Hybrid sialylated *N*-glycans are minor constituents of normal BHK-cell glycoproteins and a prominent feature in glycoproteins of some ricin-resistant cell lines

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Baby-hamster kidney (BHK) cells were labelled metabolically by growth in media containing radioactive sugars and the asparagine-linked glycopeptides (*N*-glycans) obtained by Pronase digestion of disrupted cells were fractionated by chromatography on concanavalin A–Sephadex. About 2–3% of the total [³H]galactose- or [³H]fucose-labelled glycopeptides were found to be bound tightly to the lectin column and were eluted with 500 mM-methyl α -mannoside. Further analysis of these minor components by chromatography on Bio-Gel P4, lentil-lectin–Sephadex and DEAE–Sephadex and sensitivity to α -mannosidase indicates the presence in BHK-cell glycopeptides of hybrid structures of the following form:

Similar structures were identified as major features of the glycoproteins of ricin-resistant mutants Ric^R17 and Ric^R19 as described previously for Ric^R21 cells [Hughes, Mills & Stojanovic (1983) *Carbohydr. Res.* **120**, 215–234]. The Ric^R15 cell line also produces significant amounts of hybrid *N*-glycans. The studies show that the novel *N*-glycans accumulating in ricin-resistant mutants are derived by a metabolic pathway that exists to a minor extent in normal BHK cells.

In the conversion of the asparagine-linked oligosaccharides (*N*-glycans) of glycoproteins from the high-mannose type into complex chains, a pentamannosyl product of the initial processing reactions is acted on by *N*-acetylglucosaminyl-transferase I to attach an *N*-acetylglucosamine residue, leading to removal of two additional mannose units catalysed by mannosidase II and attachment of fucose to an internal *N*-acetylglucosamine residue (Struck & Lennarz, 1980; Schachter *et al.*, 1983; Hughes, 1984). Recently the resulting *N*-glycan (Fig. 1a) has been defined as a structure underlying the carbohydrate units of glycoproteins accumulating in a ricin-resistant mutant of BHK cells, the Ric^R21 cell line (Hughes & Mills, 1983; Hughes *et al.*, 1983; Gleeson *et al.*, 1984). Elongation of the α 1 $\rightarrow$ 3-linked arm (Fig. 1a) by sequential attachment of galactose and sialic acid produces the structure shown in Fig. 1(b). A more highly branched oligosaccharide (Fig. 1c)

results from the action of *N*-acetylglucosaminyl-transferase IV, an enzyme that specifically attaches *N*-acetylglucosamine to C-4 of the α 1 $\rightarrow$ 3-mannose unit of the core sequence (Gleeson & Schachter, 1983) followed by chain elongation. These structures are called hybrid sialylated *N*-glycans in the present paper since they show features of both complex (i.e. the sialylated *N*-acetyl-lactosamine sequence) and oligomannosidic (i.e. terminal unsubstituted α -mannosyl residues) *N*-glycans. The hybrid structures account for the small degree of binding of ricin exhibited by Ric^R21 cells (Meager *et al.*, 1975, 1976; Rosen & Hughes, 1977), since 3-*O*-substituted galactose residues (Baenziger & Fiete, 1979b) have low affinity for *Ricinus* lectins.

In normal BHK cells the intermediate (Fig. 1a) is a substrate for *N*-acetylglucosaminyltransferase II, which attaches *N*-acetylglucosamine to the α 1 $\rightarrow$ 6-linked mannose unit of the core sequence.

Fig. 1. Structures of asparagine-linked oligosaccharides

(a) A product of *N*-acetylglucosaminyltransferase I; (b) and (c) the major glycopeptides of Ric^R21 cells with (b) or without (c) affinity for concanavalin A and separated by lectin affinity chromatography. The latter structures (b and c) are predominantly as shown, but minor derivatives lacking fucose or sialic acid residues are also present.

This reaction leads after chain elongation to biantennary complex *N*-glycans:

where R = NeuAcα2-3 (or 6)Galβ1-4GlcNAcβ1-2. The intermediate may also be modified further by branching on one or both of the α-mannose units initiated by *N*-acetylglucosaminyltransferases IV and V followed by attachment of galactose and sialic acid (Schachter *et al.*, 1983). The presence of terminal galactose or 6-*O*-substituted galactose residues in bi-, tri- and tetra-antennary complex *N*-glycans presumably accounts for the binding of ricin to normal BHK cells (Baenziger & Fiete, 1979b). These structures are not detectable in Ric^R21-cell glycoproteins (Gleeson *et al.*, 1984).

In the present paper we have asked whether the novel *N*-glycans detected in Ric^R21 cells (Fig. 1b and 1c) represent the products of a pathway unique to these cells or rather are derived from a minor reaction sequence present in BHK cells and accentuated in the mutant cells by selection with ricin. We show that BHK-cell glycoproteins do contain very small amounts of hybrid *N*-glycans similar to those present in Ric^R21 cells. Secondly, we show the presence of hybrid *N*-glycans as a significant feature of the glycoproteins of other ricin-resistant mutants.

