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N- and *O*-Glycosylation of Glycoprotein C Synthesized by Herpes Simplex Virus Type 1-infected Ricin Resistant Cells

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The progeny of Herpes simplex virus type 1 (HSV-1) grown in ricin-resistant 14 cells (Ric^R14) lacking *N*-acetylglucosaminyltransferase I was released in the extracellular medium at a very low rate. By using a monoclonal antibody immobilized on Sepharose we purified from HSV-1-infected Ric^R14 cells a viral glycoprotein (gC), which carries both *N*- and *O*-linked oligosaccharides. Glycopeptides obtained from [^{3}H]mannose-labeled gC by Pronase digestion were entirely susceptible to endo-β-*N*-acetylglucosaminidase H, and the major oligosaccharide released was $Man_4GlcNAc$. The accumulation of this high-mannose species was related to the enzymic defect of the host cells and to the long retention of the viral glycoprotein within the cells. The extent of *O*-glycosylation evaluated in [^{14}C]glucosamine-labeled gC from Ric^R14 cells as compared to that of gC from wild type cells did not appear to be significantly modified.

Lectin-resistant cells have been widely used to prove that the glycosylation of viral glycoproteins is dependent on host cell enzymes [1-5]. Meager *et al.* [6] isolated a clone of mutant ricin-resistant BHK cells, named Ric^R14, which is defective in *N*-acetylglucosaminyltransferase I and accumulates the *N*-linked $Man_5GlcNAc_2$ oligosaccharide [7, 8]. By using Herpes simplex virus type 1 (HSV-1)-infected Ric^R14 cells we found that some viral functions, the expression of which requires glycoproteins (such as virus-induced cell fusion) were altered because of the block in the maturation of complex type oligosaccharides [9]. Subsequently, by using different experimental systems we [10] and others [11] showed that impairment in the later steps of processing of *N*-linked glycans strongly reduced the egress of progeny virus from HSV-1-infected cells.

Abbreviations: Con A, concanavalin A; BHK cells, baby hamster kidney cells; HSV, Herpes simplex virus.

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In contrast to other viruses which acquire their envelope by budding through the plasma membrane, HSV acquires its envelope by budding through the nuclear membrane and enveloped virus particles transit from nucleus through the Golgi apparatus to the cell surface [11-13]. During this transit the glycoproteins are processed [14]. A delay in herpes virion egress results in a long persistence of the viral glycoproteins in intracellular compartments. It is possible that this condition affects further post-translational events occurring to the oligosaccharide moieties of glycoproteins, such as *O*-glycosylation.

We report now that a pronounced defect in the release of progeny virus did occur in HSV-1(F)-infected Ric^{R}14 cells. Therefore, we analyzed the oligosaccharide structure of an HSV-1 glycoprotein, glycoprotein C (gC), accumulated in Ric^{R}14 cells. Glycoprotein C is one of the major HSV-1 glycoproteins, which carries several chains *N*- and *O*-glycosidically linked to the peptide backbone [15-17]. In this study we evaluated the extent of *O*-glycosylation of gC synthesized by HSV-1 infected-Ric^{R}14 cells in comparison with that of gC synthesized by parental wild type BHK cells, and identified the structure of *N*-linked chains accumulated in gC due to the host cell deficiency in *N*-acetylglucosaminyltransferase I and of the delayed intracellular transit of the glycoprotein.

Materials and Methods

Materials

HC1 monoclonal antibodies were kindly provided by L. Pereira (California Department of Health Services, Berkeley, CA, USA). [2-^{3}H]mannose (16 Ci/mmol) and [1-^{14}C]glucosamine (56.8 mCi/mmol) were purchased from Amersham International, Amersham, UK. Pronase was from Sigma Chemical Co., St. Louis, MO, USA, and endo-β-*N*-acetylglucosaminidase H (Endo-H) from Miles Laboratories, Elkhart, IN, USA. High mannose and complex-type glycopeptides, used as markers to calibrate the Bio-Gel column, were prepared from human Tamm-Horsfall glycoprotein as described [18, 19]. *N*-Acetyl-[^{3}H]-galactosaminitol was prepared by reduction of *N*-acetylgalactosamine with tritiated potassium borohydride as previously described [17]. [^{3}H]-Galactosaminitol used as a standard in TLC was obtained by strong acid hydrolysis of the corresponding acetylated compound. Silica gel plates were from Merck, Darmstadt, W. Germany. High mannose oligosaccharides from unit A of bovine thyroglobulin (Man_5-$Man_9GlcNAc$) were kindly provided by R. Spiro (Elliott P. Joslin Research Laboratory, Boston, MA, USA). All chemicals were of reagent grade.

