Analysis by lectin affinity chromatography of *N*-linked glycans of BHK cells and ricin-resistant mutants

R. Colin HUGHES and Gary MILLS

National Institute for Medical Research, Mill Hill, London NW7 1AA, U.K.

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Normal baby hamster kidney (BHK) fibroblasts and ricin-resistant (Ric^R) mutants of BHK cells derived from them were labelled metabolically with [^3H]mannose or [^3H]fucose. Glycopeptides obtained by digestion of disrupted cells with Pronase were separated by affinity chromatography on concanavalin A–Sephadex. In the normal BHK cells major glycopeptide fractions were obtained consisting of tetra- and tri-antennary sialylated complex glycans, bi-antennary sialylated glycans, and neutral oligomannosidic chains. The majority of bi-antennary chains were shown to contain a fucosyl-($\alpha 1-6$)-*N*-acetylglucosaminyl sequence in the core region by their ability to bind to a lentil lectin affinity column. All of the mutant cell lines examined were found to accumulate oligomannosidic glycans in cellular glycoproteins: complex sialylated glycans were either absent or greatly reduced in amount. Analysis of fractions isolated from concanavalin A–Sephadex by Bio-Gel P-4 chromatography and glycosidase degradation indicated that the glycans accumulating in Ric^R14 cells have the general structure:

and derivatives having fewer α -mannosyl units. We have also analysed the glycopeptides released by trypsin treatment from the surface of the normal and mutant cells, as well as those obtained by proteolysis of fibronectin isolated from the medium. The glycopeptide profiles of the cell-surface-derived material and of fibronectin showed for the mutant cells a marked accumulation of oligomannosidic chains at the expense of complex oligosaccharide chains. Hence, the alterations in glycan structure detected in bulk cellular glycoproteins of Ric^R cells are expressed also in cell surface glycoproteins and in fibronectin, a secreted glycoprotein.

BHK cell lines selected for resistance to ricin, the toxic principle of castor beans *Ricinus communis* (Meager *et al.*, 1975, 1976) bind the toxin poorly compared with sensitive parental cells. Ricin binding to cell surface carbohydrates is an essential first step in cytotoxicity (Olsnes & Pihl, 1976). Earlier, deficiencies in glycosyltransferases involved in assembly of asparagine-linked carbohydrate moieties (*N*-glycans) of glycoproteins were detected in several of the ricin-resistant (Ric^R) cell lines (Meager *et al.*, 1975; Vischer & Hughes, 1981). In

Abbreviations used: BHK, baby hamster kidney; P_i/NaCl , phosphate buffered saline. All sugars are of the D-configuration, except fucose which is L.

one case, Ric^R14 cells were shown to lack a specific UDP-*N*-acetylglucosamine:glycoprotein *N*-acetylglucosaminyltransferase I (Fig. 1a) but in general only quantitative changes in glycosyl transferase activities were detected.

In order to extend analysis of the biochemical defects in ricin-resistant cell lines we have studied the structures of *N*-glycans that accumulate in several mutants, including Ric^R14 cells. Cells have been labelled metabolically with radioactive mannose, a specific precursor of *N*-glycans, and the glycopeptides obtained by Pronase digestion have been fractionated by affinity chromatography on concanavalin A–Sephadex (Narasimhan *et al.*, 1979;

Fig. 1. Assembly of N-glycans

(a) Transfer of a large oligosaccharide to asparagine residues is followed by glycosidase 'processing' and elongation mediated by specific glycosyltransferases to produce biantennary and (b) more complex tri- and tetra-antennary glycans. All of the sialic acid and mannose residues are in α -glycosidic linkage except the boxed mannose residue. Galactose and N-acetylglucosamine residues are in β -glycosidic linkage.

Ogata *et al.*, 1975; Krusius *et al.*, 1976; Mattila & Renkonen, 1978) and lentil lectin–Sephacrose (Kornfeld *et al.*, 1981) columns. The carbohydrate specificities of these lectins are well established (Baenziger & Fiete, 1979a; Debray *et al.*, 1981; Kornfeld *et al.*, 1981) and provide a good indication of the structures of carbohydrate moieties binding to them under appropriate conditions. Abnormal *N*-glycan assembly is readily demonstrated in several Ric^R cell lines and in some cases these alterations can be correlated with the previously reported (Meager *et al.*, 1975; Vischer & Hughes, 1981) deficiencies in glycosyltransferases.

Materials and methods

Cells

BHK C13 cells were grown at 38°C in a modified Eagle's medium supplemented with 10% (v/v) foetal calf serum, penicillin and streptomycin (Meager *et al.*, 1976). Ricin-resistant cell lines Ric^R 14, 15, 17, 19 and 21 (Meager *et al.*, 1976) were grown similarly. Cell monolayers were trypsinized as described (Meager *et al.*, 1976).