Materials and methods

Materials

D-[2-³H]Mannose (10.4 Ci/mmol), L-[5,6-³H]-fucose (45 Ci/mmol), D-[U-¹⁴C]glucosamine (213 mCi/mmol) and D-[6-³H]galactose (25 Ci/mmol) were from Amersham International. Jack-bean α-mannosidase was from Calbiochem-Behring, and β-galactosidase was purified as described by Li & Li (1972). *Vibrio cholerae* neuraminidase was from Calbiochem and Pronase from Sigma Chemical Co. A standard glycopeptide product (see Fig. 1a) synthesized from UDP-*N*-acetyl[³H]-glucosamine with purified bovine colostrum *N*-acetylglucosaminyltransferase I was kindly provided by Dr. H. Schachter, Hospital for Sick Children, Toronto, Ont., Canada. The product [³H]GlcNAc(Man)₃(GlcNAc)₂Asn (Fig. 1a) contained 66000 c.p.m./ml.

Cells and preparation of glycopeptides

BHK C13 cells and ricin-resistant mutants were grown in monolayer cultures in modified Eagle's medium supplemented with 10% (v/v) foetal-calf serum, penicillin and streptomycin (Meager *et al.*, 1976). For metabolic labelling, radioactive mannose (18.2 μCi/ml), galactose (18.2 μCi/ml), fucose (12.5 μCi/ml) or glucosamine (20 μCi/ml) was added to 24 h established non-confluent cultures in 100 mm-diameter dishes containing 10 ml of complete medium. The cells were then grown to confluency at 37°C over 2–3 days. The procedures

used to harvest the cells and to prepare glycopeptides by Pronase digestion were as described previously (Hughes & Mills, 1983).

Column chromatography

Glycopeptides (0.5 ml or less) were separated initially on a column (1 cm × 10 cm) of concanavalin A-Sepharose (Pharmacia) that had been equilibrated with 10 mM-Tris/HCl buffer, pH 7.5, containing 0.1 M-NaCl, 1 mM-MgCl₂, 1 mM-CaCl₂, 1 mM-MnCl₂ and 0.02% NaN₃ (Hughes & Mills, 1983). After a washing with buffer (24 ml), glycopeptides bound to the column were eluted with 10 mM-methyl α -glucoside (24 ml) followed with 500 mM-methyl α -mannoside (24–30 ml) heated to 60°C. Fractions (1 ml) were collected at about 12 ml/h. Samples (50–250 μ l) were counted for radioactivity in Beckman EP scintillation fluid. Selected fractions were pooled, freeze-dried and dissolved in water (0.7 or 1 ml). In some experiments analytical columns (2 ml bed volume) containing concanavalin A-Sepharose were used. Glycopeptide samples (0.1–0.5 ml) were applied and the columns were clamped for 1 h to allow complete adsorption. The columns were then washed sequentially with buffer (5 ml), 10 mM-methyl α -glucoside (9 ml) and 500 mM-methyl α -mannoside (9 ml). Fractions (1 ml) were collected into scintillation vials and mixed with Beckman EP scintillant (10 ml) for radioactivity counting. Chromatography of glycopeptide samples (0.5 ml) on a column (1 cm × 10 cm) of lentil-lectin-Sepharose (Pharmacia) was carried out initially with the above buffer (24 ml) followed by elution with 200 mM-methyl α -glucoside (24–30 ml) in the same buffer. Fractions (1 ml) were collected and treated as above. A column (1.2 cm × 72 cm) of Bio-Gel P4 (200–400 mesh) (BioRad Laboratories) was equilibrated with 0.1 M-pyridine/acetate buffer, pH 6. Glycopeptide samples (less than 0.5 ml) were applied and 1 ml fractions were eluted at 6 ml/h with pyridine/acetate buffer. Column fractions (1 ml) were collected and samples (0.1 ml) were mixed with Beckman EP scintillant (10 ml) for radioactivity counting. The column was calibrated in each run with Blue Dextran, ¹⁴C-labelled ovalbumin glycopeptide (Man)₅(GlcNAc)₂Asn and [¹⁴C]mannose (1000 c.p.m. each) as previously described (Hughes & Mills, 1983). Ion-exchange chromatography of glycopeptides diluted in 0.05 M-acetic acid (3 ml) on a column (1 cm × 30 cm) of DEAE-Sephacel (Pharmacia) was carried out (Hughes & Mills, 1983; Hughes *et al.*, 1983) by elution first with 0.05 M-acetic acid. After collection of ten 1 ml fractions the column was washed with a salt gradient (40 ml of 0.5 M-NaCl in 0.05 M-acetic acid and 40 ml of 0.05 M-acetic acid).

Identification of labelled sugars

Radioactive glycopeptides were hydrolysed in 2 M-HCl (0.1–0.25 ml), for 4 h at 100°C, dried under reduced pressure over NaOH, redissolved in water (50 μ l) and applied to Whatman 3MM paper. Standards of sugars (10 mM, 10 μ l each) were applied to parallel tracks and development was for 30 h in butan-1-ol/pyridine/0.1 M-HCl (5:3:2, by vol.). The sugar standards were located with alkaline AgNO₃ (Smith, 1960). The tracks containing the radioactive materials were cut into 1 cm segments, placed in scintillation vials and eluted with 1 ml of water for 1–2 h. Then Beckman EP scintillant (10 ml) was added, followed by counting for radioactivity. Under these conditions the migration of standard sugars was: galactose, 17 cm; glucose, 19.5 cm; mannose, 23 cm; fucose, 25.5 cm.

