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N-Acetylgalactosaminyltransferase Activity Involved in O-Glycosylation of Herpes Simplex Virus Type 1 Glycoproteins

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We report on *N*-acetylgalactosaminyltransferase (UDPacetylgalactosamine—protein acetylgalactosaminyltransferase; EC 2.4.1.41) activity in herpes simplex virus type 1 (HSV-1)-infected BHK and Ric^{R}14 cells, a line of ricin-resistant BHK cells defective in *N*-acetylglucosaminyltransferase I. The enzyme catalyzed the transfer of [^{14}C]*N*-acetylgalactosamine (GalNAc) from UDP-[^{14}C]GalNAc into HSV glycoproteins, as identified by immunoprecipitation. The sugar was selectively incorporated into the immature forms of herpesvirus glycoproteins pgC, pgD, and gA-pgB, which are known to contain N-linked glycans of the high-mannose type. The high incorporation of [^{14}C]GalNAc into endogenous acceptors of HSV-1-infected Ric^{R}14 cells was consistent with the accumulation of immature forms of HSV glycoproteins which occurs in these cells. Mild alkaline borohydride treatment of glycoproteins labeled via GalNAc transferase showed that the transferred GalNAc was O-linked and represented the first sugar added to the peptide backbone.

Herpes simplex virus type 1 (HSV-1) specifies at least four glycoproteins designated as gA-gB, gC, gD, and gE (1, 27). The occurrence of N-linked glycans in HSV-1 glycoproteins has been reported (3, 5–7, 21, 25). Specifically, the immature forms gA-pgB, pgC, and pgD were shown to contain mannose-rich chains, cleaved by endo-β-*N*-acetylglucosaminidase H, whereas the mature forms carry differentially sialylated complex-type glycans (3, 5, 12a, 20, 25, 30). Monoantennary, diantennary, and triantennary glycans are present in HSV-1 glycoproteins synthesized in BHK cells, with triantennary chains making up the predominant species (6).

The occurrence of O-linked glycans in gA-gB and gC was reported on the basis of the release of small oligosaccharides after mild alkaline borohydride treatment (17). Johnson and Spear (12a) confirmed the presence of O-linked glycans in glycoproteins gA-gB, gC, gD, and gE and suggested that the addition of O-linked oligosaccharides is a late posttranslational modification.

The biosynthesis of O-linked glycans has been extensively studied in tissues such as submaxillary glands, which produce mucins containing large amounts of O-glycosidically linked chains (9, 11, 15). It is known that the initial step involves the activity of a UDP-*N*-acetylgalactosamine:protein *N*-acetylgalactosaminyltransferase (UDPacetylgalactosamine—protein acetylgalactosaminyltransferase; EC 2.4.1.41) which transfers *N*-acetylgalactosamine (GalNAc) to serine or threonine residues in the peptide. The assembly of sugars added later involves the sequential action of specific glycosyltransferases located in the Golgi system (2, 10). GalNAc transferase activity was also described in non-mucilaginous tissues, including in vitro-cultured cells (8). Little is known about the O-glycosylation process of viral glycoproteins, since the presence of O-linked glycans has only been reported in glycoproteins of HSV-1, vaccinia virus, and coronavirus (12a, 16, 17, 26).

The purpose of the experiments described here was to study the initial step of O-glycosylation of herpesvirus glycoproteins. Specifically, we investigated the transfer of [^{14}C]GalNAc from UDP-[^{14}C]GalNAc into viral glycoproteins identified by immunoprecipitation. Since the results provided strong evidence that GalNAc was selectively transferred in O-linkage to the immature forms of HSV glycoproteins, we also examined GalNAc incorporation into viral glycoproteins made in a ricin-resistant BHK cell line (Ric^{R}14), defective in *N*-acetylglucosaminyltransferase I (28). It was previously reported that in these cells immature forms of HSV glycoproteins carrying mannose-rich glycans accumulate (3).