Solutions

P_i/NaCl, pH 7.2, contained (per litre) NaCl (8 g), KCl (0.29 g), Na₂HPO₄ (1.159 g), KH₂PO₄ (0.29 g), 2 mM-EDTA and 0.02% NaN₃. Buffer A contained 10 mM-Tris/HCl, pH 7.5, 0.1 M-NaCl, 1 mM-MgCl₂, 1 mM-CaCl₂, 1 mM-MnCl₂ and 0.02% NaN₃. Buffer B contained 50 mM-Tris/HCl, pH 7.5, 10 mM-CaCl₂ and 0.02% NaN₃.

Metabolic labelling and preparation of glycopeptides

Cells were grown to confluency over 72 h in 100 mm diameter tissue culture plates at 38°C in medium (15 ml) containing D-[2-³H]mannose (20 µCi·ml⁻¹, 10.4 Ci·mmol⁻¹, Amersham International), or in some experiments, L-[5,6-³H]fucose (20 µCi·ml⁻¹, 45 Ci·mmol⁻¹, Amersham International). The culture fluids were collected after 72 h, adjusted to 1 mM-phenylmethanesulphonyl fluoride and 5 mM-EDTA, passed through Millipore 22 µm pore filters and immediately frozen at -20°C. The cell monolayers were rinsed several times with P_i/NaCl and the washed cells were scraped off into P_i/NaCl with a rubber policeman. After two washings by centrifugation at 3000 *g* for 5 min, each cell pellet (from one plate) was suspended in buffer B, pH 7.5 (0.5 ml). The mixtures were heated at 100°C for 5 min, cooled and incubated at 38°C with Pronase [50 µl, 0.4%, (w/v) in buffer B]. Fresh additions of Pronase (50 µl portions) were made every 12 h and incubation was continued for 40–72 h in total. The mixtures were then heated at 100°C for 5 min and centrifuged at 10000 *g* for 10 min. Portions of the supernatants were taken for radioactive counting and for chromatography.

Lectin affinity chromatography

Concanavalin A–Sephacrose (Pharmacia) was washed with buffer A and packed into small columns (1 cm × 10 cm) at room temperature. Samples (up to 0.5 ml, 10⁴–10⁶ c.p.m.) were applied and eluted at room temperature and approx. 8 ml·h⁻¹. Fractions (1 ml) were collected and portions taken for radioactive counting in 10 ml of Beckman scintillation fluid EP. Elution with buffer A was continued usually until fraction 25, then elution was started with 10 mM-α-methyl glucoside in buffer A and finally after fraction 50 with 500 mM-α-methyl mannose in buffer A. In the latter the eluting solution was immersed in a 60°C water bath.

Chromatography on a column (1 cm × 10 cm) of the lentil lectin–Sephacrose (Pharmacia) was at room temperature. Samples were applied and the column washed first with buffer A followed by 10 mM-α-methyl glucoside in buffer A and then 200 mM-α-methyl glucoside in buffer A. Fractions (1 ml) were collected and analysed as before.

Ion-exchange chromatography

DEAE-Sephacel (Pharmacia) was washed with 0.05 M-acetic acid and packed into a column (1 cm × 20 cm). Glycopeptide solutions (1 ml, 10⁴ c.p.m. approx., salt concentration less than 0.025 M) in 0.05 M-acetic acid were applied to the column and washed into the gel with water (2 ml) at 2°C (Serafini-Cessi & Campadelli-Fiume, 1981). After 1 h at 2°C the column was washed with water (7 ml) and then with a linear gradient of NaCl (50 ml of 0.5 M-NaCl in 0.05 M-acetic acid and 50 ml of 0.05 M-acetic acid). Fractions (1 ml) were eluted at approx. 8 ml·h⁻¹ and their radioactivity was measured.

Gel filtration

Glycopeptide samples (0.5 ml, 10⁴–10⁵ c.p.m. applied to a Sephadex G-25 (Pharmacia) column (1.6 cm × 30 cm) were eluted with P_i/NaCl at room temperature and approx. 8 ml·h⁻¹. Fractions (1 ml) were collected and counted for radioactivity. Chromatography on a column (1.2 cm × 77 cm) of Bio-gel P-4, 200–400 mesh (BioRad) was at 2°C. Glycopeptide solutions (0.05 ml, 10⁴–10⁵ c.p.m.) were applied and eluted at 6 ml·h⁻¹ with 0.1 M-pyridine acetate, pH 6 (8 ml of pyridine and 5 ml of 1 M-acetic acid diluted to 1 litre with water). Fractions (1 ml) were collected and counted for radioactivity. Routinely the [³H]glycopeptide samples (usually 0.5 ml) were mixed with Blue Dextran (25 µl) and [¹⁴C]glycopeptide V of ovalbumin (see below) and [¹⁴C]glucosamine (approx. 1000 c.p.m. of each). Appropriate peak fractions were pooled, freeze dried and dissolved in water.