Glycosidase digests

Glycopeptide samples were dissolved in 0.1 M-sodium acetate buffer, pH 5, containing 0.3 mM-ZnSO₄ (0.1 ml), mixed with jack-bean α -mannosidase (20 μ l, 0.25 unit) and incubated at 37°C for 24 h. Fresh enzyme (20 μ l) was added every 24 h for up to 3 days. In some experiments glycopeptide incubation mixtures containing neuraminidase (0.1 unit) with or without β -galactosidase (0.16 unit) in 0.05 M-sodium phosphate buffer, pH 7, were kept at 37°C for 24 h. For subsequent α -mannosidase treatment, the incubation mixtures were freeze-dried, dissolved in 0.1 M-sodium acetate buffer, pH 5, containing 0.3 mM-ZnSO₄ (0.1 ml) and treated as above. Sialic acid was also removed from glycopeptide samples by heating at 80°C for 1 h in 0.05 M-HCl. The hydrolysates were freeze-dried, dissolved in buffer and subjected to glycosidase digestion as described above. Incubation mixtures were heated at 90°C for 5 min to inactivate enzymes before chromatography on concanavalin A-Sepharose.

Results

Isolation of radioactive glycopeptides

To detect hybrid N-glycans similar to those (Fig. 1) characterized previously (Hughes *et al.*, 1983; Gleeson *et al.*, 1984) in Ric^R21-cell glycoproteins, normal BHK cells and three ricin-resistant cell lines Ric^R15, Ric^R17 and Ric^R19 were grown in media containing radioactive sugars. Glycopeptides generated by Pronase digestion of cell pellets were then fractionated on concanavalin A-Sepharose. As Table 1 shows, the mutant cell lines contained a higher proportion of [³H]mannose-labelled glycopeptides (fraction C) binding tightly to the lectin column (72–85% of total compared with 43% for normal BHK cells). In Ric^R21-cell

Table 1. *Distribution of radioactivity in glycopeptide fractions obtained by chromatography on concanavalin A-Sepharose* BHK cells or ricin-resistant (Ric^R) cell lines were labelled by growth at 37°C for 2–3 days in the presence of the indicated sugar. Glycopeptide mixtures obtained by exhaustive proteolysis of disrupted cells with Pronase were each fractionated by affinity chromatography on concanavalin A-Sepharose as described in the text. Fractions A, B and C were eluted respectively with buffer, 10mM-methyl α -glucoside and 500mM-methyl α -mannoside.

Sugar	Cells	Radioactivity (% of total)			10 ⁻⁵ × Radioactivity (c.p.m.)	
		A	B	C	Applied to column	Recovered
[³ H]Mannose	BHK	41	16	43	3.9	3.5 (89%)
	Ric ^R 15	16	4	79	5.2	4.9 (95%)
	Ric ^R 17	25	3	72	5.8	5.3 (91%)
	Ric ^R 19	11	4	85	5.7	5.6 (97%)
	Ric ^R 21	21	3	74	2.5	2.1 (83%)
[³ H]Fucose	BHK	75	23	2	6.6	5.9 (89%)
	Ric ^R 15	46	2	52	3.5	2.6 (77%)
	Ric ^R 17	42	3	55	3.1	2.4 (77%)
	Ric ^R 21	35	1	64	3.3	2.4 (72%)
[³ H]Galactose	BHK	76	21	3	49	38 (78%)
	Ric ^R 15	74	3	23	43	34 (81%)
[¹⁴ C]Glucosamine	BHK	72	18	10	10.1	9.2 (91%)

glycopeptides, fraction C was shown (Hughes *et al.*, 1983; Gleeson *et al.*, 1984) to be a mixture of neutral high-mannose *N*-glycans, mainly a Man₆ species, and a hybrid sialylated structure (Fig. 1b). The branched hybrid *N*-glycan (Fig. 1c) was eluted (Hughes *et al.*, 1983) unretarded from the column in fraction A (Table 1). The concanavalin A-binding components were separated readily by Bio-Gel P4 chromatography, the hybrid being eluted ahead of the oligomannosidic glycopeptides (Hughes *et al.*, 1983). We performed a similar analysis of samples of [³H]mannose-labelled fraction C (Table 1) obtained from BHK and Ric^R15, Ric^R17 and Ric^R19 cells (35000, 62000, 35000 and 36000 c.p.m. respectively). A prominent peak (C1) was detected in the glycopeptides of Ric^R19 cells (Fig. 2a) and Ric^R17 cells (Fig. 2b), representing 39% and 31% respectively of total radioactivity recovered from the column. The glycopeptide fraction C of Ric^R15 cells (Fig. 2c) gave a pronounced shoulder, representing the C1 fraction (17% of total radioactivity). In each case the major peak (C2) of radioactivity was eluted in the region of a (Man)₅(GlcNAc)₂Asn glycopeptide standard (Figs. 2a–2c). In addition, a part of the [³H]mannose-labelled Ric^R19-cell glycopeptides was eluted in a peak (C3) of lower *M_r* (Fig. 2a).

After treatment of samples of the C2 and C3 fractions (approx. 2500 c.p.m.) with α -mannosidase followed by re-chromatography on Bio-Gel P4, over 90% of the radioactivity was recovered at the total volume of the column, showing the presence of oligomannosidic *N*-glycans in these glycopeptide fractions.

