Both acidic-type and neutral-type asparaginyl-oligosaccharides of host-cell glycoproteins are altered in Rous-sarcoma-virus-transformed chick-embryo fibroblasts

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In comparisons of [³H]mannose-labelled glycopeptides from chick-embryo fibroblasts infected and transformed with non-defective Prague C Rous-sarcoma virus and from untransformed fibroblasts infected with a transformation-defective derivative of Prague C Rous-sarcoma virus, we have detected transformation-dependent alterations in both the acidic-type and the neutral-type asparagine-linked oligosaccharides of cellular glycoproteins. Pronase-digested glycopeptides were analysed by the combined techniques of gel filtration, exo- and endo-glycosidase digestion and concanavalin A-agarose affinity chromatography. The transformed cell glycoproteins contained more sialic acid and were enriched for more highly branched (versus biantennary) acidic-type structures compared with the untransformed cell glycoproteins, similarly to previously reported transformation-dependent alterations. In addition, the glycopeptides from the virus-transformed cells contained several neutral-type structures that were apparently absent from the untransformed cells: small neutral-type oligosaccharides (Man₃GlcNAc₂) that were sensitive to endo- β -N-acetylglucosaminidase D but resistant to endo- β -N-acetylglucosaminidase H, and oligosaccharides with the property of 'truncated' precursor oligosaccharides (endoglycosidase-resistant, α -mannosidase-sensitive). Endoglycosidase-released oligosaccharides with the properties of hybrid-type structures were derived from the glycoproteins of both transformed and untransformed cells.

Because glycoproteins are present at the cell surface and are presumably involved in the regulation of growth and cell-cell interactions, there have been numerous investigations of alterations in the oligosaccharide moieties of cellular glycoproteins that accompany the virus-induced transformation of cells to a malignant state (Atkinson & Hakimi, 1980). Primary cultures of chick-embryo fibroblasts (CEF) that have been infected and transformed by avian Rous-sarcoma virus (RSV) offer a number of advantages as an experimental model for molecular alterations in virus-transformed cells: (i) the vast majority of CEF can be transformed within a relatively short

period of time by RSV, as opposed to the rare transformation (and non-productive infection) of mammalian cell lines by RSV or by DNA tumour viruses (Hanafusa, 1969); (ii) the biochemical basis of cellular transformation by the RSV-encoded *src*-gene product is relatively well characterized (Cooper *et al.*, 1983); (iii) appropriate comparisons can be made between untransformed CEF infected with a transformation-defective virus (td RSV) and CEF transformed by non-defective virus (nd RSV), or, alternatively, between cells infected with a temperature-sensitive mutant of RSV at the non-permissive temperature for transformation and the same cells at the permissive temperature for transformation (Lai & Duesberg, 1972; Hunt *et al.*, 1981).

We have previously demonstrated that the major envelope glycoprotein of RSV, gp85, is more extensively glycosylated in nd-RSV-transformed

Abbreviations used: CEF, chick-embryo fibroblasts; RSV, Rous-sarcoma virus. td and nd, transformation-defective and non-defective respectively.

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CEF compared with td-RSV-infected and untransformed CEF, with a higher degree of branching and greater amount of terminal sialic acid for the complex acidic-type asparaginylo-oligosaccharides synthesized in the RSV-transformed CEF (Hunt *et al.*, 1981). These differences were similar to those observed in earlier comparisons of fucose-labelled cellular glycopeptides from RSV- or polyoma-virus-transformed BHK (baby-hamster kidney) cell lines and the corresponding 'normal' BHK cell lines (Santer & Glick, 1979; Takasaki *et al.*, 1980). However, these studies with virus-transformed BHK-cell glycopeptides did not address possible alterations in the neutral-type asparagine-linked oligosaccharides (Man_nGlcNAc₂-Asn), such as the growth-dependent alterations reported for mannose-labelled glycopeptides from human diploid cells (Muramatsu *et al.*, 1976). Since we had observed minor differences in the relative amounts and the oligomannosyl core sizes of neutral-type oligosaccharides of virion glycoproteins from RSV released from transformed compared with untransformed CEF (Hunt *et al.*, 1981), we were especially interested in further examining the mannose-labelled cell-associated glycoproteins and glycopeptides from the nd-RSV-infected CEF and td-RSV-infected CEF cell cultures in order to determine whether both acidic-type and neutral-type oligosaccharide chains of cellular glycoproteins were also altered by viral transformations.

