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HYBRID, SIALYLATED *N*-GLYCANS ACCUMULATE IN A RICIN-RESISTANT MUTANT OF BABY HAMSTER KIDNEY BHK CELLS*

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ABSTRACT

Glycoproteins synthesized in a ricin-resistant mutant of BHK cells, clone RIC^{R}21, were labelled by growth of the cells in radioactive D-mannose, D-glucosamine, or L-fucose. Glycopeptides obtained from disrupted cells by exhaustive digestion with Pronase were fractionated into components binding to concanavalin A–Sepharose and nonbinding components. The binding components eluted with methyl α-D-mannopyranoside were separated by gel filtration on Bio-Gel P-4 into two main subfractions: an oligomannosidic fraction that was susceptible to Jack bean α-D-mannosidase and a fraction that became totally degraded only in the additional presence of neuraminidase, β-D-galactosidase, and *N*-acetyl-β-D-glucosaminidase. Further analysis of the latter fraction by exoglycosidase digestion together with consideration of the known pathways for the biosynthesis of asparagine-linked sugar chains of glycoproteins was consistent with a "hybrid" structure containing a NeuAc→Gal→GlcNAc sequence linked to the α-D-mannosyl-(1→3) residue of the core sequence, and a terminal α-D-mannosyl group linked to the α-(1→6) branch of the core sequence. The hybrid fraction was labelled after growth of the cells in radioactive L-fucose and was adsorbed to a lentil lectin–Sepharose column indicating the presence of core fucosylation. The novel structure represented about 30–35% of the total cellular glycopeptides of RIC^{R}21 cells and was not present in the glycopeptides of normal, ricin-sensitive BHK cells. Conversely, double-branched (biantennary) complex *N*-glycans, a prominent constituent of BHK cell glycoproteins, were absent in RIC^{R}21 cells, and analysis of the nonbinding fraction obtained from concanavalin A–Sepharose indicated that triple- and quadruple-branched (tri- and tetra-antennary), complex *N*-glycans present in normal BHK cell glycoproteins were also absent.

INTRODUCTION

Ricin, the toxic lectin of castor beans *Ricinus communis*, binds to glycoproteins having carbohydrate chains containing β-D-galactosyl residues[1,2]. Binding to

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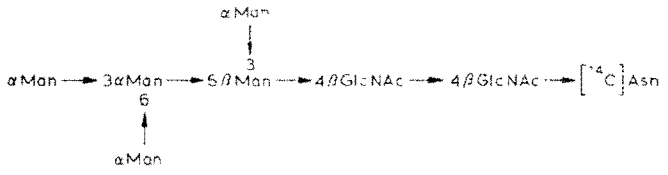
cell surface glycoproteins is an obligatory step in ricin cytotoxicity, and several ricin-resistant cell lines of mammalian fibroblasts show a greatly reduced binding of the lectin at the cell surface[3]. Furthermore, glycoproteins solubilized from the cells bind poorly to ricin-affinity columns, as compared with parental-cell glycoproteins. Direct enzymic analysis of cellular extracts has provided, in some cases, an explanation for the change in *N*-glycan structure leading to a reduced ability of cell surface glycoproteins to bind ricin. In the ricin resistant[4–6] BHK cell line RIC^R14, and in several ricin-resistant CHO cell lines[3], the enzyme *N*-acetylglucosaminyltransferase I is missing. This enzyme[7] catalyzes a key step in the conversion of a



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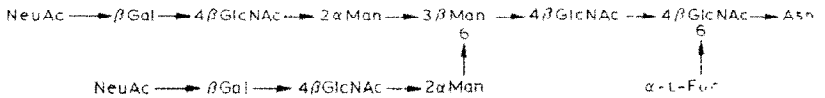

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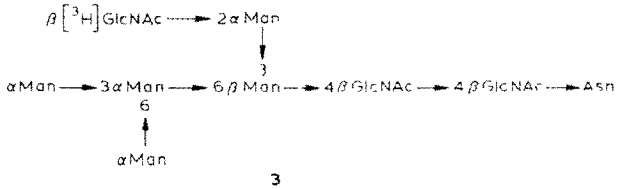

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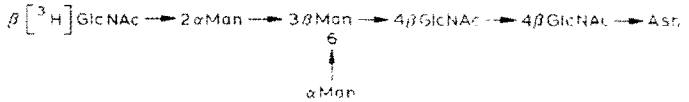

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Scheme 1. Glycopeptides of known structures used as marker substances (see Experimental section). (**1**) Ovalbumin glycopeptide, purified by ion-exchange chromatography, affinity chromatography on concanavalin A–Sepharose, and gel filtration; the monosaccharide composition of the glycopeptide was confirmed by direct analysis, the glycopeptide was labelled in the aminoacid moiety with [^{14}C]acetic anhydride. (**2**) Glycopeptide fraction obtained from Pronase-digestion products of BHK cells labelled metabolically with D-[^{3}H]mannose. The glycopeptide was purified by chromatography on concanavalin A–Sepharose and gel filtration. (**3** and **4**) Glycopeptides labelled *in vitro* with purified *N*-acetylglucosaminyltransferase I and UDP-2-acetamido-2-deoxy-D-[^{3}H]glucose

pentamannosyl-containing intermediate (**1**) to precursors of complex, sialylated *N*-glycans containing multiple repetitions of the *N*-acetyllactosamine [*O*-(β-D-galactopyranosyl-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)] residue (for example, see **2**).

Similar enzymic analysis of other ricin-resistant BHK cell lines, however, failed to show a reduction in the activity of this glycosyltransferase, although quantitative alterations in the activities of several other glycosyltransferases were detected[6]. In an attempt to correlate these relatively minor alterations in the levels of enzymes responsible for normal *N*-glycan assembly with the ricin-binding properties of the BHK cell mutants, we studied[8] the general composition of the glycopeptides obtained from cellular glycoproteins of several mutant cell-lines in comparison with the normal BHK cells. We showed that the glycopeptides derived from several mutants differed dramatically from the normal patterns, as shown by affinity chromatography on concanavalin A–Sepharose. In the present paper, we describe, in more detail, the glycopeptides obtained from one ricin-resistant BHK cell line, *i.e.*, clone RIC^R21.

EXPERIMENTAL

Radioactive precursors. — D-[2-^{3}H]Mannose (104 Ci/mmol), L-[5,6-^{3}H]fucose (45 Ci/mmol), and 2-amino-2-deoxy-D-[1-^{14}C]glucose hydrochloride (39 mCi/mmol) were from Amersham International, U.K. Aqueous samples (0.05–0.5 mL) were counted in scintillation fluid EP (10 mL, Beckman Corp.) in an Intertechnique scintillation counter which had a counting efficiency of ~60% for ^{3}H and 80% for ^{14}C.

Cells. — Baby hamster kidney cells and ricin-resistant mutants[4,5] were grown, in monolayer culture, in Glasgow-modified Eagles medium supplemented with 10% fetal calf serum at 37° as previously described. Where indicated, the cells were labelled metabolically by growth for 2–3 days at 37° in a medium containing ~20 mCi/mL of ^{3}H-labelled sugars or 5 mCi/mL of 2-amino-2-deoxy-D-[^{14}C]glucose. The cells were seeded into 100-mm diameter tissue culture plates and allowed to attach to the plates overnight at 37°. The radioactive sugars were then added and the cultures grown to confluency over 2–3 days at 37°.

Preparation of glycopeptides. — Labelled monolayer cultures were washed thoroughly with warm (37°) phosphate-buffered saline solution, pH 7.2 (P_i–NaCl), scraped from the culture plates with a rubber policeman and washed at least twice with warm P_i–NaCl. The cell pellets were suspended in 50mM Tris · HCl buffer (pH 7.5) containing 0.1M sodium chloride, mM magnesium chloride, mM calcium chloride, mM manganese chloride, and 0.02% sodium azide (0.5 mL), and immediately immersed in a vigorously boiling-water bath for 5 min. After being cooled, the mixtures were supplemented with a Pronase solution (50 μL, 0.4% in the same buffer as aforementioned) and 1–2 drops of toluene, and incubated at 37°. Additional aliquots (50 μL) of Pronase solution were added every 12 h for 2–3

days. Finally, the mixtures were again heated at 100° for 5 min to inactive the Pronase, and the mixtures were centrifuged at 10 000*g* for 10 min. Aliquots of the clear supernatant solutions were removed for counting and for chromatography.