Cells and Virus

Ric^{R}14 mutant cells and parental BHK 21/C13 cells were obtained from R.C. Hughes (National Institute for Medical Research, Mill Hill, London). The cells were grown in the Glasgow Modification of Eagles Medium supplemented with 10% fetal calf serum. Confluent (48 h) monolayers of both kinds of cells were used for all the experiments. HSV-1(F) was from B. Roizman, University of Chicago.

Virus Yield

Duplicate cell monolayers were infected at an input multiplicity of infection of 10 plaque forming units per cell. After an adsorption period of 1.5 h, the inoculum was removed and replaced with maintenance medium containing 1% serum. Cultures were incubated at 37°C and virus titer was determined by plaque assay as described [9].

Preparation of Infected Radiolabeled Cell Extracts

Two metabolic labeling conditions were used. Long-labeling: the infected cells were continuously labeled from 7 to 18 h after infection with [^{3}H]-mannose (100 μCi/ml) or [^{14}C]-glucosamine (60 μCi/ml) in medium containing 1.5 mg glucose/ml and chased for 3 h in medium containing no labeled sugar and excess unlabeled glucose. Short pulse: the infected cells were pulsed for 10 min at 10 h after infection with 250 μCi of [^{3}H]-mannose in medium containing no glucose. At the end of the labeling periods cell extracts were prepared as previously described [16]. The supernatant of cell extracts (100 000 $\times$ *g* for 1 h), which had been dialyzed against 0.01 M Tris-HCl, pH 7.5, containing 0.5 M NaCl, 0.1% Nonidet P40 and 1 mM phenylmethylsulfonylfluoride, was used to purify gC.

Purification and Electrophoresis of gC

The glycoprotein was purified from labeled cell lysates by affinity chromatography on HC1 monoclonal antibodies coupled to CNBr-Sepharose. Coupling conditions and affinity chromatography were performed as described [16]. The glycoprotein retained by the immunoadsorbent and eluted by 3 M KSCN was dialyzed against 0.025 M Tris-HCl, pH 8, containing 0.1% Nonidet P-40 and 1 mM phenylmethylsulfonylfluoride. The dialyzed glycoprotein and the cell lysate were brought to 1.2% sodium-dodecyl sulfate and 5% 2-mercaptoethanol, then heat-denaturated for 2 min and analyzed by electrophoresis on a slab gel containing 8.5% acrylamide cross-linked with N,N'-diallyltartardiamide [16]. The apparent molecular weight of the glycoprotein from infected Ric^{R}14 and BHK cells was calculated by comparing its mobility with that of molecular weight calibration standards purchased from Amersham International.

Pronase Digestion and Fractionation of Glycopeptides

Digestion of the glycoprotein with Pronase was performed at 60°C for 24 h as previously described [16]. At the end of this period the glycopeptides were separated by Bio-Gel P-10 and Con A-Sepharose chromatography. The Bio-Gel P-10 column (1 $\times$ 70 cm) was eluted with 0.1 M pyridine-acetate buffer, pH 5, and calibrated with markers of defined structure. One ml fractions were collected. The affinity chromatography on Con A-Sepharose was performed as described [10]. The glycopeptide fractions were pooled, lyophilized and desalted on a Bio-Gel P-2 column (2 $\times$ 30 cm) equilibrated and eluted with water.

Analysis of High-mannose Oligosaccharides Released by Endo-H from [^{3}H]-Mannose-labeled Glycopeptides

Glycopeptides dissolved in 100 μl of 0.1 M sodium citrate buffer, pH 5, were incubated with 5 mU of Endo-H for 20 h at 37°C. After boiling for 3 min the sample was deionized by coupled columns of Dowex 1 and Dowex 50 as previously described [16]. The oligosaccharides were then subjected to TLC on silica gel plates using 1-propanol/acetic acid/water, 3/3/2 by vol, as solvent and developed for about 40 h (system A) according to the method of Godelaine *et al.* [20]. Labeled components were detected by autoradiography and quantified by liquid scintillation counting after scraping off the gel corresponding to the radioactive spots.

Mild Alkaline Borohydride Treatment of [^{14}C]-Glucosamine-labeled Glycopeptides

Glycopeptides were treated with 1 M $NaBH_4$ in 0.1 M NaOH for 48 h at 37°C as described [16]. These conditions released 80-85% of the *O*-linked oligosaccharides by the β-elimination mechanism. At the end of the treatment the samples were acidified with acetic acid and lyophilized. The borate was removed by repeated evaporation with 0.5% acetic acid in methanol. The oligosaccharides were then subjected to Bio-Gel P-10 chromatography as described above.