Materials and methods

Growth and radiolabelling of virus-infected cells

CEF of C/E phenotype were prepared from embryonated eggs (Life Sciences, St. Petersburg, FL, U.S.A.) and infected at approximately the third passage with one of the following preparations of the subgroup C Prague strain of RSV (PrC RSV): a transformation-defective derivative obtained from Peter Vogt (td PrC RSV), agar-colony-cloned non-defective virus (nd PrC RSV) (Wright *et al.*, 1980), or uncloned virus containing both transformation-defective and non-defective virus (td/nd PrC RSV). Virus-infected cells in 75 cm² flasks were grown until nearly confluent in Eagle's minimum essential medium supplemented with 5% (v/v) foetal bovine serum (Gibco), and radiolabelling was done in Eagle's minimal essential medium with one-third the normal concentration of glucose (0.33 mg/ml) and supplemented with 2% (v/v) foetal bovine serum and 100 µCi of [2-³H]mannose (15.8 Ci/mmol; New England Nuclear Corp.)/ml. After labelling for approx. 24 h at 37°C, the medium was harvested, and fresh medium lacking radioactive sugars, but containing the normal amount of glucose and 2% foetal bovine serum, was added. The cultures were incubated for

an additional 18 h at 37°C. At the end of the second incubation, the medium was also harvested, and the virus-infected cells were collected by scraping with a rubber 'policeman' into unlabelled Eagle's minimal essential medium. Homogenates containing the cell-associated glycoproteins were prepared from these cells as previously described for vesicular-stomatitis-virus-infected HeLa cells (Hunt & Summers, 1976). The two harvests of medium for each virus-infected cell culture were combined, and radiolabelled virus was purified from clarified medium by ultracentrifugation (Hunt *et al.*, 1979).

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Analytical gel electrophoresis of samples of purified virus or cell homogenates labelled with [³H]mannose was performed in 10% polyacrylamide slab gels with the discontinuous buffer system of Laemmli (1970). After treatment for fluorography with En³Hance (New England Nuclear Corp.), the gels were dried and exposed at -70°C to Kodak X-Omat L film.

Preparation and gel filtration of Pronase-digested glycopeptides

Radiolabelled glycoproteins were extracted with butan-1-ol from Nonidet P-40-treated cell homogenates and extensively digested with Pronase (Calbiochem) as previously described (Hunt & Summers, 1976). The glycopeptides were desalted on a column of Sephadex G-15/G-50 (Pharmacia Fine Chemicals) (Hunt & Summers, 1976), and the glycopeptides were then analysed, either before or after glycosidase digestion and lectin affinity chromatography, by gel filtration through columns (120 cm × 1.5 cm) of Bio-Gel P-4 (-400 mesh; Bio-Rad Laboratories) along with unlabelled (Blue Dextran, stachyose and mannose) and ¹⁴C-labelled gel-filtration markers (Hunt & Summers, 1976; Hunt *et al.*, 1978). The internal standards were not utilized as *M_r* standards, but were useful in comparing and superimposing the profiles of different samples run separately on the same column or on different columns. Before the unknown ³H-labelled samples were run, each column was calibrated with various ³H-labelled oligosaccharides as described previously (Hunt *et al.*, 1981).

Glycosidase digestions of glycopeptides

Digestion of glycopeptides with endo-β-N-acetylglucosaminidases D and H or exoglycosidases were performed as previously described (Etchison *et al.*, 1977; Hunt *et al.*, 1978). Purified endo-β-N-acetylglucosaminidase D from *Diplococcus pneumoniae* was purchased from Miles Laborato-

ries, and purified endo- β -*N*-acetylglucosaminidase H from *Streptomyces griseus* was purchased from either Miles Laboratories or Health Research (Albany, NY, U.S.A.). Jack-bean α -mannosidase and *Clostridium perfringens* neuraminidase were purchased from Sigma Chemical Co., and a partially purified mixture of β -galactosidase and β -*N*-acetylglucosaminidase from *Clostridium perfringens* was provided by Dr. Harry Schachter (Hospital for Sick Children, Toronto, Canada). At the end of some endoglycosidase digestions, radiolabelled neutral oligosaccharide products were separated from endoglycosidase-resistant radiolabelled glycopeptides and unlabelled *N*-acetylglucosamine-peptide products by chromatography on a Dowex 1 X2 (formate form) anion-exchange column (5 cm $\times$ 0.6 cm) before gel-filtration analysis (Robertson *et al.*, 1978).