Lectin-affinity chromatography. — Columns (1 × 23, or 1 × 12 cm) of concanavalin A–Sepharose (Pharmacia, Sweden) were washed thoroughly with 10mM Tris · HCl (pH 7.5) containing 0.1M sodium chloride, mM magnesium chloride, mM calcium chloride, mM manganese chloride, and 0.02% sodium azide at room temperature. Solutions of glycopeptide sample (0.1–0.5 mL) were applied to the selected column, which was washed further with the same buffer at ~8 mL/h and room temperature. Fractions (1 mL) were collected. Subsequently the columns were washed successively with 10mM methyl α-D-glucopyranoside and 500mM methyl α-D-mannopyranoside dissolved in the buffer described earlier. During elution with methyl α-D-mannopyranoside, the eluting solution was immersed in a water bath kept at 60°. Aliquots (25–100 μL) of each eluted fraction were removed for radioactive counting. Appropriate peak fractions were pooled, freeze dried, and dissolved in water (1–2 mL).

Chromatography of glycopeptides on a column (1 × 12 cm) of lentil lectin–Sepharose (Pharmacia, Sweden) was carried out, at room temperature in the same buffer as that used for chromatography on concanavalin A–Sepharose. After application of the solution of the glycopeptide sample (0.1–0.5 mL), the flow of the column was stopped for 1 h to allow adsorption of glycopeptides to the column. Elution was commenced with buffer, followed by elution successively with 10 and 200mM methyl α-D-glucopyranoside dissolved in the same buffer. Fractions (1 mL) were collected and analyzed as described earlier.

Routinely, yields of at least 90% of the applied radioactivity were recovered from the lectin columns. No additional radioactivity could be eluted from the columns with M hydrochloric acid or 1% sodium dodecyl sulphate.

Ion-exchange chromatography. — DEAE–Sephacel (Pharmacia, Sweden) was washed with M acetic acid, packed into a column (1 × 20 cm), and washed further at 2° with 50mM acetic acid. Solutions of glycopeptide sample in water (0.5–3 mL) were applied to the column, and the flow of the column was stopped for 1 h to allow maximal adsorption of glycopeptides. The column was washed with water, and fractions (1 mL) were collected at ~6 mL/h. After collection of ten fractions, the glycopeptides were eluted with a salt gradient established by mixing a solution of 0.5M sodium chloride–50mM acetic acid (45 mL) into 50mM acetic acid (45 mL). Aliquots (50–500 μL) of fractions (1 mL) were removed for radioactive counting. Appropriate peak fractions were pooled, freeze dried, and dissolved in water (1 mL).

Gel filtration. — Bio-Gel P-4 (200–400 mesh, BioRad Laboratories, Richmond, CA) was suspended in 0.1M pyridine–acetic acid buffer (pH 6) and packed into columns (1.2 × 77, or 1 × 30 cm). The columns were washed extensively at 2° and ~5 mL/h with the same buffer. Solutions of glycopeptide samples (0.5 mL) were applied and eluted under the same conditions. Fractions (usually

1 mL) were collected and aliquots (50–500 μL) were counted for radioactivity. Appropriate fractions were pooled, freeze-dried, and dissolved in water (0.5–1 mL). In some experiments, the glycopeptide samples were supplemented with Blue dextran (~0.1 mg, Pharmacia, Sweden), D-[^{3}H]mannose (10^4 counts/min), or marker glycopeptides (10^3–10^4 counts/min) as indicated. The elution of Blue dextran was determined by measurement of the absorption at 600 nm.

Glycosidase treatments. — Neuraminidase was obtained from CalBiochem–Behring Corp. Jack bean α-D-mannosidase, β-D-galactosidase, and *N*-acetyl-β-D-glucosaminidase were purified by established procedures[9]. One unit (U) is defined as the hydrolysis of 1 μmol of substrate/min.

Glycopeptide samples dissolved in 0.1M sodium acetate buffer (pH 5) containing 0.3mM zinc sulphate (0.4 mL) were treated with α-D-mannosidase (2.5 U/mL) under toluene for 1–3 days at 37°. In other experiments, glycopeptide samples were incubated for 2–3 days at 37° with various mixtures of neuraminidase (0.1 U/mL), β-D-galactosidase (0.16 U/mL), and *N*-acetyl-β-D-glucosaminidase (0.59 U/mL) in 50mM sodium phosphate buffer (pH 7). The mixtures (0.7–0.9 mL) were freeze-dried after incubation and, in some cases, redissolved in 0.1M sodium acetate buffer (pH 5)–0.3mM zinc sulphate for treatment with α-D-mannosidase as described earlier.

Standard glycopeptides. — A glycopeptide fraction (**1**) was prepared[10] from the products of exhaustive proteolytic digestion of ovalbumin by ion-exchange chromatography. The product was purified further[11–13] by chromatography on concanavalin A–Sepharose as described earlier. The glycopeptide was bound tightly to the affinity column and was eluted with 500mM methyl α-D-mannopyranoside. Carbohydrate and amino acid analysis was consistent with the structure[14] containing five D-mannose and two 2-acetamido-2-deoxy-D-glucose per asparagine residue. In addition to asparagine, relatively small amounts (<0.2 mol/mol of asparagine) of other amino acids, especially serine and threonine, were present. This material was labelled by treatment with [1-^{14}C]acetic anhydride (2–10 mCi/mmol) as described[8], and desalted by chromatography on a column (1.6 × 29 cm) of Sephadex G-25 equilibrated with 0.1M pyridine–acetic acid buffer (pH 6). The glycopeptide fraction, well separated from salts, was freeze-dried and dissolved in water at 10^6 c.p.m./mL. Glycopeptide products synthesized[7,15] from UDP-2-acetamido-2-deoxy-D-[^{3}H]glucose with purified, bovine colostrum *N*-acetylglucosaminyltransferase I (**3** and **4**) were kindly provided by Dr. H. Schachter. The reaction products were separated by paper electrophoresis in 1% sodium tetraborate (pH 9.2) buffer and the glycopeptide fractions, located by radioactive scanning of the paper strips, were eluted with water, freeze-dried, and dissolved in water (**3**, 126 240 c.p.m./mL; **4**, 66 000 c.p.m./mL). A glycopeptide fraction containing double-branched, complex *N*-glycan (**2**) was obtained from BHK cells labelled metabolically by growth in radioactive L-fucose or D-mannose, as described in full elsewhere[8], and briefly described in the Results section.