Preparation of fibronectin

The culture fluids collected from cells labelled metabolically with [^3H]mannose as described above were applied to a column (10 ml) of gelatin-Sepharose (Pena *et al.*, 1980). The column was washed with P_i/NaCl . Fractions (1 ml) were collected and portions (0.1 ml) used to determine the level of radioactivity. When the radioactivity of the eluate reached background the column was washed with 8 M-urea in the above buffer. Peak fractions containing radioactive fibronectin were pooled and dialysed against water overnight. The fraction was then freeze dried, dissolved in buffer B (0.5 ml) and treated with Pronase as before.

Enzymes and glycopeptide standards

Jack bean glycosidases, α -mannosidase, β -galactosidase and β -*N*-acetylglucosaminidase were purified as described (Li & Li, 1972). In addition, jack bean α -mannosidase was obtained from Boehringer or Calbiochem-Behring. In all cases 1 unit of enzyme activity represents 1 μmol of substrate hydrolysed/min under assay conditions.

Glycopeptide fractions were obtained from ovalbumin (Sigma) and purified by ion-exchange chromatography (Huang *et al.*, 1970). Fraction V was purified further by chromatography on concanavalin A. Glycopeptide V (250 μg of total carbohydrate) was labelled radioactively by incubation on ice for 90 min with [^{14}C]acetic anhydride (20 μl , Amersham International) in 0.1 M- NaHCO_3 in a final volume of 0.15 ml. The reaction mixture was passed through a Sephadex G-25 column (1.9 cm $\times$ 25 cm) in 0.1 M-pyridine acetate and the glycopeptide fraction, well separated from free acetic acid, was freeze dried, dissolved in buffer A (0.2 ml) and passed through concanavalin A-Sepharose as described above. The majority of the radioactivity was recovered in fractions eluted with 500 mM- α -methyl mannoside. Peak fractions were pooled and represent the major oligomannosidic glycopeptide V, the structure of which has been thoroughly characterized as $\text{Man}_5\text{GlcNAc}_2\text{Asn}$ (Kobata, 1979).

Enzymic and chemical treatments of glycopeptides

Glycopeptides dissolved in 0.1 M-sodium acetate (pH 5.0)/0.3 mM- ZnSO_4 (0.4 ml) were incubated at 38°C with jack bean α -mannosidase (50 μl , 2 units) for 24 h. Fresh portions (25 μl) of enzyme were added every 24 h and the incubation was continued for 72 h. In some experiments glycopeptide fractions were incubated in 0.05 M-sodium phosphate buffer pH 7 (0.5 ml) with a mixture of neuraminidase (0.1 unit), β -galactosidase (0.5 unit) and β -*N*-acetylglucosaminidase (1.8 units) at 38°C for 48 h. These mixtures were then freeze dried, dissolved in 0.1 M-sodium acetate (pH 5)/0.3 mM- ZnSO_4 (0.5 ml) and

digested further with α -mannosidase as described before. Treatment of glycopeptides with neuraminidase alone was carried out in 0.1 M-sodium acetate, pH 5, (0.2 ml) containing 0.1 M- NaCl , 1 mM- MgCl_2 , 1 mM- CaCl_2 and 1 mM- MnCl_2 . Incubation at 38°C was for 24 h.

β -Elimination of carbohydrate groups linked glycosidically to serine or threonine residues was obtained by treatment (Carlson, 1968) in 1 ml of 0.8 M- NaBH_4 in 0.1 M- NaOH at 38°C for 48–72 h. The mixtures were diluted to 5 ml, adjusted to pH 5 by addition of 1 M-acetic acid and freeze dried. Periodate oxidation of glycopeptides was carried out at room temperature for 16 h with 0.1 M-sodium periodate in 50 mM-sodium acetate, pH 5, in a final volume of 200 μl . Excess periodate was destroyed by addition of glycerol (10 μl , 1 M). After 2 h at room temperature carrier mannose (20 μl of a 5 mg $\cdot$ ml $^{-1}$ solution) was added, and the samples were hydrolysed at 100°C for 4 h after addition of an equal volume of 8 M-HCl. The mixtures were diluted to 5 ml with water, passed through Amberlite MB-3 mixed bed resin (approx. 5 ml volume) and the material eluted with water (10 ml) was freeze dried. The product was dissolved in water (50 μl) and subjected to chromatography on Whatman 3MM paper in pyridine/ethyl acetate/acetic acid/water (5:5:1:3, by vol.) overnight. The mannose-containing region of the paper, revealed by AgNO_3 staining (Trevelyan *et al.*, 1950) of parallel tracks, was eluted with water and counted for radioactivity.