Lectin affinity chromatography of glycopeptides and oligosaccharides

Endo- β -*N*-acetylglucosaminidase D- and endo- β -*N*-acetylglucosaminidase H-digested glycopeptides were fractionated on columns of concanavalin A-agarose (Sigma Chemical Co.) as described in detail elsewhere (Hunt, 1982). Unbound glycopeptides and oligosaccharides were washed

through the columns with 10 mM-Tris/HCl buffer, pH 7.4, and bound glycopeptides and oligosaccharides were sequentially eluted with the same buffer containing 10 mM-, 50 mM- and 100 mM- α -methyl mannoside. The 100 mM- α -methyl mannoside-containing buffer was warmed to 60°C before addition to the top of the columns in order to elute more efficiently the tightly bound oligosaccharides containing the larger oligomannosyl core structures (Hunt, 1983). The total radiolabel recovered in the unbound and α -methyl mannoside-eluted fractions was approx. 90% of the radiolabel originally applied. Peak fractions of unbound or bound and eluted radiolabel were concentrated by freeze-drying before gel-filtration analysis.

Results

Comparison of the glycoproteins of untransformed and transformed PrC RSV-infected CEF

The two major [3 H]mannose-labelled proteins in the td-PrC RSV-infected and untransformed CEF (Fig. 1, lane A) were a very large, fibronectin-size, protein and a protein with an apparent M_r of approx. 46000, similar to the results of previous studies with [3 H]mannose-labelled and untransformed CEF (Dubbelman & Yamada, 1982). As

Fig. 1. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of RSV-infected CEF cell-associated glycoproteins and purified RSV released from these cells

[3 H]Mannose-labelled protein samples were analysed in a 10% polyacrylamide slab gel along with 14 C-labelled M_r standards (New England Nuclear Corp.). Lane A, td-PrC-RSV-infected CEF; lanes B and D, two sequentially passaged cultures of nd-PrC-RSV-infected and transformed CEF; lane C, td/nd-PrC-RSV-infected CEF; lanes E, F and G, purified PrC RSV released from the RSV-infected cultures analysed in lanes B, C and D respectively. gp85 and gp37 refer to the major and minor viral envelope glycoproteins. * indicates the position of a high- M_r glycoprotein (presumably fibronectin) absent from or greatly diminished in amount in the transformed cell cultures; ** indicates the position of a low- M_r glycoprotein that was greatly increased in amount in the transformed cell cultures. The 14 C-labelled markers were: 1, phosphorylase *b* (M_r 92500); 2, bovine serum albumin (M_r 68000); 3, IgG heavy chain (M_r 50000); 4, ovalbumin (M_r 43000); 5, carbonic anhydrase (M_r 29000); 6, IgG light chain (M_r 23500).

expected, the amount of radiolabelled fibronectin-size protein was greatly diminished in the cell-associated proteins from the nd-PrC-RSV-infected and transformed CEF (Fig. 1, lanes B and D). In addition, a new glycoprotein of M_r approx. 22000 and increased radiolabel in glycoproteins in the 90000–130000 M_r range were observed in the transformed cell-associated samples (Fig. 1, lanes B and D). The profile of radiolabelled glycoproteins from the td/nd-PrC-RSV-infected and partially transformed CEF contained amounts of fibronectin-size and 22000- M_r proteins that were intermediate between the values for the untransformed and transformed CEF (Fig. 1, lane C).

The predominant virion glycoprotein, gp85 (Fig. 1, lanes E and F), was not a predominant species in the cell-associated fraction of either transformed or untransformed CEF. The gp85 of virus released from the td/nd-PrC-RSV-infected CEF (Fig. 1, lane F) and the td-PrC-RSV-infected CEF (results not shown) had a slightly lower apparent M_r than that of virion gp85 from the nd-PrC-RSV-infected CEF (Fig. 1, lanes E and G), and the minor virion glycoprotein, gp37, exhibited a similar transformation-dependent alteration (Fig. 1, lane E compared with lane F). These electrophoretic differences have been attributed to increased processing of the oligosaccharide chains in the RSV-transformed CEF, resulting in larger acidic-type oligosaccharides with more branches and terminal sialic acid (Hunt *et al.*, 1981; Hunt, 1983). The relatively diffuse electrophoretic mobility of the virion gp85 (and also the 22000- M_r glycoprotein associated with the nd-PrC-RSV-infected CEF in Fig. 1, lanes B and D) has not been explained on a molecular basis, but may possibly represent extensive heterogeneity in carbohydrate content (number and/or structures of oligosaccharide chains) among different virion gp85 polypeptides.