Peak C1 was not noticeable during chromato-

graphy of [³H]mannose-labelled glycopeptides of normal BHK cells (Fig. 2d), indicating that hybrid structures (Fig. 1b), if present in BHK cells, form a very small part of the total fraction C (Table 1). Therefore, in order to detect hybrid *N*-glycans in BHK-cell glycopeptides, we used more sensitive methods, which are illustrated with reference to the C1 fractions detected in the ricin-resistant cells (Figs. 2a–2c).

Hybrid N-glycans of ricin-resistant cells

The [³H]mannose-labelled C1 fractions were passed through a column of DEAE-Sephacel (Figs. 3a–3c). In each case a significant proportion of the radioactive glycopeptides was bound to the column and was eluted in one major (C1-A) and two minor peaks. The flow-through fractions (C1-N) represented 30%, 17% and 12% respectively of the total radioactivity in Ric^R15-cell, Ric^R17-cell and Ric^R19-cell glycopeptides applied to DEAE-Sephacel. After treatment of samples (1500–2700 c.p.m.) of the neutral C1-N fractions with α -mannosidase at least 85% of the total radioactivity was recovered after re-chromatography on Bio-Gel P4 as free mannose (see Fig. 4b), confirming the presence of oligomannosidic *N*-glycans. Treatment of the C1 fractions with neuraminidase converted the acidic components into neutral species (Figs. 3a–3c, broken lines), showing the presence of sialic acid, as expected for hybrid *N*-glycans.

The presence of core α 1→6-fucosyl residues (see Fig. 1b) in the C1 fractions of Ric^R15, Ric^R17 and Ric^R19 cells was shown by chromatography of [³H]mannose-labelled glycopeptides on a lentil-lectin-Sepharose column. The lentil lectin binds

Fig. 2. Bio-Gel P4 chromatography of radiolabelled glycopeptide fractions C (from Table 1) on concanavalin A-Sepharose (a) Ric^R19 cells; (b) Ric^R17 cells; (c) and (e) Ric^R15 cells; (d) and (f) BHK cells. In (a)–(d) [³H]mannose-labelled glycopeptides (○) or [³H]fucose-labelled glycopeptides (● in b and c; histogram in d) were analysed. In (e) and (f) [³H]galactose-labelled glycopeptides were analysed. The arrows show the elution of Blue Dextran, ovalbumin glycopeptide (Man)₅(GlcNAc)₂[¹⁴C]Asn and [¹⁴C]mannose respectively. Fractions pooled for further analysis are indicated.

Fig. 3 DEAE-Sephacel chromatography of [^3H]mannose-labelled glycopeptide fractions C1 (see Fig. 2) of Ric^R15 cells (a), Ric^R17 cells (b) and Ric^R19 cells (c)

Glycopeptide samples were applied to the column before (○) and after (●) treatment with neuraminidase. The points at which a salt gradient was applied are indicated by the arrows. Peaks (C1-N and C1-A) were pooled for further analysis.

only oligosaccharides containing a fucose residue attached $\alpha 1 \rightarrow 6$ to the asparagine-linked *N*-acetylglucosamine of glycopeptides (Kornfeld *et al.*, 1981). When we applied a sample containing 14010 c.p.m. of [^3H]mannose-labelled Ric^R15-cell C1 fraction to the lentil-lectin affinity column, approx. 48% of the total radioactivity was bound tightly and was eluted with 200 mM-methyl α -glucoside (Fig. 5a). Similar results were obtained

with the analogous C1 fractions of Ric^R17 and Ric^R19 cells (54% and 69% respectively). Thus the C1 fractions of Ric^R15-cell, Ric^R17-cell and Ric^R19-cell glycopeptides contain a significant proportion of core fucosylated hybrid *N*-glycans. This conclusion was confirmed by analysis of the glycopeptides obtained from Ric^R cells labelled by growth in the presence of [^3H]fucose (Table 1). After acid hydrolysis of samples (2000–3400 c.p.m.) of concanavalin A-bound fractions C, essentially all (greater than 95%) of the radioactivity was present as fucose. The [^3H]fucose label of glycopeptide fractions C (Table 1) was confined almost entirely to the C1 fractions isolated by Bio-Gel P4 chromatography (Figs. 2b and 2c) and when a sample of the Ric^R15-cell-derived material (66620 c.p.m.) was applied to lentil-lectin-Sepharose, 87% of the total radioactivity was bound to the column and was eluted with 200 mM-methyl α -glucoside (Fig. 5b). Similar results were obtained with [^3H]fucose-labelled C1 fraction of Ric^R17 cells (results not shown).