BHK and Ric^{R}14 cells were grown and infected as described previously (3). Cells were harvested 12 to 15 h after infection, suspended in

TABLE 1. Requirements for GalNAc transferase activity of HSV-1(F)-infected BHK cells[a]

Incubation conditions	Enzyme activity (%)
Complete assay mixture	100
Minus incubation time	2
Minus Mn^{2+}	6
Plus EDTA (100 mM)	7
Minus Triton X-100[b]	3

[a] Supernatant fluids from cell lysates (200 μg of protein per assay) were incubated with 40 μM UDP-[^{14}C]GalNAc (61 mCi/mmol; Radiochemical Centre, Amersham, England) in 50 μl of 50 mM Tris-hydrochloride, pH 7.5, containing 40 mM $MnCl_2$. The final concentration of Triton X-100 (1.25% in the supernatant fluids) was adjusted to 0.5%. Incubation was carried out at 37°C for 1 h. The amount of labeled GalNAc incorporated into endogenous acceptors was determined by precipitating the glycoproteins with 20 volumes of 1% phosphotungstic acid in 0.5 M HCl. The precipitate was collected on glass fiber filters and then washed and counted as previously described (24). A 100% value was 6,750 dpm. Data represent the mean of two independent determinations.

[b] Supernatant fluid from a cell lysate was obtained in the absence of Triton X-100.

phosphate-buffered saline, pH 7.5, containing 1.25% Triton X-100, and lysed by freezing and thawing four times. The supernatant fluids obtained after centrifugation at 9,000 × *g* for 5 min were used to measure GalNAc transferase activity into endogenous acceptors of HSV-infected BHK and Ric^{R}14 cells. Table 1 shows the conditions and requirements of GalNAc transferase activity as measured on endogenous acceptors of HSV-1(F)-infected BHK cells. Mn^{2+} ions and Triton X-100 were essential for optimal enzyme activity, as previously reported for GalNAc transferase from other sources (9, 11, 15). Final concentrations of Triton X-100 that were higher than 0.5% reduced the overall incorporation. No difference was observed in the requirements of GalNAc transferase from infected mutant cells. For both parent and mutant cell samples, the time course of sugar incorporation followed an

TABLE 2. GalNAc transferase activity of uninfected and HSV-1-infected BHK and Ric^R 14 cells

Cells	GalNAc transferred[a] (pmol/mg of protein per h)	Wild-type cells (%)
Uninfected BHK	299	100
Uninfected Ric^R 14	278	93
HSV-1(F)-infected BHK	282 ± 19	100
HSV-1(F)-infected Ric^R 14	414 ± 59[b]	147
HSV-1(MP)-infected BHK	258 ± 45	100
HSV-1(MP)-infected Ric^R 14	398 ± 61[b]	154

[a] Supernatant fluids from cell lysates (200 μg of protein per assay) were incubated as described in Table 1, footnote *a*. Data on enzyme activity from uninfected cells represent the mean of duplicate experiments, whereas those on enzyme activity from infected cells represent the mean of five independent experiments ± standard error.

[b] Significantly different from corresponding values of wild-type cells ($P < 0.05$), as determined by Student's *t* test.

