohydrate. This type of linkage is hydrolyzed under mild alkaline conditions which do not affect *N*-glycosidically linked glycopeptides (Marks et al., 1963).

Following alkaline hydrolysis, DEAE peaks no. B7 and B8 were each converted to two smaller subunits but some of the radioactive material eluted like the unhydrolyzed material from a column of Sephadex G-50. There was not enough material present for carbohydrate analysis of each product. However, it might be speculated on the basis of the known carbohydrate compositions of the undigested material (Table III) that the glycopeptide was composed of an asparagine-linked oligosaccharide that eluted like the unhydrolyzed material and the later eluting serine- and/or threonine-linked oligosaccharide(s), both originally on a single peptide fragment which is not cleaved by Pronase digestion. After mild alkaline hydrolysis, the peptide portion remains with the asparagine-linked oligosaccharide and the O-glycosidic components are released.

Discussion

The experiments described in this paper were designed to fractionate and compare the glycopeptides derived from the membrane glycoproteins of malignant and control BHK fibroblasts grown in tissue culture. Relative to control cells, transformed cells contained significantly more large, complex glycopeptides which do not bind to Con A and correspondingly less small glycopeptides which can bind to Con A (Table I). It is clear from the data presented in Figures 3 and 4 that the cell has great diversity in its glycopeptide composition. By separating glycopeptides first on the basis of size, then by affinity to Con A, and finally by charge, we fractionated the material into an array of 40 fully or partially resolved glycopeptides. The complexity of the DEAE elution profiles was proportional to the size range of the group. Thus, the relatively small group B·Con A(+) and group C·Con A(+) glycopeptides were very well resolved (parts a and b of Figure 4), while the large glycopeptides, groups pre-A and A, showed a great deal of charge heterogeneity (parts a and b of Figure 3).

Although this complex pattern was completely reproducible, this is no assurance that the glycopeptides, even those eluting as sharp, discrete peaks, are homogeneous. Even so, the patterns of control and transformed cells are directly comparable. The existence of so many carbohydrate forms should prompt caution in interpreting compositional and structural data on less resolved material. It is of interest that similar analyses on cell surface glycopeptides derived from human and mouse fibroblasts reveal a remarkable resemblance between the DEAE-Sephadex elution profiles of glycopeptides from all three species (unpublished results). It has also been found that glycopeptides prepared from the cell pellets remaining after the treatment of cells with trypsin and subjected to the same procedure yield elution profiles similar to those obtained with trypsinate (surface) material. Thus, the degree of complexity observed in the elution profiles presented in this paper is not confined to cell surface glycopeptides derived from BHK fibroblasts.

We analyzed the major fractions from groups A·Con A(–), B·Con A(+), and B·Con A(–) for carbohydrate composition. The molar ratios of the sugars were calculated on the basis of sialic acid content for the following reasons. First, more than 95% of the glycopeptides which contained sialic acid could no longer bind to DEAE-Sephadex after the sialic acid was removed, indicating that the peptide portion of the glycopeptide contributes very little to the net negative charge of the molecule. Second, since the DEAE-Sephadex elution properties of each component were primarily due to its sialic acid content, glycopeptides of approximately the same size which coeluted from DEAE-Sephadex were expected to have the same net negative charge. Therefore, the sialic acid contents of components from control and transformed cells which eluted from DEAE-Sephadex at the same ionic strength could, for purposes of comparison, be considered equal. Third, the molecular weight of the oligosaccharide subunit calculated from molar ratios based on sialic acid content was reasonably consistent with the approximate molecular weights of the glycopeptides as determined by Sephadex G-50 column chromatography (Warren et al., 1974). The molecular weight approximations

given in the Tables II–IV are calculated solely on the basis of carbohydrate content. It is noted that the estimated molecular weights given for group A are not always larger than some of the values calculated for group B. Reliance upon gel filtration as a determinant of molecular weight for charged molecules may be misleading, since gel filtration properties can be significantly influenced by the shape and charge of the molecules (unpublished observations) (Etchison et al., 1977; Ceccarini & Atkinson, 1977).