Comparison of [3H]mannose-labelled glycopeptides and oligosaccharides from transformed and untransformed CEF infected with PrC RSV

The Pronase-digested glycopeptides derived from the total cell-associated glycoproteins of all three CEF cultures (nd-PrC-RSV-infected CEF in Fig. 1, lane B; td-PrC-RSV-infected CEF in Fig. 1, lane A; td/nd-PrC-RSV-infected CEF in Fig. 1, lane C) contained a heterogeneous array of endoglycosidase-resistant acidic-type glycopeptides and endo- β -*N*-acetylglucosaminidase D- or endo- β -*N*-acetylglucosaminidase H-released hybrid-type and neutral-type oligosaccharides (Fig. 2). There were two major differences between the glycopeptides of the transformed CEF (Fig. 2a) and the glycopeptides of the corresponding untransformed CEF (Fig. 2b): (i) the endoglycosidase-resistant glycopeptides from the transformed

CEF (fractions 40–65, Fig. 2a) were apparently larger in size than the corresponding glycopeptides from the untransformed CEF (fractions 40–65, Fig. 2b); (ii) $\text{Man}_3\text{GlcNAc}_1$ -size oligosaccharides (fractions 95–100, Fig. 2a) were present in the transformed CEF glycoproteins but absent from the untransformed CEF glycoproteins (Fig. 2b). The unusual small neutral-type oligosaccharides have been previously demonstrated in virion glycoproteins from RSV-transformed CEF (Hunt *et al.*, 1981) and in Sindbis-virus glycoproteins from virus-infected CHO (Chinese-hamster ovary) cells (Hunt, 1981; Davidson & Hunt, 1983).

The endoglycosidase-digested glycopeptides from the partially transformed CEF (Fig. 2c) had a size distribution of acidic-type glycopeptides and a relative amount of $\text{Man}_3\text{GlcNAc}_1$ -size oligosaccharides that were intermediate compared with the samples from the transformed and the untransformed CEF.

The relative amount of radiolabel in endoglycosidase-resistant glycopeptides (compared with neutral-type or hybrid-type oligosaccharides released by endo- β -*N*-acetylglucosaminidase D or H) were very similar for the three different samples, ranging from 46% in acidic-type glycopeptides for td/nd-PrC-RSV-infected CEF to 40% in acidic-type glycopeptides for td-PrC-RSV-infected CEF. These values were significantly lower than the corresponding values for purified virion glycoproteins released from transformed (66%) and untransformed CEF (53–64%) from previously published investigations (Hunt *et al.*, 1981; Hunt & Wright, 1983). One explanation for this difference was the possibility that some of the cell-associated glycoproteins contained unprocessed precursor-type oligosaccharides ($\text{Glc}_{0-1}\text{Man}_9\text{GlcNAc}_2\text{-Asn}$; sensitive to endo- β -*N*-acetylglucosaminidase H). However, the long 'chase' period following the [3H]mannose-labelling should have allowed for the complete processing and maturation of essentially all of the cell-associated glycoproteins.

Fractionation of [3H]mannose-labelled glycopeptides and oligosaccharides by concanavalin A-agarose affinity chromatography

Because the major transformation-dependent alteration in the glycosylation of PrC RSV gp85 involved the complex acidic-type structures, the endoglycosidase-digested glycopeptides from all three cell-associated glycoprotein samples were subjected to fractionation on concanavalin A-agarose columns to analyse the microheterogeneity in the acidic-type glycopeptides. Biantennary structures [$(\text{NeuAc} \pm \text{Gal-GlcNAc})_2\text{Man}_3\text{GlcNAc}_2(\pm \text{Fuc})\text{-Asn}$] should be bound to the column and eluted with α -methyl mannoside, whereas the more highly branched tri- or tetra-antennary

Fig. 2. Bio-Gel P-4 gel filtration of [^3H]mannose-labelled glycopeptides from PrC-RSV-infected CEF before and after digestion with endo- β -N-acetylglucosaminidase D and endo- β -N-acetylglucosaminidase H

(a) nd-PrC-RSV-infected and transformed CEF; (b) td-PrC-RSV-infected CEF; (c) td/nd-PrC-RSV-infected and partially transformed CEF. The corresponding profiles of untreated (○) and endo- β -N-acetylglucosaminidase D- and H-digested glycopeptides (●) were superimposed by alignment of the peak elution positions of the gel-filtration standards (indicated by vertical arrows, from left to right): Blue Dextran, ^{14}C -labelled $\text{Man}_5\text{GlcNAc}_1$ (broken arrow), stachyose and mannose. The numbers (3, 5, 6 and 7) indicate the elution positions of neutral oligomannosyl core products of endo- β -N-acetylglucosaminidase D or endo- β -N-acetylglucos-

aminidase H digestion ($\text{Man}_n\text{GlcNAc}_1$) with $n = 3, 5, 6$ and 7. X refers to the elution position of sialic acid-containing hybrid-type oligosaccharides ($\text{NeuAc-Gal-GlcNAc-Man}_5\text{GlcNAc}_1$) released by endo- β -N-acetylglucosaminidase H (Hunt & Wright, 1981). The structures of the hybrid oligosaccharide and the small neutral oligosaccharide ($\text{Man}_3\text{GlcNAc}_1$) were based upon the endoglycosidase sensitivities, lectin affinities and relative elution positions from gel-filtration columns, and, in addition, upon the results of previous sequential exoglycosidase studies (Hunt, 1981; Hunt & Wright, 1981; Davidson & Hunt, 1983).