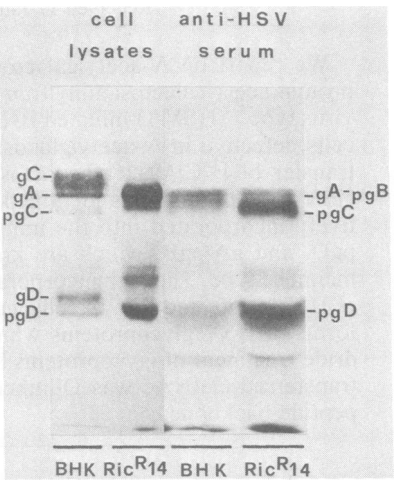

FIG. 1. Fluorography of immunoprecipitates of HSV-1(F) glycoproteins, labeled via GalNAc transferase, with anti-HSV-1(F) serum. Supernatant fluids from HSV-1(F)-infected BHK and Ric^{R}14 cell lysates (1 mg of protein for each cell type) were incubated as described in Table 1, footnote *a*, in a final volume of 0.2 ml. At the end of the incubation period, the glycoproteins were extracted with phosphate-buffered saline containing 1% Nonidet P-40, 1% sodium deoxycholate, 10^{-5} M tolylsulfonyl phenylalanyl chloromethyl ketone, and αtosyl-L-lysine chloromethylketone (buffer A). After centrifugation at 100,000 × *g* for 1 h, the supernatant fractions were removed for immunoprecipitation and incubated with 50 μl of anti-HSV-1(F) serum for 1 h at 4°C. A 5-mg portion of protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, N.J.) was added to the antigen-antibody mixture, allowed to stand for 1 h at 4°C, washed repeatedly in buffer A, and washed finally in 50 mM Tris-hydrochloride, pH 7.4, containing 10 mM NaCl. Immunoprecipitates were solubilized in 0.2% sodium dodecyl sulfate–5% 2-mercaptoethanol–0.05 M Tris-hydrochloride, pH 7, boiled for 3 min, centrifuged, and analyzed on 7.5% polyacrylamide gel slabs cross-linked with *N-N'*-diallyltartardiamide. Immunoprecipitates from HSV-1(F)-infected BHK and Ric^{R}14 cells labeled via GalNAc transferase are shown on the right. On the left, the electrophoretic pattern of viral glycoproteins synthesized in HSV-1(F)-infected BHK and Ric^{R}14 cells labeled with [^{14}C]glucosamine from 6 to 18 h after infection (cell lysates) is shown to identify the bands. Lanes showing immunoprecipitates are from a fluorogram exposed three times as long as the fluorogram used for other lanes.

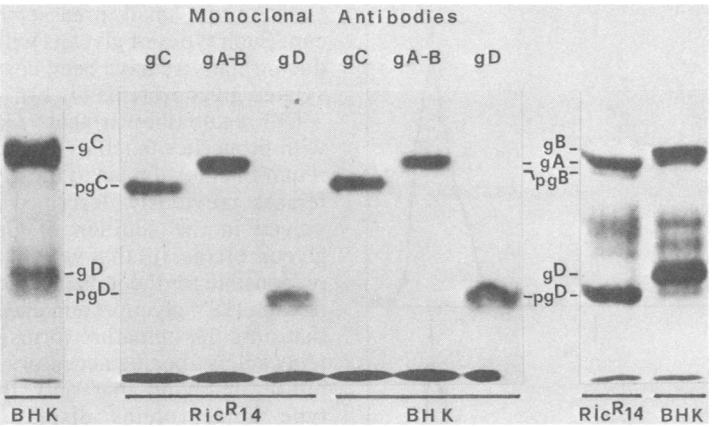

FIG. 2. Fluorography of immunoprecipitates of HSV-1(F) glycoproteins, labeled via GalNAc transferase, with monoclonal antibodies HC1, H233, and HD1 against gC, gA-gB, and gD, respectively (center panel). Experimental details were as described in the legend to Fig. 1, except that 1 to 2 μl of each monoclonal antibody was used, and the immune complex was allowed to react with rabbit anti-mouse immunoglobulin G (Miles Laboratories, Inc., Elkhart, Ind.) before the addition of protein A-Sepharose. The electrophoretic patterns of viral glycoproteins synthesized in HSV-1(F)-infected BHK cells (left panel) and in HSV-1(MP)-infected Ric^{R}14 and BHK cells (right panel) labeled with [^{14}C]glucosamine are shown to identify the bands.

asymptotic trend which reached the highest value within 1 h (subsequent increments were negligible). Although the amount of GalNAc transferred to acid-precipitable proteins was very similar in uninfected BHK and Ric^{R}14 cells, the amount of sugar incorporated into endogenous acceptors was significantly higher in HSV-1-infected Ric^{R}14 cells than in infected wild-type cells (Table 2). This argues for a higher accumulation of specific acceptors of GalNAc transferase in the mutant cells after herpesvirus infection.