RESULTS AND DISCUSSION

Fractionation of cellular glycopeptides. — As shown (Fig. 1a), concanavalin A–Sepharose chromatography of D-[^{3}H]mannose-labelled glycopeptides of normal

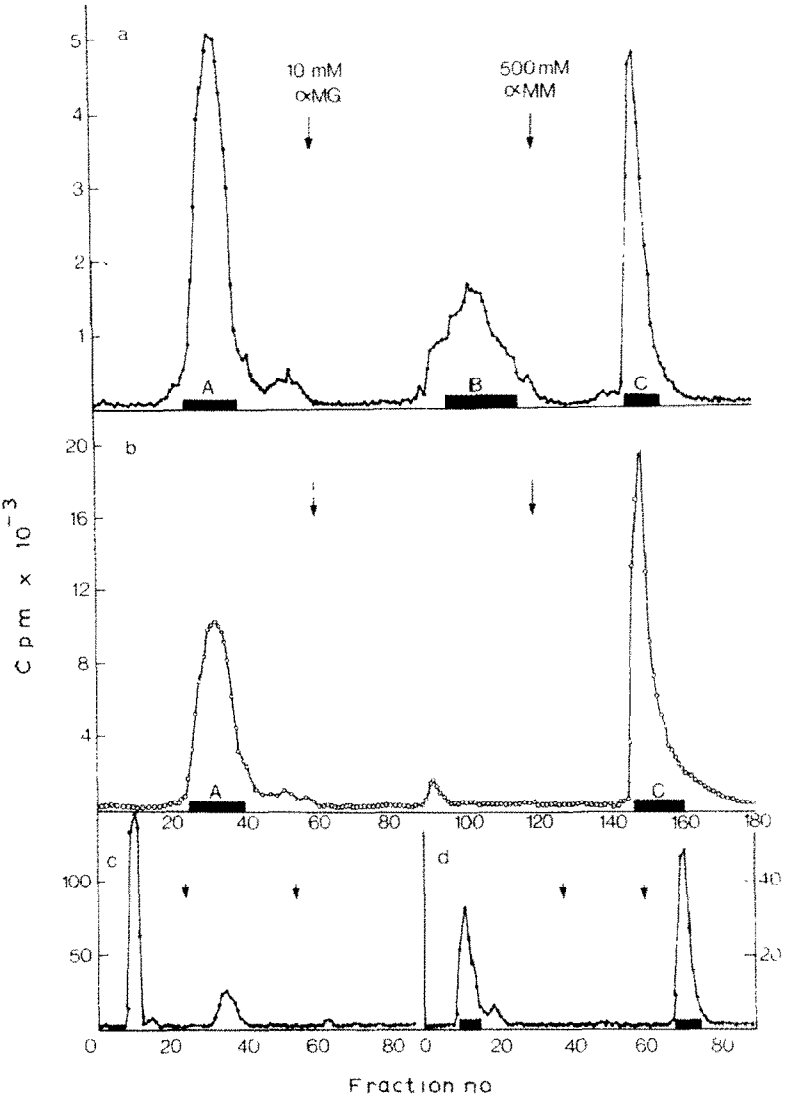

Fig 1. Separation of cellular glycopeptides on concanavalin A–Sepharose. BHK cells (a and c) or RIC^{R}21 cells (b and d) were labelled by growth in either D-[^{3}H]mannose (a and b) or L-[^{3}H]fucose (c and d), and glycopeptides obtained by Pronase treatment were fractionated on columns (1 × 23 cm, a and b; 1 × 12 cm, c and d) of concanavalin A–Sepharose. Following initial elution with buffer, fractions (1 mL) were eluted successively with 10mM methyl α-D-glucopyranoside (αMG) or 500mM methyl α-D-mannopyranoside (αMM) as shown, and aliquots (0.1 mL) of each fraction were counted for radioactivity. The major-peak fractions were pooled for further analysis as indicated by the boxes. The samples applied to the columns were: (a) 160 208, (b) 699 040, (c) 661 630, and (d) 333 760 c p m

BHK cells produced several fractions showing different affinities for the lectin. Fractions A and B represent glycopeptides containing complex *N*-glycans, whereas Fraction C consists of glycopeptides containing oligomannosidic or "high mannose" *N*-glycans. Evidence for these assignments, which are fully consistent with the affinities of glycopeptides of known structure for concanavalin A–Sepharose[11–13], are given elsewhere[8] and are also documented in part in the present paper. Typically, fractions eluted respectively with buffer, 10mM methyl α-D-glucopyranoside, and 500mM methyl α-D-mannopyranoside contained 41, 16, and 43% of the total radioactivity recovered from the column. By contrast, the relative proportions of RIC^{R}21 glycopeptides recovered by elution from concanavalin A–Sepharose (Fig. 1b) with these eluting solutions were 21, <3, and 74%, respectively. The most striking differences between the glycopeptides of parental BHK

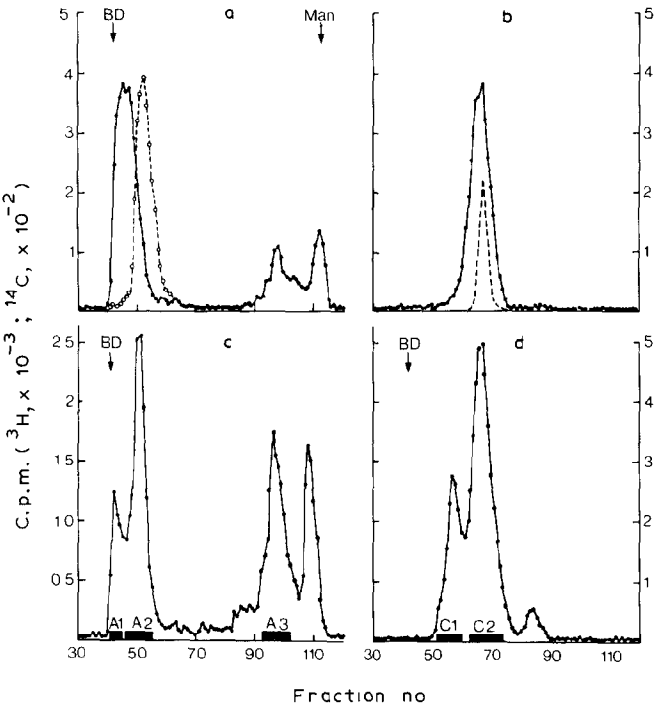

Fig. 2. Chromatography of L-[^{3}H]mannose-labelled glycopeptides on Bio-Gel P-4. Glycopeptide samples (0.5 mL) were applied to a column (1.2 × 77 cm) of Bio-Gel P-4 equilibrated with 0.1M pyridine acetate buffer, pH 6. Fractions (1 mL) were collected by elution with the same buffer and analyzed for radioactivity: (a) BHK cells glycopeptide Fraction A (—●—●—, 29 400 c.p.m.) or Fraction B (--○--○--, 12 020 c.p.m.) from concanavalin A–Sepharose chromatography (see Fig. 1a) were applied to the column in separate experiments; (b) (—●—●—) BHK cells glycopeptide Fraction C (14 000 c.p.m.) from concanavalin A–Sepharose, and (----) elution of [^{14}C]ovalbumin glycopeptide (1, 2000 c.p.m.) applied simultaneously; (c) RIC^{R}21 cells glycopeptide Fraction A (42 300 c.p.m.) from concanavalin A–Sepharose; and (d) RIC^{R}21 cells glycopeptide Fraction C (143 210 c.p.m.) from concanavalin A–Sepharose. The peak fractions indicated by the boxes were pooled for further analysis. BD indicates the elution of Blue dextran (total excluded volume of the column) and Man that of D-[^{3}H]mannose.

cell glycoproteins and RIC^{R}21 cells (Fig. 1a and b) were the drastically reduced amount of D-[^{3}H]mannose-labelled glycopeptides eluted with 10mM methyl α-D-glucopyranoside, *i.e.*, glycopeptides containing a double-branched, complex *N*-glycan structure (**3**), and secondly an accumulation of glycopeptides binding tightly to the affinity column and eluted with methyl α-D-mannopyranoside.

Further insight into the different glycopeptide profiles of the glycoproteins of RIC^{R}21 cells and BHK cells were obtained by chromatography of the concanavalin A–Sepharose fractions on Bio-Gel P-4 (Fig. 2). The BHK cell glycopeptide Fraction A was eluted from the column as a broad, heterogeneous peak close to the totally excluded region indicated by the elution of Blue dextran (Fig. 2). A minor portion of radioactive material of lower molecular-weight, including some free D-mannose, was also present. Concanavalin A-Sepharose Fractions B (Fig. 2a) and C (Fig. 2b) were eluted as relatively symmetrically peaks, the latter emerging from the column together with a marker glycopeptide$^{10\text{-}14}$ derived from ovalbumin (**1**).