Identification of Labeled Monosaccharides

When necessary, [^{14}C]-glucosamine-labeled glycopeptides or oligosaccharides were desalted on a Bio-Gel P-2 column or by coupled Dowex columns, after *N*-reacetylation. They were subjected to strong acid hydrolysis in 4 N HCl for 4 h at 100°C. The samples were dried under reduced pressure and analyzed for amino sugar composition by TLC on silica gel plates using ethanol/1-butanol/pyridine/acetic acid/water, 100/10/10/3/30 by vol, as solvent to which 1% w/v potassium tetraborate was added (system B). The chromatogram was developed twice. Labeled amino sugars and galactosaminitol were visualized by autoradiography. The radioactivity was quantified by scraping off the gel corresponding to the spots.

Results and Discussion

Yield of Infectious Virus from Ric^{R}14 and BHK Cells

Fig. 1 shows the yield of cell-associated and extracellular infectious virus from HSV-1-infected Ric^{R}14 and BHK cells. It is evident that progeny HSV-1 released into the medium from infected mutant cells in comparison with that from infected wild type cells was reduced to a much greater extent than cell-associated virus. These results indicate that a major defect of infected Ric^{R}14 cells was in the release of the progeny virus with a consequent accumulation of HSV-1 glycoproteins within the cells.

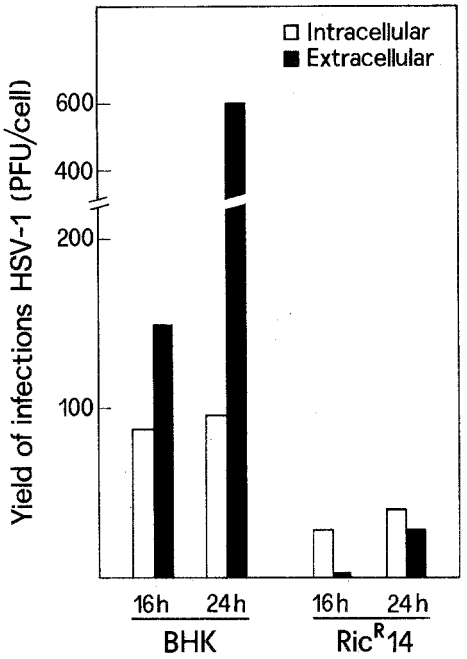


Figure 1. Yield of intracellular and extracellular infectious HSV-1 from BHK and Ric^{R}14 cells. Infected cells were incubated at 37°C for the indicated times. At the end of the incubation period the medium over the cells was collected and the extracellular virus titer determined as indicated in the text. The same amount of fresh medium was added to the monolayers and the intracellular virus titer was determined after freezing and thawing the cells three times.

Analysis of N-linked Oligosaccharides Accumulated in gC from HSV-1/Ric^{R}14 Cells

To characterize the *N*-linked chains accumulated in gC synthesized by infected mutant cells, the glycoprotein was purified from cells after a long labeling period with [^{3}H]-mannose using the immunoabsorbent, and then subjected to Pronase digestion. The mannose-labeled glycopeptides were subsequently fractionated on Bio-Gel P-10 and Con A-Sepharose columns. In both chromatographic systems the glycopeptides behaved as high-mannose species; they were eluted from the Bio-Gel column in the elution volume of a high-mannose glycopeptide marker (see Fig. 2) and from Con A-Sepharose by 300 mM α-methyl mannoside (results not shown)

The radioactive oligosaccharides were also entirely released by Endo-H (Fig. 2), an enzyme that cleaves most high-mannose oligosaccharides between the two innermost *N*-acetylglucosaminyl residues [21]. Oligosaccharides released by Endo-H treatment were analyzed by TLC (system A) and their mobility compared to that of markers of increasing size, ranging from $Man_5GlcNAc$ to $Man_9GlcNAc$. A single oligosaccharide species was found in the Endo-H-treated gC glycopeptides with a chromatographic mobility higher than that of the $Man_5GlcNAc$ marker (Fig. 3). The chromatographic mobility of this