To ascertain whether GalNAc was incorporated into HSV glycoproteins and which viral glyco- proteins served as preferential acceptors, we precipitated (19) the glycoproteins labeled via GalNAc transferase with an anti-HSV-1(F) serum and analyzed the immunoprecipitates by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Figure 1 shows that [^{14}C]GalNAc-labeled glycoproteins were recovered in immunoprecipitates with anti-HSV-1(F) serum. Strikingly, only the immature forms gA-pgB, pgC, and pgD were detected. Consistent with the results shown in Table 2, a higher degree of labeling was observed in the bands of the ricin-resistant cells than in those of BHK cells. As previously reported (3), immature forms of HSV-1 glycoproteins accumulated in [^{14}C]glucosamine-labeled Ric^{R}14 cells (Fig. 1). The higher concentration of immature forms in HSV-1-infected Ric^{R}14 cells may well account for the increased incorporation of GalNAc in this sample (Table 2).

Since immunoprecipitation with anti-HSV-1 serum did not allow a clear identification of the glycoproteins present in the pgC and gA-gB regions (particularly for the BHK cell sample), we performed immunoprecipitation with monoclonal antibodies HC1, H233, and HD1 against gC, gA-gB, and gD, respectively (18, 19). The results showed that only the immature forms pgC, pgD, and gA-pgB were specifically labeled in the in vitro reaction (Fig. 2).

Besides the GalNAc transferase mentioned above, which is involved in the initiation of O-linked glycans, another GalNAc transferase which is responsible for the addition of GalNAc as a terminal nonreducing sugar to high-molecular-weight glycans which carry the A blood group determinants has been described previously (29). To determine the nature of the linkage of GalNAc added via GalNAc transferase to the immature forms of HSV glycoproteins, we subjected [^{14}C]GalNAc-labeled glycoproteins to a mild alkaline borohydride treatment (which releases O-linked glycans by base-catalyzed β-elimination) and examined the products by gel filtration to determine the size of the released moiety. Labeled glycoproteins from incubation mixtures were separated from low-molecular-weight compounds by gel filtration on Bio-Gel P-10. Most of the radioactivity emerged in the elution position of the original UDP-GalNAc, indicating that no degradation of sugar nucleotide had occurred by pyrophosphatases or by other hydrolytic enzymes. The glycoproteins eluted in the void volume were subjected to a mild alkaline borohydride treatment and subsequently fractionated on a Bio-Gel P-4 column. A gel filtration profile of the sample from HSV-1(F)-infected BHK cells (Fig. 3A) shows that the