Labelling of hybrid *N*-glycans with [^3H]galactose

The results so far suggested strongly the presence in the C1 glycopeptide fractions of Ric^R15, Ric^R17 and Ric^R19 cells of structures similar to those established for Ric^R21-cell *N*-glycans (see Fig. 1b). This conclusion was supported by analysis of glycopeptide fractions obtained from cells labelled by growth in medium containing [^3H]galactose. This radioactive precursor labelled heavily (Table 1) the concanavalin A-Sepharose fraction C of Ric^R15-cell glycopeptides, predominantly in the C1 fraction (Fig. 2e). A portion (8190 c.p.m.) of this fraction was subjected to acid hydrolysis, and the neutral sugars were analysed by paper chromatography. Analysis showed that essentially all (>90%) of the radioactivity was present as [^3H]galactose. In particular, no radioactivity was present as glucose, mannose or fucose (results not shown). Another portion (85050 c.p.m.) was applied to a column of lentil-lectin-Sepharose (Fig. 5c). Approx. 57% of the radioactivity was bound to the column and was eluted with 200 mM-methyl α -glucoside. The bound and unbound fractions from lentil-lectin-Sepharose (Fig. 5c) were then subjected separately to ion-exchange chromatography on DEAE-Sephadex. As Fig. 6(a) shows, most (93–97%) of the radioactivity in these fractions was bound to the column, indicating the presence of anionic components. Treatment of lentil-lectin-bound or unbound fractions with neuraminidase converted at least 90% of the fractions into a neutral species (results not shown). These results show the presence of sialic acid in the majority of the galactosylated glycopeptides that bound or did not bind to lentil-lectin-Sepharose.

Fig. 4. Sensitivity to α -mannosidase of $[^3\text{H}]$ mannose-labelled glycopeptides of Ric¹⁵ cells. Fractions were treated with the enzyme at 37°C for 48 h and examined by chromatography on Bio-Gel P4. (a) Fraction C1-A; (b) fraction C1-N from DEAE-Sephacel (see Fig. 3a). In (a) the glycopeptides were desialylated before treatment with α -mannosidase as described in the text.

Fig. 5. Lentil-lectin-Sepharose chromatography of glycopeptides. Fractions derived from concanavalin A-Sepharose glycopeptides (Table 2 and Fig. 2) were analysed. The column was washed first with buffer followed by (arrows) 200 mM-methyl α -glucoside. (a)–(c) Ric¹⁵-cell glycopeptides; (d)–(f) BHK-cell glycopeptides. The radioactive precursors used are indicated.

Fig. 6. DEAE-Sepharcel chromatography of [^{14}C]galactose labelled glycopeptides (a) Ric^R15-cell fraction C1 (Fig. 2e) glycopeptides that bind (○) or do not bind (●) to lentil-lectin-Sepharose (Fig. 5); (b) BHK-cell glycopeptide fractions C1 (○) and C2 (●) from Bio-Gel P4 chromatography (Fig. 2f). The arrows indicate the start of a salt gradient.

From the quantitative data, we calculate that approx. 50% of the hybrid *N*-glycans present in the total [^3H]galactose-labelled C1 fraction of Ric^R15 cells are sialylated and core-fucosylated, about 7% are core-fucosylated but lack sialic acid and about 43% contain sialic acid but lack an $\alpha 1 \rightarrow 6$ -fucosyl residue in the core sequence. The very low proportion of non-sialylated hybrid *N*-glycans containing unsubstituted galactose units in Ric^R15 cells, as well as Ric^R21 cells (Hughes *et al.*, 1983; Gleeson *et al.*, 1984), is consistent (Baenziger & Fiete, 1979b) with the small degrees of binding of ricin to these cells (Meager *et al.*, 1976).

Hybrid N-glycans occur as minor constituents in normal BHK-cell glycopeptides

Inspection of Table 1 shows that the concanavalin A-Sepharose fraction C of normal BHK cells was labelled significantly by growth of the cells in the presence of [^3H]fucose or [^3H]galactose. The great proportion of the radioactivity was incorporated, as expected (Hughes & Mills, 1983), into fraction A, representing tri- and tetra-antennary complex *N*-glycans, and fraction B, representing bi-antennary complex *N*-glycans, as shown previously (Hughes & Mills, 1983). In three separate experiments with [^3H]galactose, the proportion of radioactivity recovered in fraction C was in the range 2–3% of the total. Portions of [^3H]galactose-labelled (4560 c.p.m.) or [^3H]fucose-labelled (1770 c.p.m.) fractions C were subjected to strong acid hydrolysis. Paper chromatography of the hydrolysates showed that at least 95% of the radioactivity was present as free galactose or fucose respectively. In separate experiments

[^3H]fucose-labelled (9870 c.p.m.) or [^3H]galactose-labelled (14500 c.p.m.) glycopeptide fractions C were fractionated by Bio-Gel P4 chromatography. Column fractions from the chromatography of [^3H]fucose-labelled glycopeptides were pooled in batches of five, freeze-dried, redissolved in water (0.7 ml) and then 70 μl portions counted for radioactivity. As shown in Fig. 2(d), the bulk of the radioactivity (6792 c.p.m., 69%) was eluted in the approximate region (column fractions 50–60) expected for a hybrid sialylated structure. Further evidence for this proposal was obtained by chromatography of a portion (2156 c.p.m.) of the [^3H]fucose-labelled fraction C1 on a lentil-lectin-Sepharose column. Most (93%) of the radioactivity was bound to the column and was eluted with 200 mM-methyl α -glucoside (Fig. 5e).