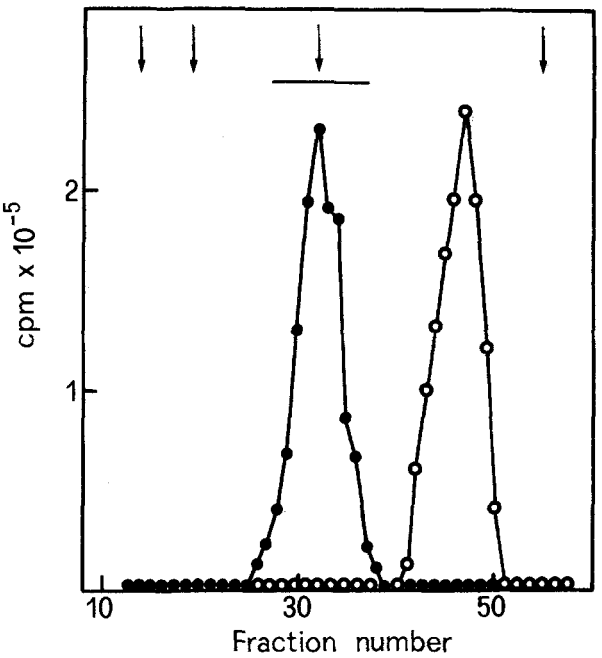
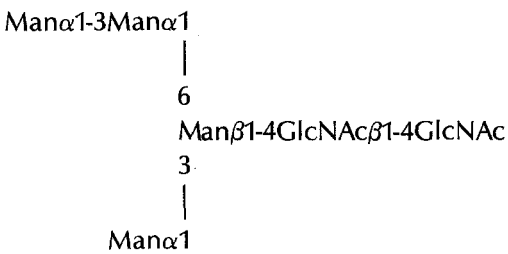


Figure 2. Bio-Gel P-10 filtration of glycopeptides of [^{3}H]-mannose-labeled gC from HSV-1-infected Ric^{R}14 cells before (●) and after (○) Endo-H digestion. The fractions were pooled as indicated by the bars and digested as described in the text. The arrows indicate (from left to right) the elution positions of Blue Dextran, complex-type glycopeptide from Tamm-Horsfall glycoprotein, high-mannose glycopeptide from the same glycoprotein, and mannose.

oligosaccharide, as calculated by plotting the mobilities of the markers, was found to be that of a $Man_4GlcNAc$. Considering the substrate specificity of Endo-H [22], we propose that the structure of the oligosaccharide in gC from HSV-1/Ric^{R}14 cells is:



To rule out that the increased occurrence of $Man_4GlcNAc_2$ chains in HSV-1-infected Ric^{R}14 cells could originate from an unusual biosynthetic pathway of *N*-linked chains, the nature of the high-mannose chain assembled on gC after a 10 min pulse with [^{3}H]-mannose was investigated. Under these conditions we expected to find a polymannoside chain already transferred to gC from the lipid donor but still unmodified by the Golgi

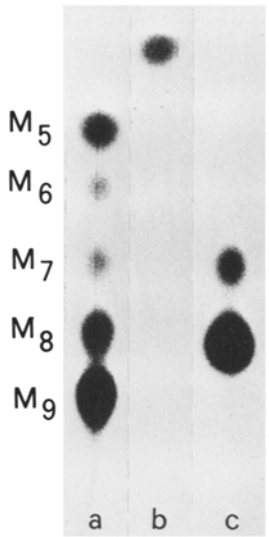


Figure 3. Migration on TLC (system A) of oligosaccharides released by Endo-H treatment from [^{3}H]-mannose-labeled glycopeptides of (b) extensively labeled gC, and (c) of 10 min pulse-labeled gC. Oligosaccharide markers obtained from unit A of thyroglobulin were run for comparison (a). About 10 000 cpm of oligosaccharides were applied. The spots were visualized by autoradiography. Radioactivity in lane b and c was determined by scintillation counting after scraping off the gel. In lane b about 95% of the radioactivity was recovered in a position corresponding to the migration of $Man_4GlcNAc$. In lane c 85% migrated as $Man_8GlcNAc$ and 15% as $Man_7GlcNAc$. Abbreviations: M_9, $Man_9GlcNAc$; M_8, $Man_8GlcNAc$; M_7, $Man_7GlcNAc$; M_6, $Man_6GlcNAc$; M_5, $Man_5GlcNAc$.

mannosidase(s). It has been shown that gC synthesized in HSV-1-infected BHK cells after a 20 min pulse with [^{3}H]-mannose contains mostl $Man_8GlcNAc_2$ and $Man_7GlcNAc_2$ structures [16]. The gC isolated from Ric^{R}14 cells after pulse-labeling with [^{3}H]-mannose for 10 min was treated as above. All the radioactivity in Pronase glycopeptides was also entirely released by Endo-H in this case and the oligosaccharides migrated as large size polymannosides when subjected to TLC (system A) (Fig. 3). $Man_8GlcNAc_2$ was by far the major component (85% in molar terms).

This result indicates that the first steps of *N*-linked oligosaccharide assembly in gC from Ric^{R}14 cells follow the well known biosynthetic pathway consisting of the transfer of $Glc_3Man_9GlcNAc_2$ from the dolichol carrier to the nascent peptide and in the sequential trimming of glucose and the first mannose residues [23].