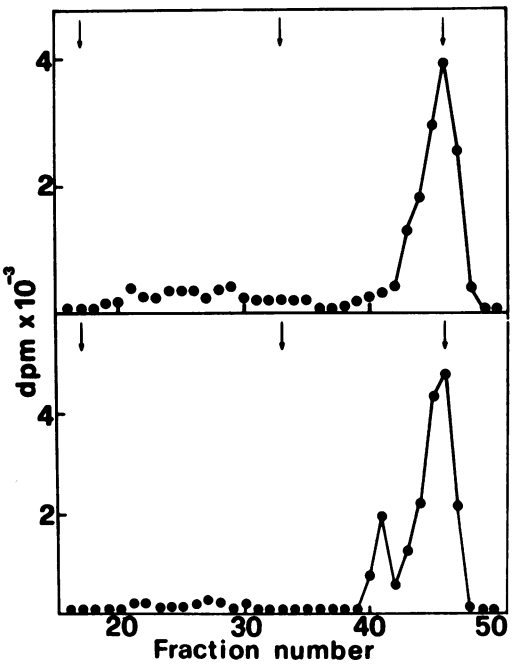

FIG. 3. Chromatography on Bio-Gel P-4 of [^{14}C]GalNAc-labeled glycoproteins from HSV-1(F)-infected BHK (top) and Ric^{R}14 (bottom) cells after a mild alkaline borohydride treatment. Supernatant fluids from infected cell lysates (500 μg of protein per 100 μl of assay) were incubated with the radioactive sugar nucleotide as described in Table 1, footnote *a*. Glycoproteins were extracted with 1% Nonidet P-40 and 1% sodium deoxycholate. After centrifugation at 100,000 × *g* for 1 h, the supernatant fractions were applied to a Bio-Gel P-10 column. The radioactive fractions of the void volume, containing the glycoproteins, were pooled, dialyzed against water, and freeze-dried. The glycoproteins were treated with 0.1 M NaOH–1 M $NaBH_4$ at 45°C for 20 h and then fractioned on a Bio-Gel P-4 column (1 by 75 cm). The three arrows represent, from left to right, the void volume and the elution positions of *N*-acetylneuraminyl lactose and GalNAc.

radioactivity was entirely recovered in the elution position of monosaccharides, indicating that the in vitro-transferred GalNAc was released as free *N*-acetylgalactosaminitol by a mild alkaline borohydride treatment. This shows that the incorporation of GalNAc into the immature forms of HSV glycoproteins represents the initial step of O-glycosylation. The gel filtration profile of the sample from infected Ric^{R}14 cells (Fig. 3B) was similar in that all the label was released from glycoproteins by base-catalyzed β-elimination. Most of it was recovered in the elution position of GalNAc. Unlike BHK cells, 20% of the radioactivity emerged as a peak slightly heavier than monosaccharides, which might have resulted from the addition of GalNAc to a small, preassembled O-linked glycan. Such types of glycans with terminal, nonreducing GalNAc have been described in O-glycosylated glycoproteins (4, 14).

Our results show (i) that a GalNAc transferase with properties (such as Triton X-100 and Mn^{2+} requirements) similar to those of GalNAc transferases previously described (9, 11, 15) is involved in the addition of GalNAc to HSV-1 glycoproteins, (ii) that very likely this enzyme is responsible for the initial step of O-glycosylation of each HSV glycoprotein class in vitro, and (iii) that only the immature forms of viral glycoproteins act as specific acceptors of GalNAc transferase, implying that only the high-mannose-type glycoproteins display the appropriate unsubstituted serine-threonine sites available for the addition of GalNAc. The inability of the mature glycoproteins which contain complex-type glycans to accept GalNAc could be explained in two ways: either GalNAc has already been attached to all the sites destined to be O-glycosylated or serine-threonine sites are not accessible to GalNAc transferase in vitro because of the folding of the mature proteins.

On the basis of the present in vitro experiments, it is difficult to infer the role played by GalNAc transferase in the assembly of HSV glycans within infected cells. The activity of this enzyme might be hindered in vivo in several ways, e.g., by the inaccessibility of the substrates to the enzyme or inadequate configuration of the acceptors due to their routing into the membranes of the Golgi apparatus. Assuming that this enzyme is involved in O-glycosylation of HSV-1 glycoproteins in vivo, the results presented here suggest that a specific, temporal sequence regulates the biosynthesis of glycomoieties of HSV-1 glycoproteins. The first step consists of the en bloc transfer of oligomannosyl chains from dolichol intermediates. Several lines of evidence indicate that this step takes place in the rough endoplasmic reticulum as a cotranslational event (10, 22). A further step consists of the addition of O-linked GalNAc to the peptide. Later steps involve the capping of O-linked chains and the branching of N-linked glycans by glycosyltransferases located in the Golgi apparatus (10, 12, 23). A similar schematic model has been proposed for the biosynthesis of glycophorin A, the major human erythrocyte glycoprotein, which contains both N- and O-linked glycans (13).

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