structures [$(\text{NeuAc} \pm \text{Gal-GlcNAc})_{3-4}\text{Man}_3\text{GlcNAc}_2(\pm \text{Fuc})\text{-Asn}$] should not be bound (Hunt, 1982). Biantennary structures with 'bisecting' N-acetylglucosamine linked to the innermost mannose residue are also expected to be unbound or only weakly retarded on concanavalin A-agarose (Harpaz & Schachter, 1980).

The profile of radiolabel that was unbound to a column of concanavalin A-agarose compared with that of radiolabel that was bound and eluted with increasing concentrations of α -methyl mannoside is shown in Fig. 3 for the glycopeptides and oligosaccharides from the nd-PrC-RSV-infected and transformed CEF. Similar profiles were also obtained for the glycopeptides and oligosaccharides from the td-PrC-RSV-infected CEF and the td/nd-PrC-RSV-infected CEF glycoproteins (results not shown).

Comparisons of the concanavalin A-unbound and concanavalin A-bound and 10mM- α -methyl mannoside-eluted glycopeptides/oligosaccharides are shown in Fig. 4 for the three samples. A significant increase in the ratio of radiolabel in the more highly branched acidic-type glycopeptides to that in the biantennary acidic-type glycopeptides was observed for the transformed cell glycoproteins (2.3:1.0, Fig. 4a) compared with untransformed cell glycoproteins (0.9:1.0, Fig. 4b). The partially transformed cell glycoproteins (Fig. 4c) had an intermediate ratio (1.5:1.0).

In addition, both the concanavalin A-unbound and concanavalin A-bound glycopeptides from the untransformed CEF were generally smaller in size than the corresponding glycopeptides from the transformed CEF, suggestive of a relative deficiency in terminal sialic acid in the glycoproteins from td-PrC-RSV-infected CEF. Analysis of the acidic-type glycopeptides after removal of terminal sialic acid (Figs. 5a and 5b) indicated that much of this apparent size difference was indeed due to additional terminal sialic acid added in the transformed CEF.

The endoglycosidase-released oligosaccharides could also be further characterized by concanava-

aminidase H digestion ($\text{Man}_n\text{GlcNAc}_1$) with $n = 3, 5, 6$ and 7. X refers to the elution position of sialic acid-containing hybrid-type oligosaccharides ($\text{NeuAc-Gal-GlcNAc-Man}_5\text{GlcNAc}_1$) released by endo- β -N-acetylglucosaminidase H (Hunt & Wright, 1981). The structures of the hybrid oligosaccharide and the small neutral oligosaccharide ($\text{Man}_3\text{GlcNAc}_1$) were based upon the endoglycosidase sensitivities, lectin affinities and relative elution positions from gel-filtration columns, and, in addition, upon the results of previous sequential exoglycosidase studies (Hunt, 1981; Hunt & Wright, 1981; Davidson & Hunt, 1983).

Fig. 3. Concanavalin A-agarose affinity chromatography of [^3H]mannose-labelled and endo- β -N-acetylglucosaminidase D- and endo- β -N-acetylglucosaminidase H-digested glycopeptides from nd-PrC-RSV-infected and transformed CEF

A portion of the endoglycosidase-digested glycopeptides shown in Fig. 2(a) was fractionated on a column of concanavalin A-agarose, and 0.1 ml samples of each 1 ml fraction were assayed for radioactivity. Bound radiolabel was eluted with increasing concentrations of α -methyl mannoside as indicated by the vertical arrows (10, 50 and 100 mM), and peak tubes of unbound or bound/eluted material were pooled for further gel-filtration analysis (Figs. 4 and 5) as indicated by the horizontal bars.