The chromatography (Fig. 2c) of the RIC^{R}21 glycopeptide Fraction A was complex: a relatively minor peak (A1) emerged with the Blue dextran marker, whereas the major peak (A2) was eluted in a position intermediate between Fractions A and B of the BHK cell glycopeptides. In addition, the lower-molecular-weight fraction (A3) was more prominent in the RIC^{R}21 glycopeptides, as compared with BHK cell components. Fractions A1, A2, and A3 represented 14, 46, and 23%, respectively, of the total radioactivity recovered from the Bio-Gel P-4 column. A new peak (C1) appeared in the chromatography profile of RIC^{R}21

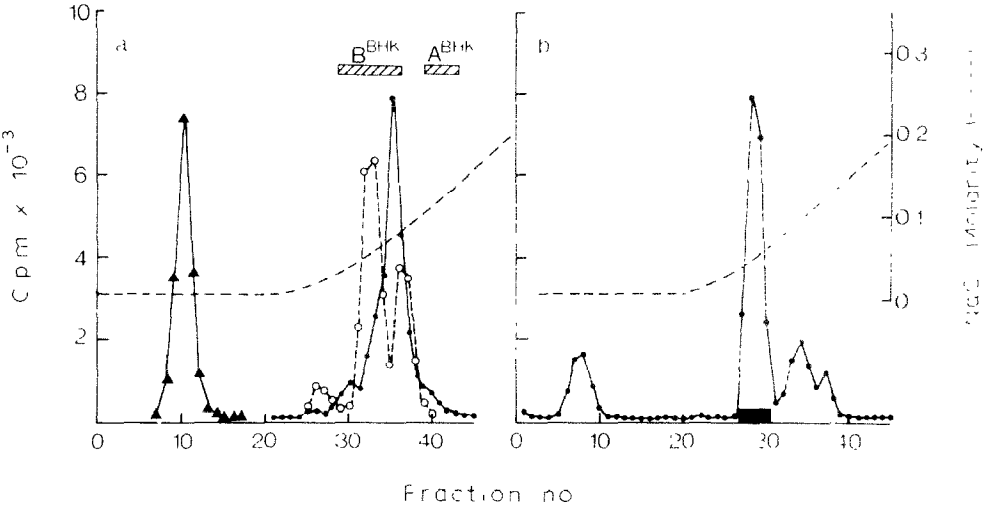

Fig. 3. Chromatography of D-[^{3}H]mannose-labelled RIC^{R}21 glycopeptides on DEAE-Sephacel Fractions (~7000 c.p.m.), isolated by chromatography on Bio-Gel P-4 (see Fig. 2), were applied separately to the ion-exchange column (1 × 20 cm) and eluted with a sodium chloride gradient (----), as described in the Experimental section: (a) Fractions A1 (●), A2 (○), and A3 (▲). The hatched boxes indicate the elution of BHK glycopeptides Fractions A and B isolated from concanavalin A-Sepharose. (b) Fraction C1. Peak fractions pooled for further analysis are indicated by the full box.

glycopeptide Fraction C (Fig. 2d). This peak migrated immediately ahead of the major fraction (C2). Typically, Fraction C1 represented 35–43% of the total radioactivity of Fraction C.

Sialic acid-containing glycopeptides. — Ion-exchange chromatography on DEAE–Sephacel readily identified the sialylated components of RIC^R21 cell glycopeptides, as shown in Fig. 3. Fractions A1 and A2 (Fig. 2c) were retained on the column and eluted with a salt gradient. By contrast, Fraction A3 was neutral and was eluted unretarded from DEAE–Sephacel. After treatment with neuraminidase, Fractions A1 and A2 were eluted from DEAE–Sephacel as neutral compounds in the region occupied by Fraction A3, thus showing that their negative charge was due largely to sialic acid (results not shown). The profiles shown in Fig. 3a were obtained reproducibly. Thus, Fraction A2 was eluted consistently in two major peaks, the more acidic of which was almost co-incident with the single peak

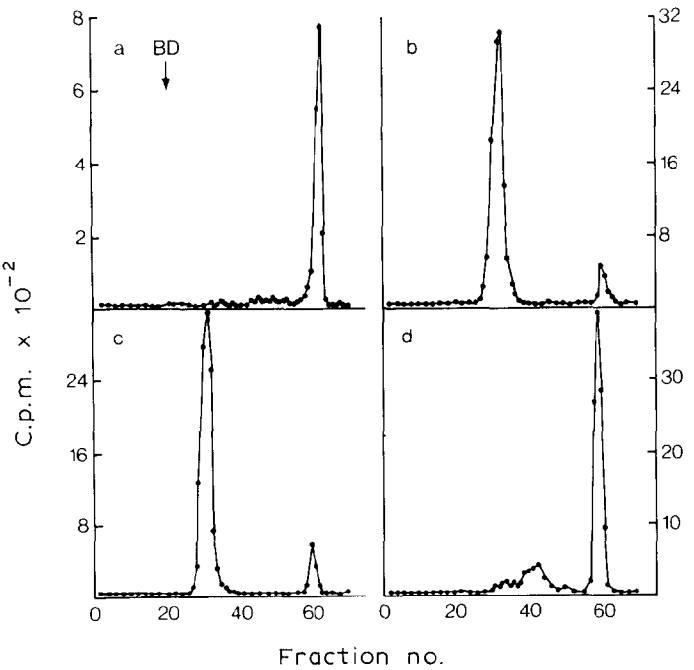

Fig. 4. Effect of glycosidases on D-[^{3}H]mannose-labelled glycopeptides. Fraction C glycopeptides isolated, by concanavalin A–Sepharose chromatography, from RIC^R21 cells were purified further by chromatography on Bio-Gel P-4. The Fraction C1 of RIC^R21 cells (see Fig. 2) was subsequently separated by ion-exchange chromatography, and the major acidic component (see Fig. 3b) used for enzymic digestion. Glycopeptide fractions were treated with purified glycosidases in various combinations for several days at 37°, as described in the Experimental section, before application separately to small columns (1 × 30 cm) of Bio-Gel P-4. Fractions (1 mL) collected by elution with 0 1M pyridine acetate buffer (pH 6) were analyzed for radioactivity: (a) Glycopeptide Fraction C2 treated with α-D-mannosidase (4395 c.p.m. applied). (b–d) Glycopeptide Fraction C1 (15 000 c.p.m. applied) treated with: (b) α-D-mannosidase alone; (c) α-D-mannosidase, *N*-acetyl-β-D-glucosaminidase, and β-D-galactosidase; and (d) α-D-mannosidase, *N*-acetyl-β-D-glucosaminidase, β-D-galactosidase, and neuraminidase.

obtained on chromatography of Fraction A1. Previously, we have shown[5] that the glycopeptide Fraction B containing double-branched *N*-glycans (see 2), isolated from normal BHK cells, was separated into two fractions by chromatography on DEAE–Sephacel. These peaks emerged at salt concentrations that were very similar to those required to elute RIC^{R}21 cell Fractions A1 and A2 (Fig. 3a). The BHK cell glycopeptides containing triple- and quadruple-branched *N*-glycans present in the concanavalin A–Sepharose Fraction A (Fig. 1a) were eluted at a significantly higher salt concentration as indicated in Fig. 3a. These results suggest that the acidic glycopeptides present in RIC^{R}21 Fraction A from concanavalin A carry similar proportions of sialic acid as the double-branched *N*-glycans of BHK cells.

