Temporal aspects of *O*-glycosylation of glycoprotein C from herpes simplex virus type-1

Franca SERAFINI-CESSI,* Fabio DALL'OLIO, Nadia MALAGOLINI and Gabriella CAMPADELLI-FIUME
Dipartimento di Patologia Sperimentale, Università di Bologna, Via San Giacomo 14, 40126 Bologna, Italy

Herpes simplex virus type-1 glycoprotein C (gC1) contains several *O*-linked oligosaccharides clustered near *N*-linked chains, and Pronase digestion produces glycopeptides carrying both oligosaccharide types. We have taken advantage of this fact to investigate the temporal relationship between the initiation of *O*-linked chains and the processing of *N*-linked oligosaccharides. gC1 was isolated from herpes-simplex-virus-infected BHK (baby-hamster kidney) cells after short labelling periods with [³H]glucosamine, and the labelled Pronase-cleaved glycopeptides fractionated on concanavalin A-Sepharose. *N*-[³H]Acetylgalactosamine, mostly convertible into free *N*-[³H]acetylgalactosaminitol on mild alkaline-borohydride treatment, was found in glycopeptides with an affinity to concanavalin A-Sepharose corresponding to that of glycopeptides carrying Man₈GlcNAc₂ or larger *N*-linked chains. Since there is evidence that the processing of *N*-linked chains up to Man₈GlcNAc₂ involves enzymes located in the rough endoplasmic reticulum, current results strongly suggest that gC1 acquires *O*-linked *N*-acetylgalactosamine before the glycoprotein routing to the Golgi apparatus. The addition of the second sugar to the nascent *O*-linked chain appeared to occur after a relatively long lag time.

INTRODUCTION

Glycoprotein C (gC1) is one of the major glycoproteins specified by herpes simplex virus type 1 (HSV-1). The *M_r* of the peptide backbone predicted by the DNA sequence analysis (Frink *et al.*, 1983; McGeoch *et al.*, 1988) is 60000, whereas the apparent size of the mature glycoprotein is 120000 (Spear, 1976; Campadelli-Fiume & Serafini-Cessi, 1984). Such a difference is due to the addition of *N*- and *O*-linked oligosaccharides. Indeed, eight potential *N*-glycosylation sites were predicted in the primary structure of the glycoprotein (McGeoch *et al.*, 1988), and all are actually glycosylated (Serafini-Cessi *et al.*, 1984; Dall'Olio *et al.*, 1986). In addition, at least 12–16 *O*-linked chains have been calculated to be present in the mature form of gC1 (Dall'Olio *et al.*, 1985, 1986). *N*-Linked oligosaccharides of mature gC1 accumulating within infected cells have the structure referred to as 'complex type' (Serafini-Cessi *et al.*, 1984), whereas among *O*-linked oligosaccharides the predominant one is the NeuAc-Galβ-GalNAc trisaccharide (Dall'Olio *et al.*, 1985). Some of the *O*-linked oligosaccharides are closely spaced in the peptide and located near *N*-linked oligosaccharides, so that Pronase digestion results in the formation of large glycopeptides carrying one *N*-linked and three or four *O*-linked chains (Dall'Olio *et al.*, 1985). For these reasons gC1 is a good model to study the biosynthesis of both *N*- and *O*-linked chains.

The aim of the present study was to ascertain the timing of the addition of the *O*-linked *N*-acetylgalactosamine (GalNAc) during glycoprotein biosynthesis. Pertinent to this study are the following considerations. (i) The degree of processing of *N*-linked oligosaccharides, namely the length of polymannoside

chains, is indicative of the location of a glycoprotein during its transit from the rough endoplasmic reticulum (RER) to the Golgi apparatus; thus, if the biosynthesis of a glycoprotein carrying both *N*- and *O*-linked chains is halted after the addition of the first *O*-linked sugar (GalNAc for gC1), the identification of the structure of its *N*-linked oligosaccharides allows one to establish the timing of the initial *O*-glycosylation. (ii) Radioactive glucosamine is incorporated into glycoproteins as *N*-*N*-acetylglucosamine (GlcNAc), GalNAc and *N*-acetylneuraminic acid; the recovery of labelled GalNAc convertible into free *N*-acetylgalactosaminitol (GalNAc-ol) by mild alkaline-borohydride treatment indicates that the *O*-glycosylation is at the first step. (iii) With a short pulse of radioactive glucosamine, *N*-linked chains incorporate labelled GlcNAc at different periods during glycoprotein biosynthesis, co-translationally, because of the transfer of the high-mannose oligosaccharide, which contains the chitobiosyl unit, post-translationally because of Golgi GlcNAc transferases initiate the assembly of side-branched chains. (iv) Pronase glycopeptides carrying *N*-linked chains bind to concanavalin A (Con A)-Sepharose according to the degree of the processing of oligomannose units, and the affinity of the high-mannose oligosaccharides increases as a function of the number of the mannose residues (Krusius *et al.*, 1976; Baenziger & Fiete, 1979).