lin A-agarose affinity chromatography, because larger oligomannosyl core structures ($\text{Man}_{7-9}\text{GlcNAc}_1$) are more tightly bound and less easily eluted with α -methyl mannoside than are smaller neutral structures ($\text{Man}_3\text{GlcNAc}_1$ and $\text{Man}_{5-6}\text{GlcNAc}_1$) or hybrid-type oligosaccharides with five-mannose cores ($\text{NeuAc} \pm \text{Gal-GlcNAc-Man}_5\text{GlcNAc}_1$) (Hunt, 1982). Distinct peaks of radiolabel in the positions of both mono-sialylated hybrid-type (X) and asialo-hybrid-type oligosaccharide (X') were observed in the concanavalin A-bound and 10 mM- α -methyl mannoside-eluted fraction from all three samples (Fig. 4). Minor lower- M_r peaks of radiolabel eluted just after the major peaks of concanavalin A-bound and sialic acid-containing hybrid-type oligosaccharides were detected in the concanavalin A-unbound fraction of all three samples (fractions 65–70 in Figs. 4a, 4b and 4c), consistent with a minor species of sialylated hybrid-type oligosaccharide with a four-mannose core (and

Fig. 4. Bio-Gel P-4 gel filtration of [^3H]mannose-labelled glycopeptides and oligosaccharides from PrC-RSV-infected CEF after fractionation by concanavalin A-agarose affinity chromatography

The conditions of gel filtration were identical with those described in Fig. 2 except for ^{14}C -labelled acidic-type glycopeptides that were included as an additional internal standard (broken arrow) in panel (a). The profiles of concanavalin A-unbound ($\circ$) and concanavalin A-bound and 10 mM- α -methyl mannoside-eluted glycopeptides and oligosaccharides ($\bullet$) are superimposed. X' indicates the peak elution position of the major asialo-hybrid-type oligosaccharide released by endo- β -N-acetylglucosaminidase ($\text{Gal-GlcNAc-Man}_5\text{GlcNAc}_1$). (a) nd-PrC-RSV-infected and transformed CEF; (b) td-PrC-RSV-infected CEF; (c) td/nd-PrC-RSV-infected CEF.

Fig. 5. Bio-Gel P-4 gel filtration of desialylated and concanavalin A-fractionated glycopeptides and oligosaccharides from nd-PrC-RSV-infected and transformed CEF

Samples of the concanavalin A-fractionated glycopeptides and oligosaccharides (shown in Figs. 3 and 4a) were desialylated by neuraminidase digestion, and the concanavalin A-bound and 10mM- α -methyl mannoside-eluted fraction was subjected to additional Dowex chromatography to separate oligosaccharides from glycopeptides. The conditions of gel filtration were identical with those described in Figs. 2 and 4 except that X in panel (b) refers to the asialo-hybrid-type oligosaccharide with a five-mannose core, and X_4 in panel (a) refers to the minor asialo-hybrid species with a four-mannose core. The ^{14}C -labelled acidic-type glycopeptides used as a standard in Fig. 4(a) and in this Figure were useful in determining the shift in elution positions of glycopeptides after removal of terminal sialic acid. (a) Concanavalin A-unbound glycopeptides; (b) concanavalin A-bound and 10mM- α -methyl mannoside-eluted glycopeptides (○) and oligo-

also asialo-hybrid-type oligosaccharides with a four-mannose core for the td-PrC-RSV-infected CEF glycoproteins; fractions 77–83 in Fig. 4b).

The unusual $\text{Man}_3\text{GlcNAc}_1$ -size oligosaccharides from the nd-PrC-RSV-infected CEF and td/nd-PrC-RSV-infected CEF glycoproteins were recovered in the 10mM- α -methyl mannoside-eluted fraction (Figs. 4a and 4c), whereas $\text{Man}_{7-9}\text{GlcNAc}_1$ -size oligosaccharides were more strongly bound and mainly recovered in the 50mM- and 100mM- α -methyl mannoside-eluted fractions (shown in Fig. 5c for the nd-PrC-RSV-infected CEF sample).

An unexpected result was the presence of minor peaks or shoulders of radiolabel between the two major peaks (X and X') of hybrid-type oligosaccharides in the 10mM- α -methyl mannoside-eluted fractions of the transformed cell and partially transformed cell samples (Figs. 4a and 4c). Because the $\text{Man}_3\text{GlcNAc}_2$ oligosaccharides on mature Sindbis-virus glycoproteins from CHO cells have been shown to be derived from 'truncated' precursor oligosaccharides (endo- β -N-acetylglucosaminidase H- and D-resistant; five instead of nine mannose residues) (Davidson & Hunt, 1983), we were interested in determining whether endoglycosidase-resistant glycopeptides ($\text{Man}_{4-5}\text{GlcNAc}_2$ -asparaginyl peptide) derived from similar truncated precursors in the PrC-RSV-transformed CEF might be present and responsible for the unexpected radiolabel. The presence of such glycopeptides was demonstrated in the transformed CEF sample after separation of endoglycosidase-resistant glycopeptides from uncharged oligosaccharides (neutral type and asialo-hybrid-type) after the removal of any terminal sialic acid (Fig. 5b). In addition to a major peak of desialylated biantennary acidic-type glycopeptides (fractions 60–70 in Fig. 5b), there were also smaller glycopeptides (fractions 75–95) in the position expected for $\text{Man}_{4-5}\text{GlcNAc}_2$ -asparaginyl peptides (Davidson & Hunt, 1983). Glycopeptides with similar properties were not detected in the untransformed td-PrC-RSV-infected CEF glycoproteins (results not shown).