As shown in Fig. 3b, a major part (~85%) of the RIC^{R}21 glycopeptide Fraction C1 was also retained on DEAE–Sephacel. The minor proportion of neutral

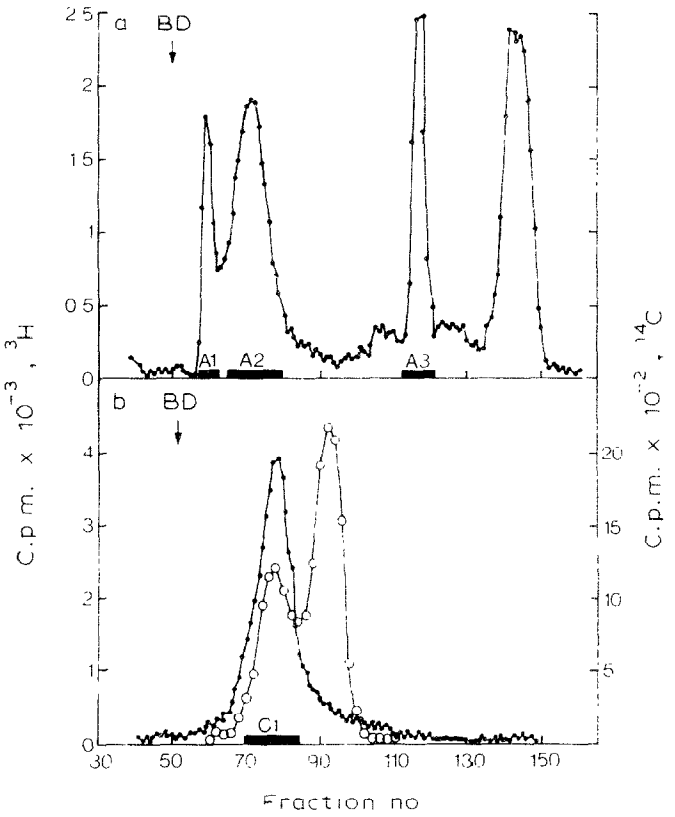

Fig. 5. Bio-Gel P-4 chromatography of L-[^{3}H]fucose- or 2-amino-2-deoxy-D-[^{14}C]glucose-labelled glycopeptide fractions of RIC^{R}21 cells. Fractions A (26 295 c.p.m., ^{3}H) and C (26 096 c.p.m., ^{3}H; 16 120 c.p.m., ^{14}C) from concanavalin A–Sepharose chromatography (see Fig. 1) were applied separately to the columns. Elution of glycopeptides was carried out as described in the legend to Fig. 3, except that smaller fractions (0.7 mL) were collected for analysis. Peak fractions were pooled as shown. BD, Blue dextran. (a) Concanavalin A–Sepharose Fraction A. (b) Fraction C. (●) L-[^{3}H]fucose radioactivity, and (○) 2-amino-2-deoxy-D-[^{14}C]glucose radioactivity.

material probably derives from contamination by Fraction C2, which was eluted totally unretarded from DEAE–Sephacel (results not shown). Other results (not shown) established that Fraction C1 was converted completely into a neutral species after treatment with neuraminidase. The major acidic component of Fraction C1 was eluted just prior to the major acidic component present in Fraction A2, with more minor components being eluted at higher salt concentrations. The main acidic fraction was isolated in a large amount for further analysis.

Structure of RIC^{R}21 glycopeptide Fraction C1. — Next, the susceptibility of glycopeptide Fraction C1 to exoglycosidase was tested by use of a D-[^{3}H]mannose-labelled preparation purified as described earlier. When the material was treated with α-D-mannosidase alone, ~7–10% of the radioactivity was released as free D-mannose (Fig. 4b). The majority of the radioactive glycopeptide was eluted from the Bio-Gel P-4 column in a position close to the point of elution of the untreated component. When the material treated with α-D-mannosidase was passed through a concanavalin A–Sepharose column, the radioactivity was quantitatively retained, and was eluted with 500mM methyl α-D-mannopyranoside, indicating that the D-mannose-rich sequences had remained intact (results not shown). By contrast, treatment of RIC^{R}21 glycopeptide Fraction C2 with α-D-mannosidase converted the component completely into lower-molecular-weight compounds, the major part (80–85%) of which was free D-mannose (Fig. 4a). Similar results were obtained for BHK glycopeptide Fraction C earlier[8], showing that these fractions consisted entirely of oligomannosidic *N*-glycans with unsubstituted D-mannosyl end-groups. Treatment of RIC^{R}21 glycopeptide C1 with mixtures of either *N*-acetyl-β-D-glucosaminidase plus α-D-mannosidase (results not shown), or β-D-galactosidase, *N*-acetyl-β-D-glucosaminidase, and α-D-mannosidase (Fig. 4c) failed to release more radioactivity than was obtained with α-D-mannosidase alone. However, after treatment with a mixture of these glycosidases and including neuraminidase, the C1 component was converted completely into lower-molecular-weight material including free D-mannose (68–73% of total), as shown in Fig. 4d. These results suggest a basic structure for RIC^{R}21 glycopeptide Fraction C1 of a core sequence $(Man)_3(GlcNAc)_2 \rightarrow Asn$ to which is attached at least one NeuAc→Gal→GlcNAc sequence. The structure was unlikely to be the usual double-branched (biantennary) complex *N*-glycan, however, in view of its smaller size and since such a glycopeptide would have been eluted from concanavalin A–Sepharose with 10mM methyl α-D-glucopyranoside. In order to determine the number of NeuAc→Gal→GlcNAc sequences present in the glycopeptide, we prepared Fraction C1 from RIC^{R}21 cells labelled metabolically with 2-amino-2-deoxy-D-[^{14}C]glucose.

Studies of D-[^{14}C]glucosamine-labelled glycopeptide Fraction C1. — 2-Amino-2-deoxy-D-glucose is a precursor of both 2-acetamido-2-deoxy-D-glucose and sialic acid units of the sugar chains of glycoproteins. The glycopeptide Fraction C1 was isolated by concanavalin A–Sepharose as described for D-[^{3}H]mannose-labelled cells in Fig. 1b, followed by Bio-Gel P-4 chromatography (see Fig. 5b). In

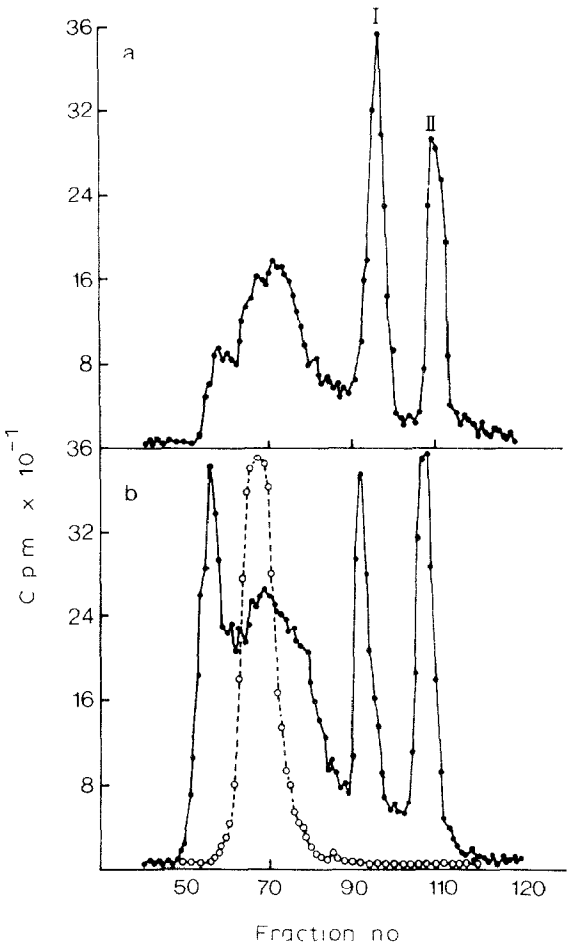

Fig 6 Release of sialic acid and 2-acetamido-2-deoxy-D-glucose from RIC^{R}21 glycopeptide fractions Glycopeptides prepared from cells labelled by growth in 2-amino-2-deoxy-D-[^{14}C]glucose were treated with a mixture of neuraminidase, β-D-galactosidase, and *N*-acetyl-β-D-glucosaminidase for 3 days at 37 °, and the reaction products were separated by chromatography on Bio-Gel P-4 as described in the legend to Fig. 2: (a) Glycopeptide Fraction A2 was purified further by DEAE–Sephacel chromatography (see Fig 3a) Column fractions 31–35 were pooled for the experiment (total, 6510 c p.m), (b) glycopeptide Fractions C1 (●) and C2 (○), Fraction C1 was purified further by DEAE -Sephacel chromatography, and column fractions 28–31 (see Fig 3b) were used for the experiment, ~10 000 and 8000 c p m , respectively, were applied to the columns. Peaks I and II were eluted coincidentally with standards of *N*-acetylneuraminic acid and 2-acetamido-2-deoxy-D-glucose, respectively