In the present study we have taken advantage of the fact that Pronase glycopeptides from gC1 carry both *N*- and *O*-linked chains to investigate the temporal relationship between the initial addition of *O*-linked GalNAc and the processing of *N*-linked oligosaccharides. gC1 was isolated from HSV-1-infected baby-hamster kidney (BHK) cells after short labelling periods with

Abbreviations used: HSV-1, herpes simplex virus type 1; gC1, glycoprotein C; RER, rough endoplasmic reticulum; HCl, monoclonal antibodies against gC1; Endo H, endo-β-*N*-acetylglucosaminidase; Con A, concanavalin A; BHK, baby-hamster kidney; Xstrongly, extra strongly.

* To whom correspondence and reprint requests should be sent.

[³H]glucosamine, subjected to Pronase digestion and the glycopeptides fractionated on Con A-Sepharose. The occurrence of radioactive *O*-linked GalNAc and the size of the labelled *O*-linked chain were determined in each glycopeptide fractionated by Con A-Sepharose. [³H]GalNAc, mostly convertible into free GalNAc-ol upon β -elimination, was found in glycopeptides with increasing affinity to the Con A, including those with a very high affinity to the lectin-gel. These results support the notion that the first step of *O*-glycosylation occurs, at least in some *O*-glycosylation sites, when the glycoprotein still carries *N*-linked oligosaccharides poorly processed by mannosidases.

EXPERIMENTAL

Materials

Monoclonal antibodies against gC1 (HC1) were a gift from Dr. L. Pereira (University of California, San Francisco, CA, U.S.A.). [³H]Glucosamine (40 Ci/mmol) and KB³H₄ (67 mCi/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. CNBr-activated Sepharose 4B and Con A-Sepharose (approx. 10 mg of Con A/ml of gel sediment) were from Pharmacia Fine Chemicals. Bio-Gel P-4 (–400 mesh) and Bio-Gel P-2 (200–400 mesh) were obtained from Bio-Rad Laboratories. Pronase was from Sigma, and endo- β -*N*-acetylglucosaminidase (Endo H) was from Miles Laboratories. HC1 monoclonal antibodies were linked to CNBr-activated Sepharose as described by Serafini-Cessi *et al.* (1984). High-mannose glycopeptides (Unit A) of thyroglobulin were prepared by Pronase digestion as described by Godelaine *et al.* (1981). [³H]GalNAc-ol and [³H]Gal β -GalNAc-ol were prepared as described by Serafini-Cessi *et al.* (1985). Cellulose- and silica-gel plates as well as the NH₂-Lichrosorb (5 μ m particle size) column (0.4 cm $\times$ 12.5 cm) were purchased from Merck. All chemicals were of Reagent grade.

Metabolic labelling and isolation of gC1

BHK-cell monolayers were infected with HSV-1(F) at an input multiplicity of infection of 10 plaque-forming units/cell. Cells were labelled at 10 h after injection for 10, 20 and 30 min with medium prewarmed to 37 °C, consisting of minimal essential medium containing no glucose and 300 μ Ci of [³H]glucosamine/ml. At the end of the pulse period the cells were washed three times with 0.02 M-sodium phosphate/0.15 M-NaCl buffer, pH 7.4, at 4 °C, and glycoproteins were extracted with detergents as previously described (Serafini-Cessi *et al.*, 1985). [³H]Glucosamine-labelled gC1 was purified by affinity chromatography on HC1-Sepharose and its purity ascertained by polyacrylamide-gel electrophoresis.

Preparation of Pronase glycopeptides

[³H]Glucosamine-labelled gC1 was incubated in 0.1 M-Tris/HCl, pH 8, with Pronase at 60 °C for 24 h. Three additions of the enzyme over the incubation period were done up to a final concentration of 0.13% (w/v). Pronase had been incubated in the same buffer for 1 h at 37 °C to inactivate contaminating enzymes. At the end of the incubation time, the digest was boiled for 3 min and applied to a Bio-Gel P-2 column (2 cm $\times$ 40 cm) equilibrated and eluted with water. The fractions eluted in the void volume for the column were pooled, freeze-dried and dissolved in 1 ml of water.