Characterization of endo- β -N-acetylglucosaminidase H-resistant neutral-type glycopeptides from nd-PrC-RSV-infected CEF and transformed CEF

In order to characterize better the composition and the relative amounts of the 'truncated' neutral-

saccharides (●); (c) concanavalin A-bound oligosaccharides eluted with 50mM- (○) or 100mM- α -methyl mannoside (●). The profile of neutral oligosaccharides in panel (b) also apparently contained a minor amount of biantennary acidic-type glycopeptides (fractions 65–75) that were not adequately retained on the Dowex column.

type oligosaccharide chains on the transformed cell glycoproteins, endo- β -*N*-acetylglucosaminidase H-resistant glycopeptides were isolated from the total population of neutral-type glycopeptides and subjected to further digestions with exoglycosidases and/or endo- β -*N*-acetylglucosaminidase D (Fig. 6). The endo- β -*N*-acetylglucosaminidase H-resistant glycopeptides represented approximately one-fourth of the radiolabel in the major peak of neutral-type glycopeptides (Fig. 6b), with an elution position corresponding to the lower- M_r side of the major peak of glycopeptides before endo- β -*N*-acetylglucosaminidase H digestion (Fig. 6a). The remainder of the radiolabel was in $\text{Man}_{5-9}\text{GlcNAc}_1$ -size neutral oligosaccharides released by endo- β -*N*-acetylglucosaminidase H (Fig. 6a).

Most of the radiolabel in the endo- β -*N*-acetylglucosaminidase H-resistant glycopeptides was converted by α -mannosidase into free mannose and smaller-size glycopeptides (Fig. 6b), consistent with neutral-type structures ($\text{Man}_n\text{GlcNAc}_2\text{-Asn}$). Endo- β -*N*-acetylglucosaminidase D digestion of these glycopeptides indicated that they were the source of $\text{Man}_3\text{GlcNAc}_1$ -size oligosaccharides (Fig. 6c). Digestion with a mixture of exoglycosidases and endo- β -*N*-acetylglucosaminidase resulted in slightly increased amounts of $\text{Man}_3\text{GlcNAc}_1$. This result suggested that these endo- β -*N*-acetylglucosaminidase H-resistant glycopeptides may also have included some oligosaccharides that could be classified as either 'monoantennary' acidic-type or hybrid-like with a three-mannose core [$\text{Gal} \pm \text{GlcNAc-Man}_3\text{GlcNAc}_2\text{-Asn}$; equivalent to the major oligosaccharide on bovine rhodopsin (Liang *et al.*, 1979)].

Discussion

The results of the present studies with the cell-associated glycoproteins and glycopeptides of untransformed and transformed CEF infected with PrC RSV have demonstrated that the glycosylation of host-cell glycoproteins is altered by viral transformation in the same way as described previously for the major viral envelope glycoprotein (gp85) of RSV released from the same type of host cells (Hunt *et al.*, 1981; Hunt, 1983). The major change involved the degree of branching of complex acidic-type oligosaccharides, as previously reported for cellular glycoproteins from BHK cells transformed with either RSV (Santer & Glick, 1979) or polyoma virus (Takasaki *et al.*, 1980): the transformed cell glycoproteins contained significantly increased amounts of the more highly branched structures (tri- and/or tetra-antennary) relative to the amount of biantennary acidic-type oligosaccharides. Although these trans-

formation-dependent alterations were observed with total cell-associated glycoproteins, these oligosaccharide differences with RSV-infected CEF were probably reflected on both cell-surface and intracellular glycoproteins, on the basis of previous comparisons involving both 'cell-surface' (removed by mild proteinase treatment) and residual cell-associated glycopeptides (Takasaki *et al.*, 1980).

The transformed cell glycoproteins also contained more sialic acid on their *N*-linked oligosaccharides, and this difference was evident even with oligosaccharide types (biantennary acidic-type or hybrid-type) with the same degree of branching and possible sites of sialic acid addition for transformed and untransformed cell glycoproteins. This alteration may have significance for tumour cells *in vivo*, since attachment of cells to fibronectin and collagen is apparently diminished by increased cell-surface sialic acid (Wagner *et al.*, 1981; Dennis *et al.*, 1982).