a preliminary experiment, the 2-amino-2-deoxy-D-[^{14}C]glucose-labelled RIC^{R}21 Fraction C was mixed with a D-[^{3}H]mannose-labelled preparation (14 940 and 10 840 c.p.m., respectively) and applied to a Bio-Gel P-4 column. Fractions C1 and C2 were isolated and assayed for radioactivity. The ratio of ^{14}C to ^{3}H in Fraction C1 was significantly higher (38%) than in Fraction C2, indicating a higher content of 2-acetamido-2-deoxy-D-glucose (and sialic acid) in the former fraction. For en-

zymic analysis, 2-amino-2-deoxy-D-[^{14}C]glucose-labelled Fraction C1 was purified further by chromatography on DEAE–Sephacel and the acidic fraction (see Fig. 3b) treated with a mixture of neuraminidase, β-D-galactosidase and *N*-acetyl-β-D-glucosaminidase. As a control, 2-amino-2-deoxy-D-[^{14}C]glucose-labelled Fraction C2 (Fig. 5b), was treated with the enzymes similarly. The reaction products were separated by chromatography on Bio-Gel P-4 (Fig. 6b). As shown, the C1 glycopeptide fraction was degraded extensively to lower-molecular-weight products, although some undegraded material was present being eluted between column fractions 51 and 59 (Fig. 6b). Both radioactive sialic acid and 2-acetamido-2-deoxy-D-glucose were released by the enzymes together with material of still high molecular weight eluted between column fractions 61 and 80 (Fig. 6b). This presumably represents the end product of enzyme degradation, containing the core sequence $(Man)_3(GlcNAc)_2Asn$. The relative recoveries of radioactivity in the pooled fractions 61–80, in free sialic acid, and in free 2-acetamido-2-deoxy-D-glucose were 5220, 1930, and 2372 c.p.m., respectively. Since the core region contains two 2-acetamido-2-deoxy-D-glucose units, these results suggest strongly that the C1 glycopeptide fraction contains one NeuAc→Gal→GlcNAc sequence substituting into the core sequence. In the control experiment using glycopeptide C2, no free radioactive sialic acid or 2-acetamido-2-deoxy-D-glucose was released (Fig. 6b), showing that the latter compound was present in the core sequence as nonterminal residues and unavailable for enzymic hydrolysis.

From the known pathways of *N*-glycan assembly[7] and the presence of normal amounts of *N*-acetylglucosaminyltransferase I in RIC^{R}21 cells[6], the most likely basic structure for the C1 glycopeptide fraction is a hybrid-type chain consisting of a NeuAc→Gal→GlcNAc sequence substituting the α-(1→3)-linked branch of a normal core region and unsubstituted D-mannosyl groups on the α-(1→6)-linked branch. The failure of Jack bean α-D-mannosidase to release these residues before degradation of the sialylated sequence is consistent with previous results obtained with the sugar chains of rhodopsin[16,17]. In rhodopsin *N*-glycans, a single 2-acetamido-2-deoxy-β-D-glucopyranosyl group is located at O-2 of the (1→3)-linked α-D-mannopyranosyl residue of the core region, and 3, 4, and 5 α-D-mannopyranosyl residues are attached to the α-(1→6)-linked α-D-mannopyranosyl residue of the core. From the extent of degradation of D-[^{3}H]mannose-labelled RIC^{R}21 glycopeptide Fraction C1 by mixed glycosidases (Fig. 4d), the major component appears to be a trimannosyl compound, indicating that the processing pathway catalyzed by Golgi α-D-mannosidases[7] had proceeded. However, the amount of D-mannose released (68–73% of total) is rather higher than the theoretical (66%) expected for a trimannosyl structure, and consequently some processing intermediates containing additional D-mannose units may be present.

Comparison with standard glycopeptides. — The structures proposed for RIC^{R}21 glycopeptide Fraction C1 are similar to those predicted from the action of *N*-acetylglucosaminyltransferase I on the pentamannosyl intermediate[7,15]. Through the generosity of Dr. H. Schachter, we obtained authentic samples of

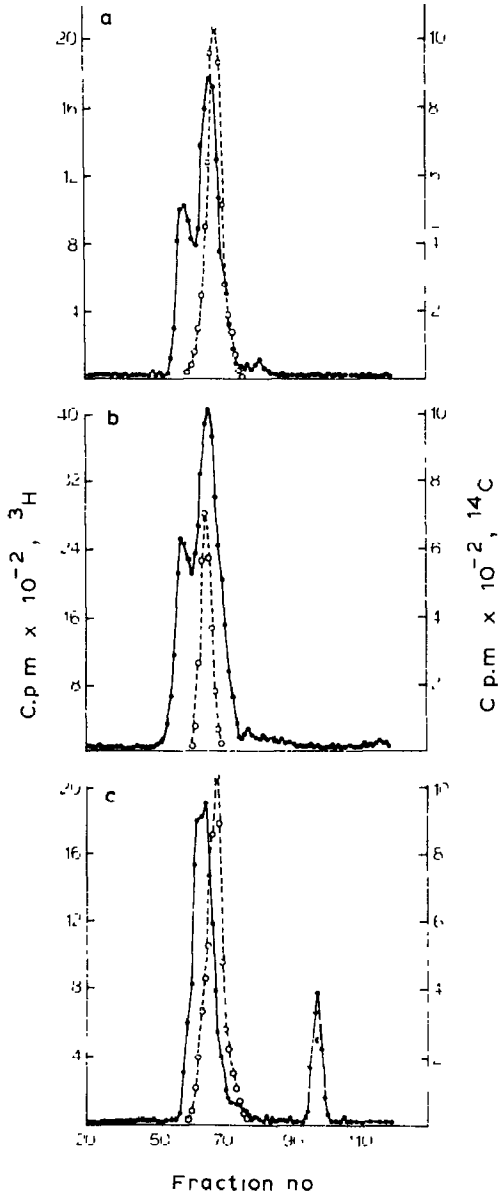

Fig 7 Cochromatography of 2-amino-2-deoxy-D-[^{14}C]glucose-labelled glycopeptides (—●—●—) from RIC^{R}21 cells and 2-amino-2-deoxy-D-[^{3}H]glucose-labelled marker glycopeptides 3 and 4 (--□--□--) Glycopeptides obtained from RIC^{R}21 cells, labelled metabolically with 2-amino-2-deoxy-D-[^{14}C]glucose, were fractionated on concanavalin A–Sepharose. Samples (7485 c p m , ^{14}C) of Fraction C (see Fig 1) were mixed with either: (a) Compound 4 (16 500 c p m , ^{3}H) or (b) Compound 3 (31 560 c p m , ^{3}H), and applied to a Bio-Gel P-4 column treated as described in the legend to Fig 2 Column fractions were analyzed for radioactivity (c) RIC^{R}21 glycopeptide Fraction C1 (6440 c p m) was treated with a mixture of neuraminidase and β-D-galactosidase as described in the text, mixed with compound 4 (16 500 c.p.m , ^{3}H), and then applied to the column The lower-molecular-weight ^{14}C radioactivity comigrated with authentic *N*-acetylneuraminic acid

structures analogous to those proposed (**3** and **4**) which had been prepared, *in vitro* by the addition, in the presence of a purified transferase, of 2-acetamido-2-deoxy-D-[^{3}H]glucose from UDP-2-acetamido-2-deoxy-D-glucose to D-mannoside glycopeptides of defined sequence containing three or five D-mannose units. In order to compare the chromatographic properties of the RIC^{R}21 glycopeptide Fraction C1 with these biosynthetic, [^{3}H]-labelled glycopeptides, we used RIC^{R}21 glycopeptides labelled with 2-amino-2-deoxy-D-[^{14}C]glucose.