A comparison of the carbohydrate composition of group A·Con A(−) glycopeptides from control cells with that from transformed cells (Table II) revealed some interesting differences between the two cells. Most components from the transformed cell had a higher content of *N*-acetylglucosamine than did the corresponding components from the control cell (i.e., DEAE peaks no. A2, A3, A5, and A7, Table II). Several components from the transformed cell also had a higher galactose content (i.e., DEAE peaks no. A2, A3, A4, and A7). These differences in composition may reflect a qualitative difference between the two cells. Alternatively, each peak may represent an unresolved mixture of the same glycopeptides with a quantitative increase in one or more of the components of the mixture.

The data in Table II suggest that the glycopeptides from group A·Con A(−) may have had a core region containing three to four mannose residues. These values are in agreement with the findings of others for the mannose content of the core region of the G protein of vesicular stomatitis virus (Etchison et al., 1977; Reading et al., 1978), for a number of glycoproteins found in serum (Kornfeld & Kornfeld, 1976; Kornfeld et al., 1971; Spik et al., 1975; Toyashima et al., 1972), and for proposed structures of glycopeptides isolated from transformed BHK cells (Santer & Glick, 1979; Ogata et al., 1976). Core structures containing three to five mannose residues have been found in the acidic glycopeptides isolated from Rous sarcoma virus, Prague C, grown in chick embryo fibroblasts (Hunt et al., 1979).

When the group B·Con A(−) glycopeptides were analyzed for carbohydrate composition following the standardization of sialic acid content, the core regions of several components appeared to contain four to six mannose residues (DEAE peaks no. B2, B3, and B4 from both BHK_{21}/C_{13} and C_{13}/B_4 cells and no. B5 and perhaps no. B6 from BHK_{21}/C_{13} cells, Table III). The remaining group B·Con A(−) glycopeptides contained fewer than 3 mannose residues/glycopeptide; however, in these latter components, all of which require a high ionic strength for elution from DEAE-Sephadex, a significant amount of *N*-acetylgalactosamine was also present. As the amount of *N*-acetylgalactosamine increased, mannose content decreased (DEAE peaks no. B5, B6, B7, and B8, Table III). *N*-Acetylgalactosamine is usually found in association with O-glycosidically linked material, which frequently contains galactose and sialic acid as well (Thomas & Winzler, 1969; Spiro & Bhoyrov, 1974; Finne, 1975; Codington et al., 1975). The presence of mannose and *N*-acetylgalactosamine in the same peak suggests that the material may contain both N-glycosidic and O-glycosidic components, possibly on the same polypeptide chain. Our data support this notion and are similar to those of Kornfeld (1978) with a glycopeptide from calf thymocytes. The two oligosaccharides may have been spaced too closely together to permit cleavage by Pronase.

The carbohydrate composition of components of the group B·Con A(+) fraction also indicated the possibility of a core region containing four to six residues of mannose (Table IV). Unlike the carbohydrate composition of group B·Con A(−) glycopeptides, in which the content of *N*-acetylglucosamine and galactose as well as of mannose was high, group B·Con A(+) glycopeptides contained relatively little *N*-acetylglucosamine and galactose. Since Con A binding requires the presence of α-mannose residues which are unsubstituted on the C3, C5, and C6 hydroxyls (Goldstein et al., 1965; Poretz & Goldstein, 1970; Kornfeld & Ferris, 1975), it is possible that the inability of group B·Con A(−) glycopeptides to bind to Con A is due to the substitution of *N*-acetylglucosamine and galactose on these sites.