The most obvious, as well as previously unreported, alteration in the neutral-type oligosaccharides of transformed cell glycoproteins was the presence of the small endo- β -*N*-acetylglucosaminidase D-sensitive oligosaccharides (Fig. 7b). The $\text{Man}_3\text{GlcNAc}_1$ oligosaccharides were present in lesser amounts in the endo- β -*N*-acetylglucosaminidase D digest of the partially transformed cell (td/nd-PrC-RSV-infected CEF) glycopeptides, but absent from the corresponding digest from the untransformed cells (td-PrC-RSV-infected CEF). Because of both the resistance to endo- β -*N*-acetylglucosaminidase H and the oligomannosyl core size equivalent to that of complex acidic-type oligosaccharides, these oligosaccharides could not appropriately be designated as 'high-mannose', as is commonly done for the $\text{Man}_{5-9}\text{GlcNAc}_2$ structures that are cleaved by endo- β -*N*-acetylglucosaminidase H (Kornfeld & Kornfeld, 1980). We have previously detected identical oligosaccharide chains on the glycoproteins of Sindbis virus replicated in CHO cells, and these mature structures were derived from 'truncated' precursor oligosaccharides (Fig. 7), rather than by some abnormal α -mannosidase processing of full-size precursor oligosaccharides (Hunt, 1981; Davidson & Hunt, 1983). The endo- β -*N*-acetylglucosaminidase D- and H-resistant glycopeptides detected in the glycoproteins of the PrC-RSV-transformed CEF (Figs. 5 and 6) presumably contained less-processed structures ($\text{Man}_{4-5}\text{GlcNAc}_2$) derived from the truncated precursor oligosaccharides.

Truncated precursor oligosaccharides have also been reported for uninfected CHO cells subjected to glucose starvation (Gershman & Robbins, 1981; Rearick *et al.*, 1981). The effects of the *src*-gene product on glucose transport and glycolysis in the

Fig. 6. Bio-Gel P-4 gel filtration of endo- β -N-acetylglucosaminidase H-resistant neutral-type glycopeptides from nd-PrC-RSV-infected and transformed CEF

The conditions of gel filtration were the same as in Fig. 2. (a) The radiolabel from the major peak of neutral-type glycopeptides (indicated by cross-hatched horizontal bar) in a preparative gel-filtration profile (○) was subjected to endo- β -N-acetylglucosaminidase H digestion, and the endo- β -N-acetylglucosaminidase H-released oligosaccharides (●) were separated by Dowex chromatography from the endo- β -N-acetylglucosaminidase H-resistant glycopeptides; (b) samples of these endo- β -N-acetylglucosaminidase H-resistant neutral-type glycopeptides were analysed before (○) and after digestion with α -mannosidase (●); (c) samples of the same endo- β -N-acetylglucosaminidase H-resistant neutral-type glycopeptides were analysed after digestion with endo- β -N-acetylglucosaminidase D alone (○) or with *Cl. perfringens* β -galactosidase and β -N-acetylglucosaminidase plus endo- β -N-acetylglucosaminidase D (●).

(a) Structure of the precursor oligosaccharides (Li *et al.*, 1978), with the truncated precursor oligosaccharide lacking the four mannose residues in the dashed box (Rearick *et al.*, 1981; Kornfeld *et al.*, 1979); (b) structure of the most highly processed neutral-type oligosaccharide, with the mature oligosaccharide that was derived from the truncated precursor structure lacking the two mannose residues in the dashed box (Davidson & Hunt, 1983).

transformed CEF (Kletzien & Perdue, 1975; Cooper *et al.*, 1983) may therefore have been at least partially responsible for the apparent synthesis and processing of truncated precursor oligosaccharides. Whether or not these possible alterations in the initial stages of glycosylation were also responsible for any of the major alterations in the more highly processed acidic-type oligosaccharides on the mature glycoproteins in the RSV-transformed CEF was unknown, but Kornfeld *et al.* (1979) have demonstrated that these truncated precursor oligosaccharides can be processed by an 'alternative pathway' into tri- and bi-antennary-acidic-type oligosaccharides on the vesicular-stomatitis-virus glycoprotein in vesicular-stomatitis virus-infected mutant lymphoma cells that were incapable of synthesizing full-size precursor oligosaccharides. Further investigations will be necessary to determine the relative contributions of alterations in the synthesis of precursor oligosaccharides, compared with alterations in the latter stages of oligosaccharide processing, to the overall changes in both complex acidic-type and neutral-type oligosaccharides on the cell-associated glycoproteins of RSV-transformed CEF.

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