Samples of 2-amino-2-deoxy-D-[^{14}C]glucose-labelled RIC^{R}21 glycopeptide Fraction C were mixed with the 2-amino-2-deoxy-D-[^{3}H]glucose-labelled biosynthetic glycopeptides and chromatographed on Bio-Gel P-4. As shown (Fig. 7a,b) neither biosynthetic product was eluted together with Subfraction C1. After treatment of the purified Fraction C1 with a mixture of neuraminidase and β-D-

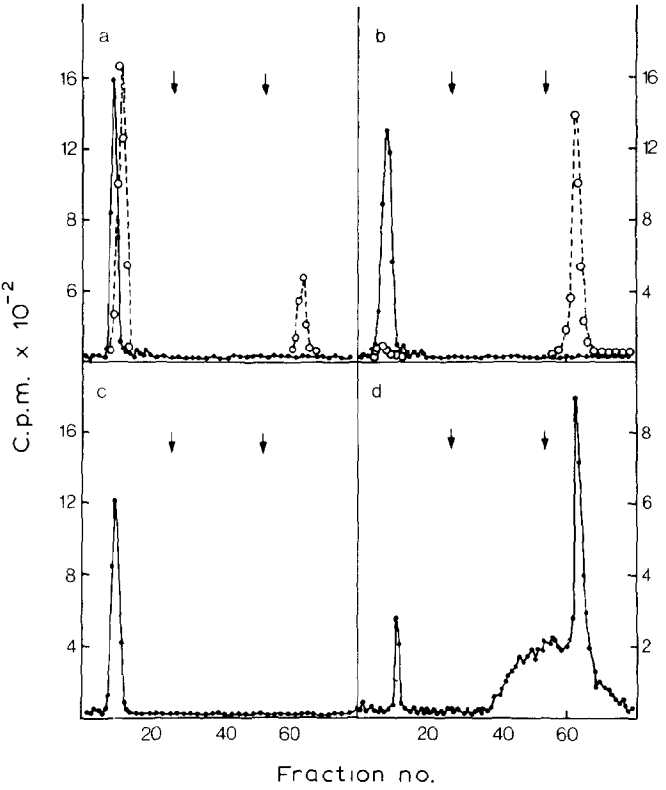

Fig. 8. Chromatography of L-[^{3}H]fucose-labelled glycopeptides on lentil–Sepharose (—●—●—). RIC^{R}21 glycopeptide Fractions A1 (a), A2 (b), A3 (c), and C1 (d) (see Fig. 5) were applied separately (each ~5000 c p.m.) and eluted successively with buffer, followed by 10 and 200mM methyl α-D-glucopyranoside (arrows). Column fractions (1 mL) were analyzed for radioactivity. The separate chromatographies of L-[^{3}H]fucose-labelled Fractions A (7320 c.p.m.) and B (5000 c.p.m.), obtained by concanavalin A–Sepharose chromatography of BHK glycopeptides (Fig. 1c), are shown (--○--○--) in (a) and (b), respectively.

galactosidase, and cochromatography with the trimannosyl biosynthetic compound (**4**), there was a closer correspondence of the peaks (fig. 7e), but the RIC^{R}21-derived material still migrated significantly ahead of the biosynthetic product, indicating the presence of additional sugar residues. As expected, there was release of radioactive sialic acid from the RIC^{R}21 glycopeptide fraction by neuraminidase and β-D-galactosidase. This material (Fig. 7e) represented 17–21% of the total radioactivity, in reasonable agreement with the presence of one sialic acid unit in a structure containing three 2-acetamido-2-deoxy-D-glucose units. The theoretical value would be 25% of released radioactivity assuming equal labelling of sialic acid and 2-acetamido-2-deoxy-D-glucose units during growth of the cells with the 2-amino-2-deoxy-D-[^{14}C]glucose precursor.

L-Fucose content of RIC^{R}21 glycopeptides. — As shown in **3** and **4**, **and** 2-amino-2-deoxy-D-[^{3}H]glucose-labelled glycopeptides synthesized *in vitro* lack L-fucose units. *In vivo* L-fucosylation of core sequences of *N*-glycans is allowed after action of *N*-acetylglucosaminyltransferase I on the pentamannosyl intermediate[18]. Since our proposals for the structures of RIC^{R}21 glycopeptide Fraction C1 include the sequence synthesized by this enzyme, it seemed likely that the slightly greater molecular size of the glycosidase-degraded glycopeptide C1 of RIC^{R}21 cells, as compared with the biosynthetic compounds, may be due in part to the attachment of L-fucose residues. This prediction was strengthened by the results shown in Fig. 1d, 5b, and Fig. 8.

BHK cells and RIC^{R}21 cells were grown in medium containing L-[^{3}H]fucose. The glycopeptides produced by exhaustive Pronase treatment of disrupted cells were fractionated by chromatography on concanavalin A–Sepharose (Fig. 1c,d). In the BHK cell glycopeptides, the complex *N*-glycans present in Fractions A and B were labelled, whereas the oligomannosidic *N*-glycans present in Fraction C were not labelled. By contrast, Fraction C of the RIC^{R}21 cellular glycopeptides was heavily labelled (65% of the total). In addition, an L-fucose-labelled Fraction A was eluted unretarded from the column. It is of interest that no radioactivity was eluted by 10mM methyl α-D-glucopyranoside in the region of Fraction B of BHK cell glycopeptides, thus confirming the absence of double-branched *N*-glycans in RIC^{R}21 cell glycopeptides. The location of L-[^{3}H]fucose labelling in Fraction C of RIC^{R}21 glycopeptides was confined to the hybrid C1 glycopeptides, as shown by Bio-Gel P-4 chromatography (Fig. 5b). Thus, the results confirm the presence of L-fucose in the hybrid structures. The biosynthetic control of core L-fucosylation elucidated *in vitro* by Schachter and assoc.[7,18] is also shown as operating *in vivo* by this experiment, which further places the L-fucopyranosyl groups of the RIC^{R}21 glycopeptides in α-(1→6) linkage to the chitobiosyl sequence of these glycopeptides.

Additional evidence for this assignment was obtained by affinity chromatography of L-[^{3}H]fucose-labelled glycopeptides on lentil lectin–Sepharose (Fig. 8). Recently, the specific requirements for binding to this lectin have been determined[19,20]. The presence of an L-fucosyl group attached to the core chitobiosyl se-

quence of glycopeptides is essential for high-affinity binding. In addition to L-fucose, two α-D-mannosyl residues that may be nonreducing, terminal groups or substituted at O-2 are required. The BHK cellular glycopeptide Fraction B from concanavalin A–Sepharose fulfils all of these requirements. Thus, the L-[^{3}H]fucose (Fig. 1c) or D-[^{3}H]mannose-labelled (Fig. 1a) glycopeptide Fraction B was almost completely adsorbed to the lentil-lectin column and was eluted only by high concentrations of a hapten (Fig. 8b). By contrast, only a small portion of the L-[^{3}H]fucose-labelled Fraction A from BHK cell glycopeptide was tightly bound to the lentil-lectin column (Fig. 8a). According to Kornfeld *et al.*[19], substitution of terminal α-D-mannosyl groups at O-2 and -4 abolishes affinity for the lectin, whereas substitution at O-2 and -6 does not affect binding. Thus, the adsorbed glycopeptides probably carry triple-branched (triantennary) *N*-glycans with an α-D-mannosyl residue bisubstituted at O-2 and -6, and the unadsorbed material represents triple- and quadruple-branched *N*-glycans with α-D-mannosyl units bisubstituted at O-2 and -4. When L-[^{3}H]fucose-labelled RIC^{R}21 glycopeptide Fraction C1 was passed through the lentil-lectin column, the majority (90–95%) of the radioactivity was bound and was eluted with hapten sugar (Fig. 8d). The heterogeneity in the Fraction C1 revealed by lentil lectin–Sepharose chromatography is consistent with the conclusions obtained from glycosidase degradation described earlier. Thus, a hybrid structure containing only the three α-D-mannosyl residues of the core region would be expected, from the published data[19,20], to have high affinity for lentil lectin, whereas structures containing additional α-D-mannosyl residues substituting at O-3 and -6 of a core-sequence α-D-mannosyl residue may show lesser or no affinity. Unfortunately, glycopeptides of these defined structures were not available for direct comparison.