The results of [^3H]galactose-labelling of normal BHK cells were more complex. Chromatography on Bio-Gel P4 of the [^3H]galactose-labelled C fraction from concanavalin A-Sepharose produced a definite peak (51% of total radioactivity) in the region (C1, Fig. 2f) expected for hybrid sialylated *N*-glycans with or without fucose (fraction C1). However, approx. 39% of the radioactivity was eluted (C2, Fig. 2f) in later fractions. In addition, a small peak (10% of total radioactivity) was eluted in the void column. This latter peak was present also to a similar extent in [^3H]galactose-labelled glycopeptide fraction C of Ric^R15 cells (Fig. 2e) and presumably represents partially digested glycopeptides. [^3H]Galactose-labelled fractions C1 and C2 (Fig. 2f) were passed separately through lentil-lectin-Sepharose. As Fig. 5(f) shows, 28% of fraction C1 was bound to the column, indicating

the presence of core fucosylation. Most (92%) of this fraction was also bound to DEAE-Sephacel (Fig. 6b) in a neuraminidase-sensitive manner (results not shown), showing that sialic acid residues are also present. By contrast, only 5% of the radioactivity present in the [^3H]galactose-labelled C2 fraction (Fig. 2f) had affinity for lentil lectin (results not shown), and DEAE-Sephacel chromatography showed that at least 96% of the radioactive material was neutral (Fig. 6b).

These results are consistent with the presence of galactosylated, sialylated and core-fucosylated *N*-glycans in the C1 fraction of normal BHK cells. In addition, galactosylated *N*-glycans lacking both sialic acid and fucose were present in fraction C2. Thus, of the total galactosylated *N*-glycans present in fraction C of normal BHK cells, 17% represents sialylated and core-fucosylated *N*-glycans, 40% represents sialylated non-fucosylated chains, 5% represents core-fucosylated chains without sialic acid, and 38% represents non-sialylated chains lacking fucose.

Further evidence for hybrid structures in BHK-cell glycopeptide fraction C1, and in particular sialic acid groups, was obtained by using cells labelled metabolically with [^{14}C]glucosamine, a precursor of sialic acid as well as *N*-acetylglucosamine residues of glycoproteins (see Hughes, 1984). Glycopeptide fraction C (Table 1) obtained by concanavalin A-Sepharose chromatography of [^{14}C]glucosamine-labelled BHK-cell glycopeptides was de-salted by chromatography on Bio-Gel P4. The radioactivity was eluted similarly to that shown for [^3H]mannose-labelled glycopeptide fraction C (Fig. 2d). No prominent leading peak C1 was observed. When the whole de-salted fraction C (19775 c.p.m.) was passed through a lentil-lectin-Sepharose column a small, but significant, peak (2109 c.p.m.) was detected in the bound fraction (Fig. 5d). Sufficient amounts (total 8436 c.p.m.) of this material were accumulated by lentil-lectin-Sepharose chromatography for further analysis. Samples (2500 c.p.m.) were either heated in 0.05 M-HCl at 80°C for 1 h or treated with neuraminidase at 37°C for 16–20 h. The freeze-dried reaction mixtures were redissolved in 0.1 M-pyridine/acetate buffer, pH 5, and passed again through the Bio-Gel P4 column under standard conditions. Approx. 15–17% of the total radioactive material was eluted from the column (fractions 93–108) in a position expected (Hughes *et al.*, 1983) for free *N*-acetylneuraminic acid. The remaining radioactive material was eluted from the column (fractions 55–77) as material of greater molecular size, presumably representing the desialylated glycopeptides. No material that was eluted as free sialic acid was detected without prior treatment of the glycopeptide fraction with acid or neuraminidase.

Degradation of hybrid *N*-glycans by α -mannosidase

The hybrid sialylated structures (Figs. 1b and 1c) established previously for Ric^R21-cell glycopeptides (Gleeson *et al.*, 1984) contain a single unsubstituted α -mannosyl residue. Paradoxically, however, we showed earlier that the simpler hybrid *N*-glycan (Fig. 1b) obtained from Ric^R21-cell glycopeptides was very resistant to the action of jack-bean α -mannosidase (Hughes *et al.*, 1983). Similar results for other hybrid *N*-glycans have been described by others (Liang *et al.*, 1979; Yamashita *et al.*, 1980; Yoshima *et al.*, 1980; Hunt & Wright, 1981). We have re-examined this result by using a standard glycopeptide (Fig. 1a) labelled with an *N*-acetyl[^3H]glucosamine residue linked to the $\alpha 1 \rightarrow 3$ -mannosyl unit of the core sequence. Other results suggest that the terminal α -mannosyl residue of this structure may be released slowly by jack-bean α -mannosidase (Tulsiani & Touster, 1983). The release of the terminal $\alpha 1 \rightarrow 6$ -mannosyl unit by α -mannosidase was measured by the conversion of the labelled glycopeptide from a compound binding tightly to concanavalin A-Sepharose into a non-binding product. Concanavalin A shows highest affinity for sequences containing two interacting α -mannosyl residues (Baenziger & Fiete, 1979a), such as those present in the biosynthetic product (see Fig. 1a) and the hybrid sialylated *N*-glycan of Ric^R21 cells (Fig. 1b). Portions of the *N*-acetyl[^3H]glucosamine-labelled biosynthetic glycopeptide (see Fig. 1a) were treated at 37°C with high concentrations of jack-bean α -mannosidase, and the product was separated from unchanged glycopeptide by chromatography on concanavalin A-Sepharose. As shown in Fig. 7, there was a slow progressive hydrolysis of the biosynthetic glycopeptide standard (t_4 approx. 40 h at 37°C). An essentially complete conversion into the non-binding glycopeptide [^3H]GlcNAc $\beta 1 \rightarrow 2$ Man $\alpha 1 \rightarrow 3$ Man $\beta 1 \rightarrow 4$ GlcNAc $\beta 1 \rightarrow 4$ GlcNAc-Asn, and presumably free mannose, was reached after 80 h incubation.