Analysis of O-linked Oligosaccharides of gC from HSV-1/Ric^{R}14 Cells

Purification of gC from HVS-1-infected Ric^{R}14 cells was performed after extensive labeling with [^{14}C]-glucosamine. Under these conditions *O*-linked chains with *N*-acetylgalactosamine at their reducing end also become metabolically labeled. The glycopeptides obtained from Pronase digestion of glucosamine-labeled gC were analyzed by Bio-Gel P-10 and separated by Con A-Sepharose. Of the glucosamine-labeled glycopeptides 34% were eluted from the Bio-Gel column as components larger than high-mannose

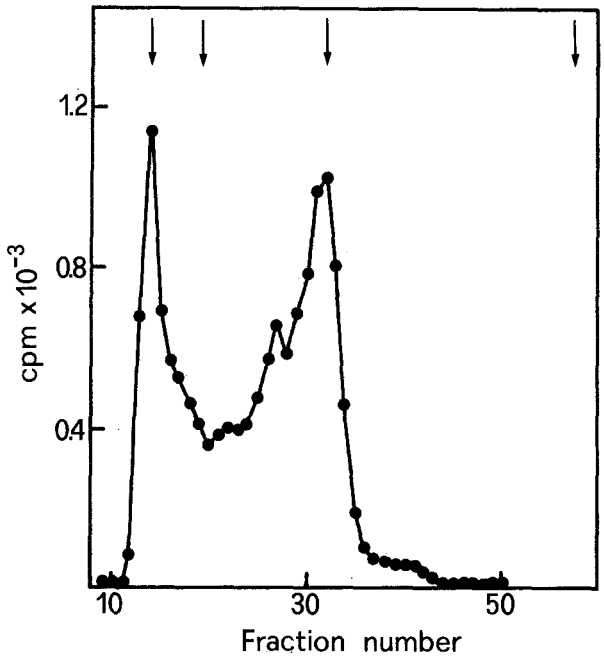


Figure 4. Bio-Gel P-10 filtration of Pronase-digested [^{14}C]glucosamine-labeled gC from HSV-1-infected Ric^{R}14 cells. Arrows as in Fig. 2.

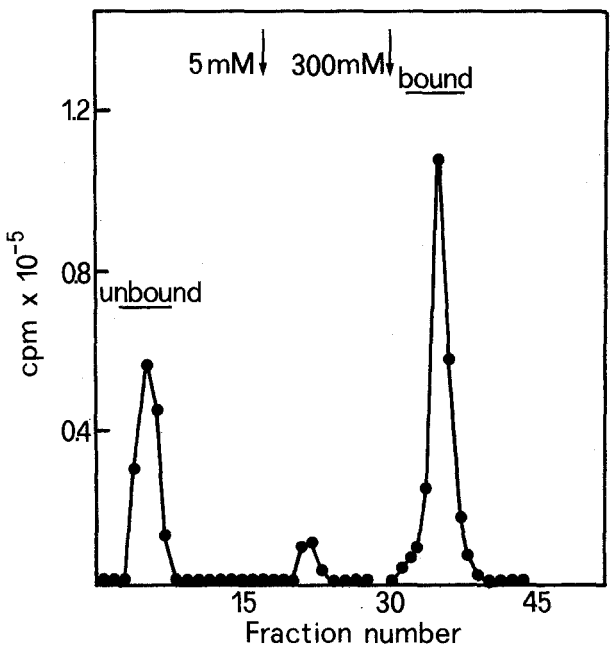


Figure 5. Affinity chromatography on Con A-Sepharose of Pronase glycopeptides from [^{14}C]-glucosamine-labeled gC from HSV-1-infected Ric^{R}14 cells. The arrows indicate the change of eluent (5 mM and 300 mM methyl α-mannoside). The fractions were pooled as indicated by the bars.

glycopeptides (Fig. 4). Accordingly, 37% were recovered as unbound material from Con A-Sepharose (Fig. 5). Both results suggested that a relevant portion of the radioactivity present in glucosamine-labeled gC was in the *O*-linked chains.

To evaluate the amino sugar composition of the glycopeptide species separated by Con A-Sepharose (Con A-unbound and Con A-bound fractions in Fig. 5), they were subjected to strong acid hydrolysis and analyzed by TLC. After hydrolysis, *N*-acetylglucosamine and *N*-acetylgalactosamine were recovered as the corresponding deacetylated compounds, glucosamine and galactosamine, respectively. As expected, *N*-acetylglucosamine was the predominant labeled amino sugar of the fraction which behaved as a high-mannose glycopeptide (Con A-bound fraction) whereas *N*-acetylgalactosamine was by far the major component present in the fraction thought to contain the *O*-linked chains (Con A-unbound fraction) (Fig. 6).