Column chromatography

The Con A-Sepharose column (0.5 cm $\times$ 10 cm) was equilibrated in 20 mM-Tris/HCl, pH 7.5, containing 0.15 M-NaCl, 0.02% CaCl₂, 0.02% MnCl₂ and 0.02% MgCl₂ (Hughes & Mills, 1983). The column was eluted with the equilibrating buffer and subsequently with 5 and 300 mM- α -methyl mannoside dissolved in the equilibrating buffer. The peak fractions were pooled, freeze-dried and applied to a Bio-Gel P-2 column in order to remove salts and the sugar. Bio-Gel P-4 filtration of oligosaccharides released by mild alkaline-borohydride treatment was performed on a column (1 cm $\times$ 80 cm) equilibrated and eluted with 0.1 M-acetate/pyridine buffer, pH 5.

Mild alkaline-borohydride treatment

Desalted glycopeptides were treated with 1 M-NaBH₄ in 0.1 M-NaOH for 72 h at 37 °C (final volume 1 ml) in sealed tubes. At the end of this period the samples were acidified with acetic acid and freeze-dried. Methyl borate was removed by several evaporations in methanol/acetic acid.

Endo H treatment of unit A of thyroglobulin. Unit A (high-mannose glycopeptide) of thyroglobulin was fractionated on Con A-Sepharose as detailed above. The fractions eluted immediately and in a retarded manner by 300 mM- α -methyl mannoside were separately pooled, desalted on a Bio-Gel P-2 column, freeze-dried and subjected to Endo H treatment (Serafini-Cessi *et al.*, 1984). The oligosaccharides released by the enzyme were incubated with tritiated borohydride in order to label the reducing end and were fractionated by t.l.c. with the method described by Godelaine *et al.* (1981).

T.l.c. The composition in radioactive amino sugars of glycopeptides was performed after acid hydrolysis in 4 M-HCl, for 4 h at 100 °C. After removal of acid by evaporation under reduced pressure, the hydrolysed samples were applied to silica-gel plates and developed with a solvent containing borate, which allows a good separation of glucosamine and galactosamine (Dall'Olio *et al.*, 1985) (system A). Separation of GalNAc-ol and Gal β -GalNAc-ol was performed by t.l.c. as described by Edge & Spiro (1984) (system B).

H.p.l.c.

Labelled GalNAc-ol was separated from GlcNAc-ol, GalNAc and GlcNAc by a Waters h.p.l.c. apparatus equipped with a NH₂-Lichrosorb (12.5 cm $\times$ 0.4 cm) column. Chromatography was performed isocratically with a solvent mixture of acetonitrile/15 mM-KH₂PO₄ (17:3, v/v) as mobile phase, at a rate of 0.5 ml/min (Bergh *et al.*, 1981).

RESULTS

O-Glycosylation of gC1 labelled with a 10, 20 or 30 min pulse of [³H]glucosamine

Since in mature gC1 GalNAc has been only found at the terminal reducing end of *O*-linked chains (Dall'Olio *et al.*, 1985), the identification of labelled GalNAc in [³H]glucosamine-pulsed gC1 may be assumed as an indication of the assembly of *O*-linked chain. In

Fig. 1. T.l.c. of labelled components generated upon β -elimination from gC1 isolated from 10 min- (a), 20 min- (b) and 30 min- (c) [^3H]glucosamine-pulsed HSV-1-infected BHK cells

[^3H]Glucosamine-labelled gC1 isolated as described in the text was subjected to Pronase digestion and chromatographed on a Bio-Gel P-2 column. Labelled glycopeptides eluted in the void-volume fractions were freeze-dried, dissolved in 1 ml of water and an aliquot subjected to mild alkaline-borohydride treatment as described in the text. The samples were then reappplied to the same Bio-Gel P-2 column. The labelled components recovered in the included volume were applied to coupled columns of Dowex 50 (H^+ form) and Dowex 1 (formate form) to remove salts. The flow-through fractions were freeze-dried and applied to cellulose plates. T.l.c. was developed as described in the text (system B). The areas of the cellulose were scraped off of the plates and radioactivity was determined by liquid-scintillation counting. The percentage of [^3H]Gal β -GalNAc-ol was calculated by taking the radioactivity recovered in the migration position of Gal β -GalNAc-ol (arrow 1) and GalNAc-ol (arrow 2) as 100%.