Results

Glycopeptides of BHK cells

Glycopeptides prepared from BHK cells metabolically labelled with [^3H]mannose were fractionated by chromatography on a column of concanavalin A-Sepharose (Fig. 2a). The pattern of fractionation was very reproducible and the radioactivity applied was usually recovered quantitatively from the column. No additional radioactivity was eluted from the column with 1% sodium dodecyl sulphate or with 0.1 M-HCl. Four major glycopeptide classes were obtained (for evidence see later Discussion). Fraction A passed unretarded through the column and represents at least in part tri- (Fig. 1b, Tri I and Tri II) and tetra- (Fig. 1b) antennary complex *N*-glycans. Fraction B was bound weakly to the column and was eluted with 10 mM- α -methyl glucoside: it represents bi-antennary complex glycans (Fig. 1a). The most tightly bound material was eluted from the column with 500 mM- α -methyl mannoside and typically showed a skewed peak with extensive trailing. The material so eluted was divided into a main fraction C and the trailing fraction D. Both fractions represent oligomannosidic glycans (Fig. 1a).

Fig. 2. *Chromatography of [^3H]mannose-labelled glycopeptides on concanavalin A-Sepharose* Cellular glycopeptides produced by Pronase digestion were applied to the column and eluted with Tris buffer, pH 7.5, followed by 10 mM α -methyl glucoside/Tris buffer and 500 mM α -methyl mannoside/Tris buffer (arrows). Fractions 10–13, 32–42, 62–68 and 69–81 were pooled separately (A–D).

The identities of the fractions A–D separated by concanavalin A-Sepharose chromatography were examined further by using [^3H]fucose-labelled glycopeptides. Fucose substituents found on *N*-glycans of the complex type occupy sites either in the core

di-*N*-acetylchitobiosyl sequence, where it is substituted at C-6 of the *N*-acetylglucosamine residue attached to asparagine, or at peripheral sites on galactose or *N*-acetylglucosamine residues (Montreuil, 1980). [^3H]Fucose-labelled glycopeptides

were separated on concanavalin A–Sephadex into a major unretarded fraction and a minor fraction coincident with the [^3H]mannose-labelled peak B. By contrast the oligomannosidic fraction C was not labelled (Fig. 3a). The [^3H]fucose-labelled peak B from concanavalin A–Sephadex was excluded from Sephadex G-25 (Fig. 3c) and was completely retarded on a lentil lectin column (Fig. 3d). The specificity of lentil lectin requires a core fucose-(α 1-6)-*N*-acetylglucosamine as well as peripheral *N*-acetylglucosamine residues (Kornfeld *et al.*, 1981). Since [^3H]mannose-labelled peak B from concanavalin A–Sephadex was also quantitatively retained on a lentil lectin column (results not shown) we conclude that all of the biantennary complex *N*-glycans of BHK cells carry the core fucosyl substituent.

The [^3H]fucose-labelled glycopeptide fraction A from concanavalin A–Sephadex (Fig. 3a) is clearly heterogeneous. Gel filtration on Sephadex G-25 gave a major excluded peak, two included peaks and a very small amount of free fucose (Fig. 3b). The majority of the [^3H]fucose was present on alkali-stable *N*-glycans (Fig. 3b). When the alkali-stable major peak excluded from Sephadex G-25 was passed through a lentil lectin affinity column a small but significant proportion of the radioactivity was retained and was eluted by α -methyl glucoside (Fig. 3d). The majority (approx. 70%) of the radioactive glycopeptides were eluted unretarded from the column. The unadsorbed glycopeptides may represent complex *N*-glycans carrying fucose substituents on peripheral sequences, or alternatively tetra-antennary glycans (Fig. 1b) or tri-antennary glycan

Fig. 3. Chromatography of [^3H]fucose-labelled BHK cell glycopeptides

(a) Chromatography on concanavalin A. (b) Chromatography of fraction A from (a) on Sephadex G-25 before (solid line) or after (broken line) treatment with alkaline borohydride. (c) Chromatography of fraction B from (a) on Sephadex G-25. (d) Chromatography on lentil lectin–Sephadex of alkali-treated fraction A (broken line) and fraction B (solid line). The high molecular weight fractions separated on Sephadex G-25 [column fractions 21–29 in (b) and (c) respectively] were used. The arrows indicate elution with 10mM- and 200mM- α -methyl glucoside respectively.

carrying a 2- and 4-*O*-linked mannose unit (Fig. 1*b*, Tri II). According to Kornfeld *et al.*, (1981) the tri-antennary glycan Tri I (Fig. 1*b*) retains affinity for lentil lectin. It presumably represents the material adsorbed to the column in our experiments. Very similar results were obtained by affinity chromatography on lentil lectin of [^3H]mannose-labelled glycopeptide fraction A from concanavalin A-Sepharose, suggesting that the majority of the tri-antennary *N*-glycan Tri I (Fig. 1*b*) carry core fucose substituents.