Our laboratory has compared the glycopeptides derived from several individual pairs of purified, homologous membrane glycoproteins from both control and transformed cells. Carbohydrate alterations were found in 20 of 24 pairs examined (Tuszynski et al., 1978). These findings are in agreement with data presented here, suggesting that the increased amount of group A glycopeptides observed in transformed cells relative to control cells was neither the result of a selective increase in the synthesis of a glycoprotein containing group A glycopeptides nor due to an inability of the transformed cell to synthesize any of the glycopeptides normally synthesized in control cells. Rather, there appears to be an overall shift in populations of carbohydrate groups toward the synthesis of larger, more complex glycopeptides which, while present in the control cell, represent a smaller proportion of the overall complement of carbohydrate. It should be noted that differences in relative amounts of glycopeptides in transformed and control cells appear to involve whole series of glycopeptides simultaneously.

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Phosphatidylcholine Exchange Protein Catalyzes the Net Transfer of Phosphatidylcholine to Model Membranes†

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ABSTRACT: 2-Stearoyl spin-labeled phosphatidylcholine (PC*) has been introduced into the phosphatidylcholine exchange protein from bovine liver and its electron spin resonance (ESR) spectrum determined. The spin-labeled group in the PC*–exchange protein complex was strongly immobilized. Addition of sodium deoxycholate micelles released PC* from its binding site, producing a mobile signal. This was also observed when micelles of lysophosphatidylcholine and vesicles of phosphatidic acid were added, indicating that the exchange protein can insert its endogenous PC* into interfaces devoid of phosphatidylcholine. ESR spectroscopy was used to measure transfer of PC* from spin-labeled "donor" vesicles to unlabeled "acceptor" vesicles as described by Machida & Ohnishi [Machida, K., & Ohnishi, S. (1978) *Biochim. Biophys. Acta 507*, 156–164]. The donor vesicles consisted of PC* and phosphatidic acid (75:25 mol %) and the acceptor vesicles of phosphatidylethanolamine and phosphatidic acid (81:19 mol %). Addition of exchange protein catalyzed a net tranfer of PC* from donor to acceptor vesicles. This transfer proceeded until the acceptor vesicles contained ~2 mol % of PC*. A spontaneous transfer of PC* was not observed. As for the mode of action, it appears that the exchange protein, after insertion of its endogenous PC* into the acceptor, leaves the interface without a bound phospholipid molecule yet continues to shuttle PC* from donor to acceptor.

The phosphatidylcholine exchange protein from bovine liver is widely used to determine both the size of the PC[1] pool in the outer monolayer of membranes and the rates of transbilayer movement of PC (Zilversmit, 1978; Rothman et al., 1976; Shaw et al., 1979; Van den Besselaar et al., 1978; de Kruijff & Wirtz, 1978). Essential to these studies are the absolute specificity of the protein for PC (Kamp et al., 1977) and its ability to equilibrate PC among "donor" and "acceptor" membranes without changing the size of the PC pools involved (Demel et al., 1973). As for its mode of action, the protein parts with its bound PC molecule upon interaction with a membrane (Kamp et al., 1977). Upon disruption of the protein–membrane complex the free protein contains another PC (Demel et al., 1973). So far, it has not been established whether the protein can leave the interface devoid of PC. This would be required if the protein is involved in net transfer of PC within the cell (Wirtz, 1974).

Recently, ESR spectroscopy has been introduced to monitor continuously the exchange protein catalyzed transfer of 2-acyl spin-labeled (PC*) from PC* vesicles to unlabeled phospholipid vesicles (Machida & Ohnishi, 1978). Evidence was provided that as a putative intermediate in the transfer process a PC*–exchange protein complex was formed characterized by an immobilized spectrum (Devaux et al., 1977; Machida & Ohnishi, 1978). In the present study ESR spectroscopy has

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[1] Abbreviations used: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PA, phosphatidic acid; LPC, lysophosphatidylcholine; PC*, 1-acyl-2-(16-doxylstearoyl)-*sn*-glycero-3-phosphocholine; PE*, 1-acyl-2-(16-doxylstearoyl)-*sn*-glycero-3-phosphoethanolamine; ESR, electron spin resonance; cmc, critical micelle concentration.

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