hydrolysates of 10, 20 or 30 min-pulsed gC1, [^3H]galactosamine was detected and the ratio [^3H]galactosamine/[^3H]glucosamine was found to be 0.19, 0.25 and 0.26 respectively. The fact that from 10 min- to 30 min-pulsed gC1 there was only a small increase in the relative amount of [^3H]galactosamine strongly suggests that most of the potential O-glycosylation sites of gC1 have been glycosylated within the 10 min of labelling period. To ascertain the extent of elongation of

Fig. 2. Con A-Sepharose chromatography of Pronase glycopeptides from [^3H]glucosamine-labelled gC1 (10 min-pulsed), and their amino-sugar composition

Pronase glycopeptides from gC1 isolated from HSV-1-infected BHK cells pulsed with [^3H]glucosamine for 10 min were obtained as described in the legend to Fig. 1. (b) Con A-Sepharose chromatography was performed as described in the text, and the arrows indicate the change of eluant (5 mM- and 300 mM- α -methyl mannoside). Fractions under each peak were pooled as indicated by the horizontal bars, freeze-dried and desalted by Bio-Gel P-2 filtration. An aliquot of each sample was subjected to strong acid hydrolysis (4 M-HCl; 4 h at 100 $^{\circ}\text{C}$) and analysed for amino-sugar composition by t.l.c. (system A). (a) The radioactivity recovered in the migration position of glucosamine (open column) and galactosamine (filled column) was determined by scraping off the gel from the plate and subjecting it to liquid-scintillation spectrometry.

O-linked chains in the three gC1 preparations, their total Pronase glycopeptides were subjected to mild alkaline-borohydride treatment and the released labelled oligosaccharides separated by t.l.c. As Fig. 1 shows, 12, 20 and 24% of the total radioactivity released as O-linked oligosaccharides from 10, 20 and 30 min-pulsed gC1 were found to migrate as Gal β -GalNAc-ol and the remainder as GalNAc-ol. These results indicate that, particularly in the shortest-pulse experiment, the biosynthesis of gC1 O-linked oligosaccharides involved in most cases the addition only of the first GalNAc residues. Since the increase in the labelling period did not result in a proportional elongation of GalNAc to Gal-GalNAc, it may be inferred that there is a significant lag time before the addition of the second residue in O-linked chains of gC1.

Fractionation by Con A-Sepharose chromatography and labelled-amino-sugar analysis of Pronase glycopeptides from 10 min-pulsed gC1

Since in 10 min-pulsed gC1 the O-glycosylation was found to be mostly halted at the stage of the GalNAc

Fig. 3. T.l.c. of high-mannose oligosaccharides released by Endo H treatment from unit A of thyroglobulin fractionated on Con A-Sepharose

Unit A of thyroglobulin was subjected to Con A-Sepharose chromatography as described in the legend to Fig. 2. Fractions promptly eluted by 300 mM- α -methyl mannoside (A) and those eluted in a retarded manner (B) were separately pooled, freeze-dried, desalted on a Bio-Gel P-2 column and digested with Endo H as described in the text. The released oligomannosides were labelled with KB^3H_4 , desalted by coupled columns of Dowex 50 (H^+ form) and Dowex 1 (formate form) and fractionated on t.l.c. as described by Godelaine *et al.* (1981). The labelled oligosaccharides were revealed by autoradiography. Abbreviations are as follows: M_9 , $\text{Man}_9\text{GlcNAc}$; M_8 , $\text{Man}_8\text{GlcNAc}$; M_7 , $\text{Man}_7\text{GlcNAc}$; M_6 , $\text{Man}_6\text{GlcNAc}$; M_5 , $\text{Man}_5\text{GlcNAc}$.

addition, further experiments were performed on this preparation. Glycopeptides carrying *O*-linked chains and *N*-linked oligosaccharides of triantennary type are unretained by Con A-Sepharose (Con A-unbound glycopeptides). A low concentration (5 mM) of α -methylmannose eluted biantennary *N*-linked chains (Con A-weakly-bound glycopeptides), whereas, to remove glycopeptides carrying high-mannose chains, a high concentration of α -methylmannose is required (Con A-strongly-bound glycopeptides) (Krusius *et al.*, 1976). The Con A-Sepharose elution pattern of [^3H]glucosamine-labelled glycopeptides from 10 min-pulsed gC1 is shown in Fig. 2(b). In Fig. 2(a) the labelled-amino-sugar composition of each glycopeptide peak is reported. The highest amount of [^3H]glucosamine-derived radioactivity was found in the Con A-unbound glycopeptides, as well as the highest relative percentage of [^3H]galactosamine. The last result was expected, because many *O*-linked chains of gC1 are not clustered near any *N*-linked chain, thereby they were unretained by Con A-Sepharose. An easily detectable amount of [^3H]galactosamine was found in hydrolysates of all glycopeptides bound to Con A-Sepharose, confirming that the Pronase digestion results in the production of peptides fragments carrying