As shown in Fig. 4, peaks A and B from concanavalin A-Sepharose are negatively charged due to sialic acid substituents. The net negative charge of glycopeptides present in peak A appears greater than that of peak B as shown by the higher salt concentration required to elute the material from DEAE-Sephacel. Fraction B appears to be hetero-

geneous; the major part was eluted at a significantly lower salt concentration than peak A indicating the presence of partially sialylated bi-antennary glycans in the fraction. After removal of sialic acid with neuraminidase both fractions A and B were eluted by a low salt concentration (Figs. 4*b* and 4*d*) and in a position identical with that found on DEAE-Sephacel chromatography for the oligomannosidic fractions C and D (results not shown).

Confirmation of the structural assignments for fractions A–D from concanavalin A-Sepharose was obtained by the experiments shown in Fig. 5. Using [^3H]mannose-labelled glycopeptides, chromatography on Bio-Gel P-4 showed that fraction A was of highest molecular weight and was eluted (Fig. 5*a*) just after a Blue Dextran marker for the totally excluded volume of the column. Fraction B was eluted (Fig. 5*c*) slightly later, indicating a smaller

Fig. 4. DEAE-Sephacel chromatography of [^3H]mannose-labelled glycopeptides

Normal BHK cells: fraction A from concanavalin A-Sepharose (a, b); fraction B from concanavalin A-Sepharose (c, d). Ric^R14 cells: fraction A from concanavalin A-Sepharose (e, f). The fractions were applied in Tris buffer/0.025 M-NaCl before (a, c, e) or after (b, d, f) treatment with neuraminidase. The points at which a linear NaCl gradient (broken line) was started are indicated by the arrows.

Fig. 5. Bio-Gel P-4 chromatography of [^3H]mannose-labelled glycopeptides of BHK cells

Fractions A (a, b), B (c, d) and C (e, f) isolated by chromatography on concanavalin A-Sepharose (Fig. 2a) were analysed by gel filtration with (b, d, f) or without (a, c, e) prior treatment with glycosidases. In (f) α -mannosidase was used and in (b, d) a mixture of α -mannosidase, β -galactosidase, β -N-acetylglucosaminidase and neuraminidase was used. Co-chromatography of ^{14}C -labelled ovalbumin glycopeptide and [^{14}C]glucosamine is indicated by the broken line (e) and the arrows (a-d, f). The void volume of the column was determined by the elution of Blue dextran (BD). Fractions pooled for further analysis are indicated (A1, A2, B1, B2).

molecular weight but significantly larger than the oligomannosidic peak C (Fig. 5e) or peak D (results not shown). When the peak fractions isolated by Bio-Gel P-4 chromatography of fractions A and B were treated with α -mannosidase the profiles of radioactivity obtained by re-chromatography on Bio-Gel P-4 was unchanged. However, radioactive mannose was released from the fractions after incubation with a mixture of neuraminidase, β -galactosidase, β -N-acetylglucosaminidase and then

by α -mannosidase (Figs. 5b and 5d). Essentially all of the radioactivity was removed from the glycopeptides of relatively high molecular weight and two new major radioactive peaks were found. The most retarded peak of radioactivity (A2 and B2) was identified as free [^3H]mannose by paper chromatography. A smaller peak (A1 and B1) was eluted just ahead of the free mannose and represents the end product of glycosidase degradation namely, $\text{Man}(\beta 1-4)\text{GlcNAc}(\beta 1-4)[\pm \text{Fuc}(\alpha 1-6)]\text{GlcNAc}$

Asn. This identification was supported by the results of periodate oxidations. Fraction A1 from glycosidase-treated peak A (Fig. 5b) was freeze dried and dissolved in water (0.2 ml). One portion (0.1 ml, 3350 c.p.m.) was treated with sodium metaperiodate followed by NaBH_4 and subjected to strong acid hydrolysis. In parallel, 0.1 ml of the untreated fraction was also hydrolysed. After paper chromatography of the hydrolysates 2790 c.p.m. were recovered as mannose in the untreated portion and only 395 c.p.m. were recovered as unchanged mannose in the oxidized and reduced material. Periodate oxidation would be expected to degrade mannose residues with free *cis*-glycol groups as in a terminal β -mannose unit of the theoretical end product of glycosidase treatment of complex *N*-glycans.

The ratio of radioactivity in free mannose (A2 and B2) to the resistant end products (A1 and B1) was 60% and 56% for fractions A and B respectively. Assuming a core sequence for complex *N*-glycans (Fig. 1):

complete removal of α -mannosyl residues by glycosidase treatment represents 66% of total radioactivity, in reasonable agreement with the experimentally determined values.

Treatment of [^3H]mannose-labelled fraction C from concanavalin A-Sepharose (Fig. 2a) with α -mannosidase alone released 85–89% of the total radioactivity as free mannose with production of a small fraction with the properties expected for the end product of glycosidase digestion, $\text{Man}(\beta 1-4)\text{GlcNAc}(\beta 1-4)\text{GlcNAc-Asn}$ (Fig. 5f). The untreated fraction C was eluted from Bio-Gel P-4 in the region at which authentic glycopeptide $\text{Man}_5\text{GlcNAc}_2\text{Asn}$ from ovalbumin was eluted. However, a substantial part of the radioactivity was eluted before the ovalbumin glycopeptide (Fig. 5e) indicating a structure containing more than five mannose residues. This conclusion is consistent with the proportion of mannose released by α -mannosidase treatment. The theoretical value for $\text{Man}_5\text{GlcNAc}_2\text{Asn}$ is 4 residues/mol or 80%.