Confirmation that the Con A-unbound fraction contained oligosaccharides linked to hydroxyamino acids was obtained by subjecting it to mild alkaline borohydride treatment. As shown in Fig. 7, a change in the chromatographic profile was observed after β-elimination, indicating that *O*-linked oligosaccharides had been cleaved. The occurrence in the Con A-bound fraction of some radioactive *N*-acetylgalactosamine suggests that the peptide fragment carried *O*-linked chains in addition to a high-mannose oligosaccharide. This was confirmed by the observation that after β-elimination a portion of the radioactivity was released as oligosaccharides that no longer bound to Con A-Sepharose (data not shown). The oligosaccharides, released by mild alkaline borohydride treatment from both Con A-unbound and -bound fractions, were subjected to strong acid hydrolysis and analyzed by TLC (system B). This system separates galactosaminitol from galactosamine and glucosamine. Most of the radioactivity from both hydrolysates was recovered in the migration position of authentic galactosaminitol.

Altogether these results indicated that the vast majority of the *N*-acetylgalactosamine present in gC from HSV-1-infected Ric^R14 cells was located at the reducing end of *O*-linked chains. The small amount of radioactive *N*-acetylglucosamine recovered in the Con A-unbound fraction may be associated with *O*-linked chains or, more probably, with some tri-antennary *N*-linked chains which had "escaped" from the enzymatic block of *N*-acetylglucosaminyltransferase I. Consistent with an incomplete defect in the enzyme was the occurrence of small amounts of glycopeptides weakly bound to Con A-Sepharose (fractions 20-24 in Fig. 5), to which a di-antennary *N*-linked structure can be assigned.

In a previous study [17], we reported that in gC from HSV-1-infected BHK cells the vast majority of the *N*-acetylgalactosamine was present as the innermost sugar of *O*-linked chains, while *N*-acetylglucosamine was exclusively present in di- and tri-antennary *N*-linked chains. Therefore, the content of *N*-acetylgalactosamine accounts for the number of *O*-linked chains in gC from both mutant and wild type cells. Since we know the degree of *N*-linked oligosaccharide processing for both forms of gC, we can calculate the difference in *N*-acetylglucosamine content of the two glycoprotein forms. In fully glycosylated gC from HSV-1-infected BHK cells [16, 17] about half of the *N*-linked glycans have a di-antennary structure (four *N*-acetylglucosamine residues) and half have a tri-antennary structure (five *N*-acetylglucosamine residues). Therefore, the average number of *N*-acetylglucosamines in each *N*-linked unit is 4.5, whereas partially glycosylated gC from mutant cells carries high-mannose chains with two *N*-acetylglucosamine residues. Thus the content of labeled *N*-acetylglucosamine in fully glycosylated gC is more

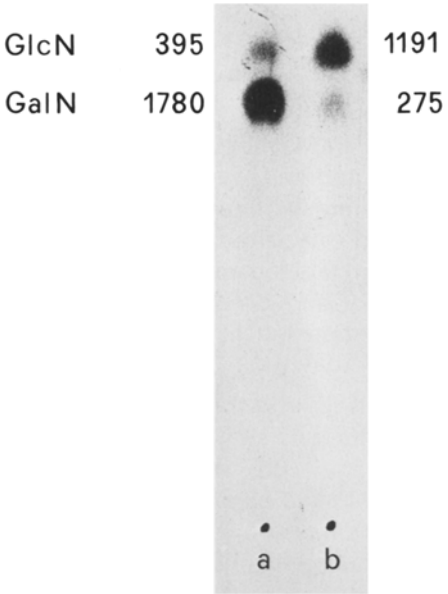


Figure 6. TLC (system B) of the strong acid hydrolysates of Con A-unbound (lane a) and Con A-bound (lane b) glycopeptides from Fig. 5. The spots were visualized by autoradiography. The numbers indicate the amount of radioactivity (in cpm) quantified by liquid scintillation counting after scraping off the gel corresponding to the spots.

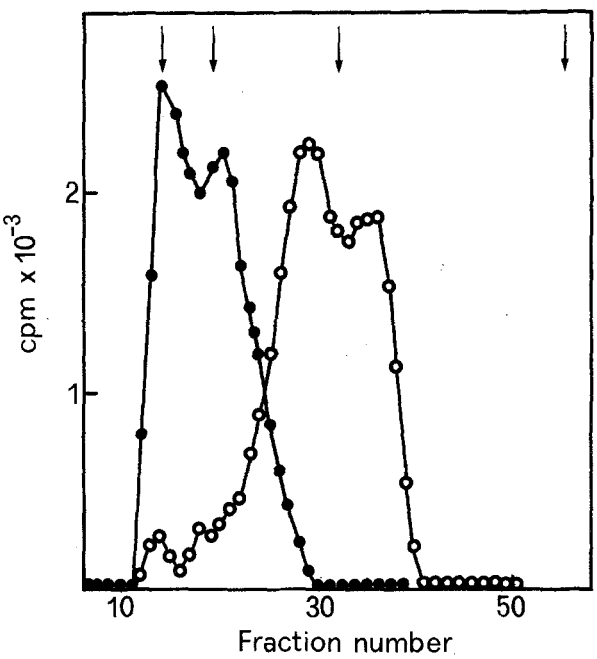


Figure 7. Bio-Gel P-10 filtration of Con A-unbound fractions from Fig. 5 before (●) and after (○) mild alkaline borohydride treatment. The treatment was performed as described in the text. Arrows as in Fig. 2.