Fig. 4. Bio-Gel P-4 filtration profiles of glycopeptides from [^3H]glucosamine-labelled gC1 (10 min-pulsed) after mild alkaline-borohydride treatment

Glycopeptide fractions separated as shown in Fig. 2 were subjected to mild alkaline-borohydride treatment as described in the text and applied to a Bio-Gel P-4 column equilibrated and eluted with 0.1 M-pyridine/acetic acid buffer, pH 5. Arrows indicate the void volume (Blue Dextran) and the elution position of $\text{Gal}\beta\text{-GalNAc-ol}$ (1) and GalNAc (2). (a) Con A-weakly-bound glycopeptides; (b) Con A-strongly-bound glycopeptides; (c) Con A-Xstrongly-bound glycopeptides.

both *N*- and *O*-linked chains. A portion of high-mannose glycopeptides were promptly eluted by 300 mM- α -methyl mannoside (Con A-strongly-bound glycopeptides), but a significant amount was removed by the column in a retarded manner. These latter fractions were pooled separately from those promptly eluted and were designated 'Con A-X (extra) strongly-bound glycopeptides'. A reasonable explanation for such a high affinity to Con A-Sepharose could be that these glycopeptides carry oligomannosides with a high number of mannose residues, such as those not yet trimmed by the first mannosidase(s). To prove this point, we fractionated on the same column a glycopeptide preparation carrying oligosaccharides ranging from Man_5 to $\text{Man}_9\text{GlcNAc}_2$ (unit A of thyroglobulin). Consistent with our expectations, only Man_5 and $\text{Man}_8\text{GlcNAc}_2$ chains were found in the glycopeptides eluted as Con A-Xstrongly-bound components (Fig. 3).

Fig. 5. Identification by h.p.l.c. of [^3H]GalNAc-ol generated upon β -elimination from Con A-Xstrongly-bound glycopeptides

Fractions in Fig. 4(c) eluted in the elution volume of a monosaccharide were pooled and indicated by the horizontal bar and freeze-dried. To remove salts, the sample was applied to double columns of Dowex 50 and Dowex 1, and the flow-through fractions were freeze-dried, dissolved in 0.2 ml of deionized water and injected on a NH_2 -Lichrosorb column. Chromatography was performed as described in the text. Fractions were collected and counted for radioactivity. Retention times of standard components are indicated by arrows as follows: 1, GlcNAc; 2, GalNAc; 3, GalNAc-ol; 4, GlcNAc-ol.

Characterization of the products generated by mild alkaline-borohydride treatment of glycopeptides bound to Con A-Sephrose

Con A-weakly-bound, -strongly-bound and -Xstrongly-bound glycopeptides were applied to a Bio-Gel P-4 column. The fractions eluted in the void volume and contiguous region were pooled and freeze-dried. These samples were then subjected to mild alkaline-borohydride treatment and reapplied to the same column. The treatment released two labelled components from Con A-weakly-bound glycopeptides, the larger was eluted in the elution volume of Gal-GalNAc-ol and the smaller in that of monosaccharides (Fig. 4a); by contrast, only the smaller component was generated upon β -elimination of the two glycopeptides, with higher affinity to Con A-Sephrose column (Figs. 4b and 4c). By means of an h.p.l.c. analysis which separates GalNAc-ol from GlcNAc-ol, GalNAc and GlcNAc, the smaller component generated from all three glycopeptides was identified as GalNAc-ol (a representative h.p.l.c. fractionation is shown in Fig. 5). Consistent with its elution volume the larger labelled oligosaccharide generated upon β -elimination from Con A-weakly-bound glycopeptides migrated on t.l.c. as Gal β -GalNAc-ol. These results support the notion that, within the 10 min of metabolic labelling: (i) the initial O-glycosylation of gC1, at least in some hydroxyamino acids, occurs when N-linked chains still have an oligomannoside unit poorly processed by early glycosidase; (ii) the addition of the second sugar to O-linked GalNAc takes place when the vicinal N-linked chain has been processed up to the initial addition of sugars in side-branched chain(s).