It should be noted that chromatography of charged glycopeptides on Bio-Gel P-4 may not accurately reflect their relative size. This is shown by the difference in elution of mannose and glucosamine (see Figs. 5b, 5d and 5c). Furthermore, the end product of α -mannosidase treatment of ovalbumin glycopeptide V labelled by [^{14}C]acetic anhydride on the primary amino group of asparagine was eluted ahead (peak fractions 77–80) of the $\text{ManGlcNAc}_2\text{Asn}$ product obtained from the BHK glycopeptides.

Concanavalin A-Sepharose chromatography of mutant cell glycopeptides

Ricin-resistant cell lines Ric^R 14, 15, 17, 19 and 21 were labelled metabolically with [^3H]mannose and the glycopeptides obtained by Pronase digestion were passed through concanavalin A-Sepharose under the standard conditions (Figs. 2b–2f). Marked differences in the patterns of elution of glycopeptides were found in comparison with the results described previously for the glycopeptides of parental, ricin-sensitive BHK cells. In each ricin-resistant cell line the proportion of radioactivity recovered in the biantennary complex glycan fraction B was greatly reduced, particularly in the Ric^R 15 cell line (Fig. 2d). The radioactive peak A that was eluted unretarded from the column was also significantly reduced, particularly in the Ric^R 14, 15 and 19 cell lines. The majority of the radioactive glycopeptides was recovered in fractions C and D, i.e. oligomannosidic glycans.

In general the glycopeptide profiles obtained by chromatography on concanavalin A-Sepharose suggests that in the Ric^R cells *N*-glycan assembly (Fig. 1a) is defective at stages subsequent to the transfer of the precursor oligosaccharide $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ to protein and prior to the conversion of these glycans into bi-, tri-, and tetra-antennary complex glycans.

Glycopeptides of Ric^R 14 cells

This conclusion was confirmed by further analysis of the glycopeptides of one ricin-resistant cell line, Ric^R 14. Analysis of other Ric^R cell lines will be described elsewhere.

Fraction C (Fig. 2b) representing 81% of the total radioactivity recovered from concanavalin A-Sepharose chromatography of Ric^R 14 cell glycopeptides was eluted from a calibrated Bio-Gel P-4 column in a major peak almost coincident with the ovalbumin glycopeptide but skewed to a lower molecular weight range (Fig. 6b). After treatment with α -mannosidase, the radioactive glycopeptides were degraded to free mannose and the fraction previously described as the end product of α -mannosidase digestion, $\text{Man}(\beta 1-4)\text{GlcNAc}(\beta 1-4)\text{GlcNAc-Asn}$. The amount of free mannose was 72–76% of the total, values in reasonable agreement with the value (80%) expected for complete α -mannosidase digestion of $\text{Man}_5\text{GlcNAc}_2\text{Asn}$. The lower value may indicate the presence of glycopeptides containing fewer α -mannose residues or incomplete digestion. We favour the former explanation in view of the shape of the elution profile of fraction C compared with that of the ovalbumin glycopeptide. Similar results were obtained for fraction D (Fig. 6c) although the glycopeptides in this minor fraction (3% of total radioactivity) migrated ahead of the marker ovalbumin glyco-

Fig. 6. Bio-Gel P-4 chromatography of [^3H]mannose-labelled glycopeptides of Ric^R14 cells. Fractions A (a), C (b) and D (c) from concanavalin A-Sepharose (see Fig. 2b) were chromatographed with (○) or without (●) prior treatment with α -mannosidase; see Fig. 5 for other details.

peptide indicating more than five mannosyl residues per mol. However, α -mannosidase treatment released only about 70% of the radioactivity as free mannose for reasons that are not understood.

Fraction A (Fig. 2b) representing 11% of the total radioactivity recovered from concanavalin A-Sepharose was clearly not equivalent to the glycopeptides unretarded on the column during chromatography of normal BHK cell glycopeptides. Peak A from the Ric^R14 glycopeptides was neutral as shown by chromatography on DEAE-Sephacel (Fig. 4e) and was unchanged by neuraminidase treatment (Fig. 4f). Bio-Gel P-4 chromatography showed that the radioactive components in this fraction were

Fig. 7. Chromatography on concanavalin A-Sepharose of [^3H]mannose-labelled surface glycopeptides of BHK cells (a) and Ric^R15 cells (b).