Glycan structures of RIC^{R}21 glycopeptides Fraction A. — As Fig. 8 (a–c) shows, none of the L-[^{3}H]fucose-labeled glycopeptides present in RIC^{R}21 Fraction A (Fig. 5a) had any affinity for the lentil lectin. One interpretation of these results for the acidic components (see Fig. 3a) of Fractions A1 and A2 is that these glycopeptides contain hybrid structures with two NeuAc→Gal→GlcNAc sequences substituting at O-2 and -4 of the core α-D-mannosyl residue linked α-(1→3) to the β-linked D-mannosyl residue. If this interpretation is correct, it implies that the specific *N*-acetylglucosaminyltransferase IV responsible for the addition of a 2-acetamido-2-deoxy-D-glucosyl residue to O-4 of the α-(1→3)-linked D-mannosyl residue[21] may utilize a hybrid structure formed by the action of *N*-acetylglucosaminyltransferase I on the oligomannoside intermediate.

Some evidence in support of the structures proposed is shown in Fig. 6a. 2-Amino-2-deoxy-D-[^{14}C]glucose-labelled Fractions A1 and A2 were prepared by concanavalin A–Sepharose chromatography, followed by Bio-Gel P-4 chromatography. The acidic glycopeptides of each fraction were isolated by elution from DEAE–Sephacel (see Fig. 3a), treated with neuraminidase, β-D-galactosidase, and *N*-acetyl-β-D-glucosaminidase, and rechromatographed on Bio-Gel P-4. Fig. 6a shows the results obtained with one of the acidic components (column fractions 31–

39, Fig. 3a) of Fraction A2. The material was extensively degraded by glycosidase action, producing free sialic acid, 2-acetamido-2-deoxy-D-glucose, and radioactive glycopeptides of lower molecular-weight than the untreated fraction (column fractions 62–80). The recovery of radioactivity in this fraction was 1754 c.p.m., compared with 1614 and 1462 c.p.m. in the sialic acid and 2-acetamido-2-deoxy-D-glucose peaks, respectively. From the ratio of counts in the three fractions, it is calculated that there are, respectively, 1.84 and 1.66 residues of sialic acid and 2-acetamido-2-deoxy-D-glucose units per core sequence (column fractions 62–80) containing two 2-acetamido-2-deoxy-D-glucosyl residues. Since the glycopeptide did not adsorb to concanavalin or lentil-lectin columns, the two NeuAc→Gal→GlcNAc sequences may be placed most probably as suggested earlier, at O-2 and O-4 of the D-mannose unit linked α-(1→3) in the core sequence.

Similar analysis of A1 glycopeptide and the second acidic glycopeptide of Fraction A2 (see Fig. 3a) gave similar results (results not shown). Approximately two residues of 2-acetamido-2-deoxy-D-glucose were released. However, these fractions apparently contain an excess of sialic acid, since 2.4–2.7 times as much radioactivity was recovered in the free sialic acid peak compared with the free 2-acetamido-2-deoxy-D-glucose peak. The placement of these additional sialic acid groups in the glycopeptide structures is unknown.

CONCLUSIONS

The present analysis of the structure of RIC^R21 glycopeptides must be confirmed by further work, *e.g.*, by using oligosaccharides to eliminate the complication of a peptide contribution to the chromatographic properties of the fractions, and by other methods. Nevertheless, our results are consistent with the following proposals. In RIC^R21 cells, processing of nascent *N*-glycans, after transfer to polypeptide, proceeds normally to give a structure containing a penta-D-mannosyl block (see **1**). This structure is modified by: (a) *N*-acetylglucosaminyltransferase I, which is present[6] at normal levels in RIC^R21 cells; (b) processing by α-D-mannosidase II to produce, as the major species, a three D-mannosyl residues core; (c) addition of a 2-acetamido-2-deoxy-D-glucosyl residue to C-4 of the (1→3)-linked-α-D-mannosyl residue of the core sequence in a very minor fraction; (d) elongation by attachment of D-galactose and sialic acid to the 2-acetamido-2-deoxy-D-glucose units added by *N*-acetylglucosaminyltransferase I and IV; and (e) core L-fucosylation.

The biosynthetic reasons for the dramatic shift in *N*-glycan structure in RIC^R21 cells, compared with normal BHK cells, remains to be established. However, direct analysis of the levels of glycosyltransferases in RIC^R21 revealed[6] a reduction (70%) in the activity of *N*-acetylglucosaminyltransferase II, measured *in vitro* by use of model glycopeptide substrates. This transferase acts on the processed product of *N*-acetylglucosaminyltransferase I to start the second branch of the double-branched complex chains[7]. It seems unlikely that our earlier finding[6] can

explain the remarkable absence of normally branched, complex *N*-glycans in RIC^R21 cell glycoproteins, and other possibilities must be considered. One difficulty of determining glycosyltransferase activities *in vitro* is that any model substrate may function as an acceptor for more than one glycosyltransferase. A complete absence of a transferase activity, *e.g.*, *N*-acetylglucosaminyltransferase II, may then be missed because of the presence of the other activities in normal amounts. The apparent paradox may, therefore, result from the use of an insufficiently specific enzyme assay. Alternatively, the abnormal assembly of *N*-glycans in RIC^R21 cells may be due to the substrate specificity of *N*-acetylglucosaminyltransferase II. The preferred substrate, as determined *in vitro*[7], is **4**. Attachment of a D-galactosyl to the terminal 2-acetamido-2-deoxy-D-glucosyl group may limit action of the transferase[22]. Therefore, even if a low activity of transferase II is present in RIC^R21 cells, elongation of the NeuAc→Gal→GlcNAc sequence on the α-(1→3)-linked branch of the core sequence catalyzed by the specific glycosyltransferase that are expressed normally in RIC^R21 cells[6] may occur rapidly to obscure its action.

Structures of the type proposed for RIC^R21 glycoproteins have not previously been described as major features of normal cellular glycoproteins. As described earlier, rhodopsin *N*-glycans have similar structures[16,17] but the single 2-acetamido-2-deoxy-D-glucose unit attached to the α-(1→3)-linked branch of the core sequence is a nonreducing terminal group. More recently, structures similar to that proposed for glycopeptide fraction C1 of RIC^R21 cells have been identified as very minor components of human chorionic gonadotropin and secretory components of human milk[23,24].

Finally, the relationship of the *N*-glycan structures of RIC^R21 cell glycoproteins to the poor ricin-binding properties of these cells (~10% of normal cells)[4,5] should be mentioned. Since the major part of the D-galactose-containing sequence appears to be present in hybrid structures, it seems that ricin shows reduced affinity for glycoproteins carrying such carbohydrate chains, as compared with normal cellular glycoproteins containing double-, triple-, and quadruple-branched complex chains. In agreement with this conclusion, Baenziger and Fiete[1] have shown that the presence of one, two, or three *N*-acetyllactosamine units in glycopeptides results in a steep, progressive increase in the association constants for ricin. Similarly, Debray *et al.*[2] have shown that a carbohydrate chain containing only one NeuAc→Gal→GlcNAc sequence inhibits ricin-induced haemagglutination less well (six-fold) than a structure containing two such sequences. It is possible, therefore, that the altered *N*-glycan structure of RIC^R21 cellular glycoproteins is sufficient to reduce ricin binding and confer resistance to the cells. These general structural alterations may be a rather common feature of ricin-resistant cells. Preliminary analysis of two other resistant BHK cell lines, namely RIC^R17 and 19, but not others (for example RIC^R14 and 15), have shown that a glycopeptide fraction similar in properties to Fraction C1 of RIC^R21 cells is present as a major constituent.

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