DISCUSSION

The subcellular site of initial O-glycosylation of glycoproteins is not yet firmly established. By contrast,

several lines of evidence indicate that the first step of N-glycosylation is a co-translational event, consisting of the transfer of $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ oligosaccharide from the lipid carrier to the nascent peptide. In many cell types the processing of this oligosaccharide is initiated in RER by the action of glycosidases, which remove the three glucose residues and at least one mannose residue. At this stage of the oligosaccharide biosynthesis glycoproteins are routed to the Golgi apparatus. The subsequent trimming of mannose residues is catalysed by a Golgi mannosidase(s), which still yields a high-mannose chain ($\text{Man}_5\text{GlcNAc}_2$); this step of N-linked processing seems to occur in the *cis* or *medial* cisternae of the Golgi apparatus, as well as the addition of GlcNAc residues to the side-branched chains (for references, see Kornfeld & Kornfeld, 1985).

The results presented here show that gC1 carrying $\text{Man}_8\text{GlcNAc}_2$ or less-processed N-linked chains already contains GalNAc, at least at some O-glycosylation sites. Thus, assuming that, even in cells synthesizing gC1, the first steps of N-linked oligosaccharide processing occurs as described above, our data indicate that the addition of O-linked GalNAc to some serine or threonine residues of gC1 is a very early event occurring before or during the routing of the protein from RER to the *cis* part of the Golgi apparatus.

Various reports indicate as the site of initial O-glycosylation of glycoproteins either the RER (Strous, 1979; Patzelt & Weber, 1986) or the Golgi apparatus, and for the latter compartment proposals ranged from the *cis*, *medial* and *trans* part of the stack (Cummings *et al.*, 1983; Hanover *et al.*, 1982; Johnson & Spear, 1983; Elhammer & Kornfeld, 1984; Roth, 1984; Abeijon & Hirschberg, 1987; Tooze *et al.*, 1988). Major sources of differences may be related to both the various cell systems and the extent of O-glycosylation of the glycoprotein studied in each case. Indeed, heavily O-glycosylated proteins, such as secreted mucins, appear to require an earlier initiation than glycoproteins with a low extent of O-glycosylation (Spielman *et al.*, 1987). In this context it is important to note that gC1, though it is a membrane glycoprotein, carries many O-linked oligosaccharides (Dall'Olio *et al.*, 1985, 1986) which are for the most part closely spaced in the peptide backbone.

The present results also show an heterogeneous pattern of N-linked oligosaccharides near the nascent O-linked chain, inasmuch as some gC1 glycopeptides (Fig. 4a) with a non-elongated GalNAc residue carry N-linked chains already processed by Golgi enzymes, as shown by their reduced affinity to Con A-Sephrose. Two explanations may account for this result. (i) A long lag time elapses before the addition of the second sugar to GalNAc. The time course of the appearance of Gal-GalNAc in the differently pulsed gC1 (Fig. 1) is in agreement with this explanation, as well as the finding that the enzyme responsible for the addition of galactose residues is located in the distal part of the Golgi stack (Elhammer & Kornfeld, 1984). (ii) The initial step of O-glycosylation is a non-synchronous event that varies at individual sites. The lack of synchrony may be caused by a different accessibility of each O-linked site to the peptidyl GalNAc-transferase. Since the present results account for the O-glycosylation of sites clustered near one N-linked chain, even the degree of N-linked chain processing may be relevant in facilitating the assembly of O-linked chains at individual O-glycosylation sites.

In a previous paper we showed that UDP-GalNAc:peptidyl *N*-acetylgalactosaminyltransferase of HSV-infected cells is active *in vitro* towards endogenous viral glycoproteins only when they were of precursor type, i.e. still carrying high-mannose chains (Serafini-Cessi *et al.*, 1983). In the case of glycoprotein G from HSV-2 (gG2), which carries 25 *O*-linked chains for each *N*-linked oligosaccharides (Serafini-Cessi *et al.*, 1985), we found that a precursor species with Man₈GlcNAc₂ as major *N*-linked chain was already heavily *O*-glycosylated (Dall'Olio *et al.*, 1987). Altogether these results support the notion that HSV glycoproteins, such as gC1 and gG2, both containing a relatively high number of *O*-linked chains, initiate the *O*-glycosylation when the *N*-linked oligosaccharides have been processed only by RER enzymes.

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