With these conditions of enzymic treatment, approx. 80% of [^3H]mannose- or [^3H]fucose-labelled hybrid sialylated glycopeptide (Fig. 1b) of Ric^R21 cells was found to retain affinity for concanavalin A even after incubation with α -mannosidase for 80 h, as found previously (Hughes *et al.*, 1983). This result suggests that the susceptibility of the terminal $\alpha 1 \rightarrow 6$ -mannosyl unit to the exo-glycosidase is controlled markedly by the nature of the sequence substituted on to the $\alpha 1 \rightarrow 3$ -linked mannose residue. This conclusion was supported by the results of mixed glycosidase treatments. Thus, after removal of sialic acid, the Ric^R15-cell/Ric^R21-cell hybrid glycopeptides became susceptible to α -mannosidase, as shown

(Figs. 4a and 7). The galactose substituent on the $\alpha 1 \rightarrow 3$ -linked arm seemed to bear a lesser influence, since treatment of the desialylated glycopeptide with β -galactosidase did not appreciably increase the rate of conversion (37% and 89% after 24 h and 72 h respectively).

The degradation of the hybrid *N*-glycans of normal BHK cells by α -mannosidase is shown in Fig. 7. For these experiments, we used the [3 H]galactose-labelled glycopeptides showing highest affinity for concanavalin A (Table 1) and eluted from Bio-Gel P4 in the region expected for hybrid *N*-glycans (fractions 52–62, Fig. 2f). As shown in Fig. 7, the glycopeptide fraction after desialylation was degraded to a product without affinity for concanavalin A, indicating the presence of terminal α -mannosyl residues. This is consistent with the presence of the hybrid structure shown in Fig. 1(b). However, the proportion of radioactivity present in fractions retaining affinity

for concanavalin A at various times was consistently higher by 15–20% compared with the standard hybrid glycopeptides (Fig. 7). This result suggests that the minor hybrid *N*-glycans of BHK cells may be a mixture containing terminal α -mannosyl units on the $\alpha 1 \rightarrow 6$ -linked arm of the core sequence and a core sequence in which both the $\alpha 1 \rightarrow 3$ - and $\alpha 1 \rightarrow 6$ -linked arms are substituted by residues other than α -mannosyl residues, to produce structures resistant to α -mannosidase.

Discussion

The ricin-resistant Ric^R21 variant of BHK cells (Meager *et al.*, 1975, 1976) represents a novel type of mutation affecting protein glycosylation. No complete deficiency in the major enzymes active in normal *N*-glycan assembly was detected (Vischer & Hughes, 1981). However, the activity *in vitro* of *N*-acetylglucosaminyltransferase II (Scheme 1) was diminished by about 70% and the activity of this enzyme relative to $\beta 1 \rightarrow 4$ -galactosyltransferase was decreased about 4-fold compared with normal BHK cells (Vischer & Hughes, 1981; Narasimhan *et al.*, 1984). Earlier we had shown (Hughes & Mills, 1983; Hughes *et al.*, 1983; Gleeson *et al.*, 1984) that these changes in glycosyltransferase activities were correlated with a profound switch in *N*-glycan assembly from complex chains (Scheme 1, pathway B) to the novel hybrid structures (Scheme 1, pathway A). The present paper extends these findings to show that similar hybrid *N*-glycans are present as major components in the glycoproteins of two other ricin-resistant variants of BHK cells, namely Ric^R17 and Ric^R19 cells. Although enzymic analyses of these cells is less complete than for Ric^R21 cells (Vischer & Hughes, 1981), it seems likely that the production of hybrid *N*-glycans results from similar lesions in glycosyltransferase activities. By contrast, the Ric^R15 cell line appears to be significantly different from those previously examined, since the proportion of hybrid *N*-glycans as a total of the glycopeptides binding tightly to concanavalin A–Sephadex is lower than determined for Ric^R17, Ric^R19 and Ric^R21 cells, suggesting other as yet undefined abnormalities in the processing of nascent *N*-glycans.

The main point made in the present paper is that hybrid *N*-glycans with properties very similar to those identified in Ric^R21-cell glycoproteins (Hughes *et al.*, 1983; Gleeson *et al.*, 1984) are present in normal BHK-cell glycoproteins. The proportion of total *N*-glycans represented by the hybrid structures in BHK cells is best estimated from the amounts (2–3%) of [3 H]galactose- or [3 H]fucose-labelled glycopeptides that bind tightly to concanavalin A (Table 1). However, these

Fig. 7. Hydrolysis of glycopeptides by jack-bean α -mannosidase

The glycopeptide samples (2000–3000 c.p.m.) were incubated at 37°C with glycosidase as described in the Materials and methods section. After various times of incubation the mixtures were heated to inactivate the enzyme, applied to concanavalin A–Sephadex and the proportion of total radioactivity bound to the column was determined. Two independent experiments (○ and ●) were carried out with [3 H]GlcNAc $\beta 1 \rightarrow 2$ Man $\alpha 1 \rightarrow 3$ [Man $\alpha 1 \rightarrow 6$]Man $\beta 1 \rightarrow 4$ GlcNAc $\beta 1 \rightarrow 4$ GlcNAc–Asn standard glycopeptide; ▲, desialylated hybrid glycopeptides of Ric^R21 cells labelled with [3 H]mannose; △, BHK cells labelled with [3 H]galactose.