Table 1. Distribution of radioactivity between aminosugars obtained from strong acid hydrolysates of gC synthesized by HSV-1-infected BHK and Ric^{R}14 cells.[a]

Source of gC	Radioactivity (cpm)[b]		
	GlcN	GalN	$\frac{[^{14}C]GlcN}{[^{14}C]GalN}$
HSV-1-infected BHK	3981	1532	2.6
HSV-1-infected Ric^{R}14	2360	2159	1.1

[a] [^{14}C] Glucosamine-labeled gC from both virus-cell systems was digested with Pronase as described in the text. Pronase-digests were loaded on a Bio-Gel P-2 column equilibrated with water and the glycopeptides eluted in the void volume were lyophilized and subjected to strong-acid hydrolysis. The dried samples were then analyzed by TLC (system B).

[b] Areas of the gel corresponding to the migration position of GlcN and GalN were scraped off the plate and the radioactivity determined by scintillation counting.

than twice (2.25 ×) that of partially glycosylated gC. By assuming that the specific activity of the two amino sugars was identical, because of the long labeling period with [^{14}C]-glucosamine, the extent of *O*-glycosylation may be calculated for each glycoprotein by the relative distribution of radioactivity between *N*-acetylglucosamine and *N*-acetylgalactosamine. Supposing that the same number of *O*-linked chains are carried by fully and partially glycosylated gC, we should expect a [^{14}C]-GlcNAc/[^{14}C]-GalNAc ratio 2.25 fold higher in fully glycosylated gC than in partially processed gC because of the difference in the *N*-acetylglucosamine content. Table 1 shows that the differences in the distribution of radioactivity between the two amino sugars were very close to the value calculated above.

Our results indicate that the extent of *O*-glycosylation was very similar for the two forms of gC and support the conclusion that the impairment of *N*-glycosylation did not change significantly the extent of *O*-glycosylation in gC synthesized in Ric^{R}14 cells.

Electrophoretic Mobility of gC from HSV-1-Infected Ric^{R}14 Cells

Fig. 8 shows the electrophoretic mobility of gC from HSV-1-infected Ric^{R}14 and BHK cells. The apparent molecular weight of the two glycoprotein preparations was 108 000 for the former and 120 000 for the latter, indicating that the block in the processing of *N*-linked chains significantly reduced the M_r of the glycoprotein. The results reported above show that the *N*-linked chains carried by gC from mutant cells are almost exclusively the hexasaccharide $Man_4GlcNAc_2$ with a molecular weight of 1 050, whereas the oligosaccharides carried by gC from parental wild type cells are di- and tri-antennary oligosaccharides with a molecular weight of 2 200 and 2 850, respectively. Therefore, it can be calculated that the conversion of each *N*-linked chain from high-mannose to antennary type accounts for an average mass increment of 1 400 Da. The difference in the apparent M_r between fully and partially glycosylated gC was 12 000; dividing this value by the mass increment occurring at each *N*-linked chain (about 1 400 Da) indicates that eight oligosaccharides were fully processed in gC synthesized by BHK cells. This number is consistent with the value obtained by DNA sequencing studies [24] that predicted

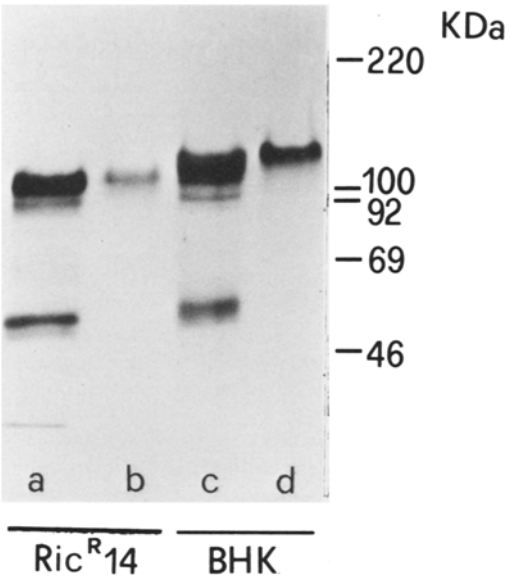


Figure 8. Autoradiography of sodium dodecylsulfate gel electrophoresis of [^{3}H]-mannose-labeled HSV-1 glycoproteins from BHK (lane a and b) or Ric^{R}14 cells (lane c and d). Lanes a and c; total cell lysates; lanes b and d; gC isolated by HC1-Sepharose affinity chromatography. The numbers on the right indicate the molecular weights of the reference substances.