Large glycopeptides obtained from metabolically labelled cells by mild trypsinization were digested with Pronase and applied separately to the column. See Fig. 2 for details.

exclusively of low molecular weight (Fig. 6a), including free mannose and material migrating as $\text{Man}(\beta 1-4)\text{GlcNAc}(\beta 1-4)\text{GlcNAc-Asn}$. Too little material was available to analyse these products further.

Surface glycopeptides of BHK cells and ricin-resistant mutants

The finding of significant differences between the cellular glycopeptides of BHK cells and ricin-resistant cell lines revealed by chromatography on concanavalin A-Sepharose prompted us to examine whether any similar alterations were expressed in the carbohydrate structures of cell surface glycoproteins of the mutant cells. Fig. 7 shows the concanavalin A-Sepharose chromatography of [^3H]mannose-labelled glycopeptides obtained from cell surface material released by mild trypsinization. The pattern obtained for normal BHK cells (Fig. 7a) was similar to the fractionation of the total cellular glycopeptides (Fig. 2a). However, fraction C was quantitatively diminished relatively to the complex glycan fractions A and B, suggesting that glycoproteins expressed at the cell surface are enriched in fully assembled complex *N*-glycans. By contrast, the

Fig. 8. Glycopeptides of fibronectins isolated from the culture medium of BHK cells (a, c, d), Ric^R15 cells (b, broken line) or Ric^R21 cells (b, solid line)

Cells were labelled metabolically with [³H]mannose (a, b) or [³H]fucose (c, d) and fibronectin was isolated from the medium by gelatin-Sepharose chromatography. Glycopeptides obtained by Pronase digestion were applied to concanavalin A-Sepharose (a, b, c) or to lentil lectin-Sepharose (d). In (d) the chromatography of [³H]fucose-labelled peaks A (broken line) or B (solid line) from concanavalin A-Sepharose chromatography are shown. The arrows indicate the points at which elution with sugar haptens was started. See Figs. 2 and 3 for details.

surface glycopeptides released from Ric^R14 and 15 cells were largely of the oligomannosidic type. The patterns obtained for Ric^R15 cells (Fig. 7b) and for Ric^R14 cells (results not shown) were very similar to those obtained using total cellular glycopeptides (Figs. 2b and 2d).

Glycopeptides of fibronectin

Fibronectin secreted by cells including BHK cells and the ricin-resistant mutants (Pena & Hughes, 1978) contains several asparagine-linked carbohydrate chains (Fukuda & Hakomori, 1979). The alterations in *N*-glycan structure found in the bulk cellular glycopeptides of the Ric^R mutant cells were also expressed in the *N*-glycans of fibronectin. The [³H]mannose-labelled (Fig. 8a) or [³H]fucose-labelled (Fig. 8c) glycopeptides of fibronectin secreted by normal BHK cells consisted primarily of material that passed unretarded through concanavalin A-Sepharose, presumably tri- and tetra-antennary complex chains, and bi-antennary chains

that were eluted with 10mM- α -methyl glucoside. Essentially all of the latter were adsorbed to a lentil lectin affinity column (Fig. 8d) showing substitution of the asparagine-linked *N*-acetylglucosamine with fucose. Approx. 20% of the [³H]mannose- or [³H]fucose-labelled material unretarded on concanavalin A was retained by the lentil lectin column and presumably represents fucosylated tri-antennary chains. By contrast, the *N*-glycans of Ric^R15 or Ric^R21 cell fibronectins appeared to be almost entirely of the oligo-mannosidic type (Fig. 8b).

We conclude that the defects of *N*-glycan assembly present in ricin-resistant cell lines are expressed in the carbohydrate structures of a secreted glycoprotein.

Discussion

The recently determined specificities of concanavalin A (Baenziger & Fiete, 1979a; Ogata *et al.*, 1975; Narasimhan *et al.*, 1979; Krusius *et al.*, 1976;

Mattila & Renkonen, 1978) and lentil lectin (Kornfeld *et al.*, 1981) for glycopeptides have provided a powerful tool for the preliminary classification of the *N*-glycan structure of glycoproteins available in small amounts.

The major oligosaccharides which accumulate in the cellular glycoproteins of parental BHK cells are complex chains: biantennary *N*-glycans which appear to be fully fucosylated in the core region, tri-antennary chains and tetra-antennary chains. The bi-antennary chains represent approx. 45% of the total complex chains, in good agreement with previous data (Takasaki *et al.*, 1980). The presence of oligomannosidic *N*-glycans in BHK cellular glycoproteins presumably represents in part nascent glycoproteins not fully processed into complex *N*-glycans. Thus, these chains are featured less prominently in secreted fibronectin, the *N*-glycans of which are predominantly of the bi-antennary type with smaller amounts of more highly branched *N*-glycans. The structure of the glycans of hamster fibronectin as determined by others (Fukuda & Hakomori, 1979; Carter & Hakomori, 1979) is consistent with the present results, although the existence of tri- and tetra-antennary chains has not previously been recognized for fibronectin. Complex *N*-glycans also predominate in the glycopeptides released from the BHK cell surface by mild trypsinization. However, a significant amount of oligomannoside chains are clearly present at the cell surface. This finding may have functional significance, since mannose-binding lectins have been identified at the surface of cells (Grabel *et al.*, 1979) including BHK cells (D. Stojanovic & R. C. Hughes, unpublished results) and BHK cells adhere to inert surfaces coated with concanavalin A (Aplin & Hughes, 1981) and α -mannosidase (Rauvala & Hakomori, 1981), reagents binding preferentially to oligomannosidic *N*-glycans.