Scheme 1. Simplified schemes of N-glycan assembly

Allowed reactions (full arrows) and non-allowed reactions (broken arrows) are based on assay performed *in vitro* (see Schachter *et al.*, 1983). See the text for further discussion. Non-standard abbreviations used: M, Mannose; F, fucose; Gn, N-acetylglucosamine; Ga, galactose; Sa, sialic acid; GlcNAc-TI, N-acetylglucosaminyltransferase I; GlcNAc-TII, N-acetylglucosaminyltransferase II; Fuc-T, fucosyltransferase; Gal-T, galactosyltransferase; Sa-T, sialyltransferase.

fractions are clearly heterogeneous and a maximum of 17% only of the tightly bound [^3H]galactose-labelled fraction from concanavalin A-Sepharose exhibits properties expected for the structure shown in Fig. 1(b). The majority appear to be derivatives lacking either sialic acid or fucose, or both sugars. By contrast, in the hybrid N-glycans present in Ric^R21-cell glycoproteins, representing 63% of total N-glycans (Gleeson *et al.*, 1984) with the remainder being oligomannosidic chains, over 80% of the hybrid structures contain both sialic acid and fucose residues. The reason for these differences in hybrid structures in BHK cells and Ric^R21 cells are unknown.

The clearest evidence available for the presence of hybrid structures in BHK-cell glycoproteins comes from the action of exo- α -mannosidase. The conversion of [^3H]galactose-labelled glycopeptides from a concanavalin A-binding fraction into a non-binding fraction can only be explained by the presence of a terminal α -mannosyl residue linked to the core sequence, and this provides a more sensitive assay for such residues than previous analyses (Tulsiani & Touster, 1983). Biosynthetic

evidence (Schachter *et al.*, 1983) fixes the α -mannosidase-sensitive residue on the $\alpha 1 \rightarrow 6$ arm of the core sequence, as shown in Scheme 1. It may be noted, however, that conversion of the desialylated [^3H]galactose-labelled hybrid fraction of BHK cells into a fraction without affinity for concanavalin A is less complete than is the hydrolysis of the Ric^R21-cell hybrid N-glycan (Fig. 7). A part (about 12–15%) of the BHK-cell hybrid fraction appears to be insensitive to α -mannosidase, even after prolonged incubation. The two interacting α -mannosyl units essential for concanavalin A-binding (Baenziger & Fiete, 1979a) may be terminal or 2-O-substituted residues. This binding specificity explains the high affinity of the structure shown in Fig. 1(b) for concanavalin A. However, structures containing a $\beta 1 \rightarrow 2$ -linked N-acetylglucosamine residue on the $\alpha 1 \rightarrow 6$ arm of this sequence also retains high affinity for the lectin (Baenziger & Fiete, 1979a; Narasimhan *et al.*, 1979). Only when this N-acetylglucosaminyl residue is substituted by a $\beta 1 \rightarrow 4$ -galactosyl unit does the binding become weaker (Paquet *et al.*, 1984). Hence we suggest that the α -mannosidase-resistant

portion of the hybrid fraction of [^3H]galactose-labelled BHK-cell glycopeptides may be substituted with *N*-acetylglucosamine on the $\alpha 1 \rightarrow 6$ -linked arm of the core sequence. This basic structure is present in several intermediates in the normal pathway of assembly of complex *N*-glycans (*c*, *e* and *h* in Scheme 1).

The hybrid galactosylated *N*-glycans carrying an unsubstituted α -mannosyl residue detected as minor components in BHK-cell glycopeptides cannot be intermediates in a normal pathway of assembly of complex *N*-glycans (Scheme 1). Recent evidence (Schachter *et al.*, 1983) from assays performed *in vitro* with acceptor glycopeptides of known structure has shown that the addition of galactose to a terminal *N*-acetylglucosamine unit linked to the $\alpha 1 \rightarrow 3$ arm of the core structure prevents the action of *N*-acetylglucosaminyltransferase II (Scheme 1). Hence pathways A and B (Scheme 1) are mutually exclusive; no interconversion of structures appearing below the broken line in Scheme 1 seems to be allowed. The data presented here and elsewhere (Hughes *et al.*, 1983; Gleeson *et al.*, 1984) show that pathway B (Scheme 1) is by far the dominant one in normal BHK cells, whereas only pathway A operates in certain ricin-resistant mutants, e.g. Ric^R21. We do not understand at present how this remarkable switch in metabolic pathways A and B (Scheme 1) relates to the different activities of enzyme activities, e.g. *N*-acetylglucosaminyltransferase II in normal BHK and Ric^R21 cells (Narasimhan *et al.*, 1984).

Finally, these studies illustrate the practical value of lectin affinity chromatography (Cummings & Kornfeld, 1982*a,b*) for the fractionation of quantitatively minor glycopeptides. The use of specific labelling with sugar precursors and the action of exo-glycosidases of well-defined specificities leads to reasonable structural assignments that would be very difficult to establish by other means, such as n.m.r. analysis.

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