eight *N*-glycosylation sites in the peptide backbone of gC. Therefore, the difference in mobility between fully and partially processed gC indicates that all oligosaccharides in the eight sites underwent complete processing in gC synthesized by HSV-1/BHK cells.

In conclusion we report that in Ric^{R}14 cells the processing of the *N*-linked oligosaccharides of HSV-1 glycoproteins is halted at the stage of high-mannose chains. This results in a delay in virion egress, which, in turn, may induce a longer persistence of the viral glycoproteins, including gC, within the cells. Current results show that these conditions do not affect the extent of *O*-glycosylation.

Consistent observations [25-27] have been reported in the literature that addition of the first residue of the *O*-linked chains is an early post-translational event occurring after the *en block* transfer of high-mannose oligosaccharides to the nascent peptide. By using a cell-free system we found that the *N*-acetylgalactosaminyltransferase responsible for initiation of *O*-linked chains in HSV-1 glycoproteins made in BHK and Ric^{R}14 cells acts exclusively on the precursor forms of the viral glycoproteins which carry high-mannose chains [28]. On this basis, we proposed that the biosynthesis of HSV-1 glycoproteins followed the schematic model proposed for other *N*- and *O*-glycosylated proteins [25-27]. According to this scheme, after the addition of high-mannose oligosaccharides to the nascent peptide, an early event is the transfer of *O*-linked *N*-acetylgalactosamine to the peptide, followed by extension of *O*-linked chains and branching of *N*-linked oligosaccharides.

The present results are consistent with our previous proposal that *N*-acetylgalactosaminyltransferase acts prior to maturation of *N*-linked oligosaccharides, since absence of complex type chains in gC from Ric^R14 cells had no effect on the amount of *O*-linked glycans added. The extent of *O*-glycosylation of gC in the mutant Ric^R14 cells did not increase in spite of the high number of serine and threonine residues available in the outward facing portion of gC, as deduced from DNA sequencing studies [24]. Altogether the results suggests a strict control on the initiation of the *O*-linked chains. It is possible that the folding of gC, a consequence of its primary structure and to the addition of high-mannose chains, results in a constraint upon the activity of UDP-GalNAc:protein *N*-acetylgalactosaminyltransferase, so that only a fraction of the serine/threonine residues are available for the addition of *N*-acetylgalactosamine.

Detailed analyses of the high-mannose oligosaccharides carried by Sindbis virus and vesicular stomatitis virus glycoproteins synthesized by a mutant clone of chinese hamster ovary cells (clone 15) lacking *N*-acetylglucosaminyltransferase I have been reported [5, 29]. A $Man_5GlcNAc_2$ chain was the oligomannoside prevalent in the glycoproteins extracted from extracellular virions whereas $Man_4GlcNAc_2$ chains were present only in trace amounts (4-5%). The acumulation of $Man_5GlcNAc_2$ structures was consistent with the cell enzyme defect, since this high-mannose chain is the substrate for *N*-acetylglucosaminyltransferase I [30]. Enzymatic transfer of *N*-acetylglucosamine to $Man_5GlcNAc_2$ is usually required for the removal of the last two mannose residues by Golgi mannosidase II [29].

In the present study we found that gC extracted from HSV-1-infected Ric^R14 cells, which also lack *N*-acetylglucosaminyltransferase I, contains $Man_4GlcNAc_2$ chains. The accumulation of this oligosaccharide in such quantities implies that HSV-I-infected Ric^R14 cells are able to cleave an α1-6Man residue from $Man_5GlcNAc_2$. An α(1-6)-mannosidase with activity towards $Man_5GlcNAc_2$ has been postulated to be responsible for the catabolism of high-mannose oligosaccharides accumulated in swainsonine-treated animals [31]. In fact, Daniel *et al.* [31] found a large amount of $Man_4GlcNAc_2$ (probably derived from $Man_5GlcNAc_2$) in urine of sheep intoxicated with locoweed (which contains swainsonine). Under our conditions the prolonged persistence of viral glycoproteins within the cells and the cytopathic action of the virus might facilitate the activity of this mannosidase so that the $Man_5GlcNAc_2$-chains are converted to $Man_4GlcNAc_2$-chains.

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