The major finding reported here is that five ricin-resistant BHK cell lines (Meager *et al.*, 1976) show significant alterations in *N*-glycan structure of cellular glycoproteins including those present at the cell surface and in cell secretions. This result is consistent with deficiencies (Meager *et al.*, 1975; Vischer & Hughes, 1981) in glycosyltransferases involved in *N*-glycan assembly present in these cells. However, studies using various glycopeptide acceptors in general have not established unequivocally the exact biochemical lesion leading to ricin resistance. Many glycopeptide substrates used in enzymic analysis may serve as acceptors for several glycosyltransferases (Schachter *et al.*, 1978). Hence a complete block in any one step in *N*-glycan assembly is difficult to demonstrate. The present results show unequivocally that enzymic blocks must occur in the ricin-resistant cell lines at steps prior to the assembly of bi-, tri- or tetra-antennary

complex *N*-glycans (Fig. 1a). This conclusion is consistent with the greatly reduced ricin binding to these cells (Meager *et al.*, 1975; Rosen & Hughes, 1977).

Ricin binds to galactose-containing sequences present in complex *N*-glycans (Baenziger & Fiete, 1979b). The preferred structures for maximum binding are tri- and tetra-antennary chains and bi-antennary chains. For Ric^R14 cells an enzyme, *N*-acetylglucosaminyltransferase I, catalysing the first step in conversion of oligomannosidic chains into complex chains (Fig. 1a, step B) is missing (Meager *et al.*, 1975; Vischer & Hughes, 1981). Consequently, the oligosaccharide chains which accumulate in the glycoproteins of Ric^R14 cells are predominantly the preferred acceptor for this enzyme, namely Man₅GlcNAc₂ (Schachter *et al.*, 1978). Similar results have been reported (Li & Kornfeld, 1978) for ricin-resistant Chinese hamster ovary cell line 15B which also lacks *N*-acetylglucosaminyltransferase I. In addition, chains of lower molecular size containing fewer α -mannosyl residues accumulate in Ric^R14 cells. Schachter *et al.* (1978) have shown that the action of *N*-acetylglucosaminyltransferase I triggers the removal of two α -mannosyl residues (Fig. 1a, step C) to produce the preferred acceptor for *N*-acetylglucosaminyltransferase II (Fig. 1a, step D). The present results suggest that the processing α -mannosidase involved in these steps can act to remove one or two mannose residues even in the absence of *N*-acetylglucosaminyltransferase I. It is possible however that this represents an abnormal metabolic pathway resulting from an accumulation of the Man₅GlcNAc₂ intermediate in Ric^R14 cells. It appears from the present results that some further degradation of the intermediate occurs in Ric^R14 cells to produce small oligosaccharides, possibly even the core sequence ManGlcNAc₂ with no affinity for concanavalin A. Presumably this degradation occurs in lysosomes and is not directly relevant to normal processing reactions occurring during *N*-glycan assembly.

The exact biochemical lesions in other ricin-resistant BHK cells remain to be determined. For example, the Ric^R15 cell line clearly is abnormal in *N*-glycan assembly. Oligomannosidic chains accumulate and complex chains are virtually absent. Nonetheless, *N*-acetylglucosaminyltransferase I is present in these cells although at a somewhat reduced level compared with normal BHK cells. *N*-Acetylglucosaminyltransferase II and galactosyltransferase activities approach wild type levels (Vischer & Hughes, 1981). The enzymic analysis gives no clue therefore to the reason for the block in *N*-glycan assembly. On the basis of previous data and the present results one can conclude that *N*-glycan assembly in these cells is prevented at an

early step, e.g. in one or more of the processing steps leading to the Man₅GlcNAc₂ intermediate (Fig. 1a). Detailed analysis of the glycans accumulating in these and other Ric^R cells should define more exactly the biochemical lesions resulting in altered carbohydrate structures and loss of ricin binding sites at the cell surface.

In conclusion, we have correlated the previously known loss of ricin-binding sites on the surface of the ricin-resistant cell lines with alterations in N-glycan structure. The direct demonstration of altered carbohydrate chains on the surface of these cells is of interest in regard to their reduced adhesiveness (Edwards *et al.*, 1976; Hughes *et al.*, 1979) and provides some support for models implicating carbohydrate specificity in adhesive interactions.

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