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Glycosylation of Herpes Simplex Virus Type 1 gC in the Presence of Tunicamycin

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The presence of *O*-glycosidic linkages on herpes simplex virus type 1 (HSV-1) glycoproteins was indicated by the synthesis and glycosylation of HSV-1 glycoproteins in the presence of tunicamycin. Monospecific antiserum to HSV-1 gC immunoprecipitated a 92,000-molecular-weight protein synthesized in the presence of tunicamycin and isotopically labeled with glucosamine or galactose. Anti-gAB did not immunoprecipitate a carbohydrate-labeled HSV-1 protein synthesized in the presence of tunicamycin. The purified glucosamine-labeled 92,000-molecular-weight protein synthesized in the presence of tunicamycin and the fully glycosylated forms of gAB and gC were tested for their sensitivity to mild alkaline hydrolysis. Purified gAB was resistant to mild alkaline hydrolysis, whereas gC and the 92,000-molecular-weight protein were both sensitive to mild alkaline hydrolysis. These results suggest that *O*-glycosidic linkages are associated with the HSV-1 gC glycoprotein.

The carbohydrate-peptide linkages which occur between specific amino acid residues of glycoproteins and their oligosaccharide side chains may be classified into the two main types: *N*-glycosidic and *O*-glycosidic linkages. The carbohydrate-peptide linkage which occurs between *N*-acetylglucosamine and asparagine is of the *N*-glycosidic type. The anomeric carbon atom of *N*-acetylglucosamine is linked glycosidically via a β linkage to the amide group of asparagine. During the synthesis of *N*-linked glycoproteins, the high-mannose oligosaccharide is first synthesized on the dolichol beginning with *N*-acetylglucosamine, and then it is transferred en bloc to the nascent polypeptide (13, 30).

The *O*-glycosidic linkages include those glycoproteins with covalent linkage of *N*-acetylgalactosamine to serine or threonine. In this case, the anomeric carbon atom of *N*-acetylgalactosamine is linked glycosidically via an α linkage to the hydroxyl group of serine or threonine. In contrast to *N*-glycosidic linkages, synthesis of *O*-glycosidically linked glycoproteins occurs by the direct sequential addition of the carbohydrates to the nascent polypeptide. The occurrence of both *N*-glycosidic and *O*-glycosidic linkages within the same glycoprotein molecule is not uncommon and has been observed in immunoglobulins (1, 2), fetuin (17, 26), erythrocyte membrane glycoproteins (6), and glomerular basement-membrane glycoproteins (29).

Recently, the presence of *O*-glycosidically linked oligosaccharides has been described for virus-specific glycoproteins. A mouse coronavirus glycoprotein has been shown to contain *O*-linked oligosaccharides exclusively (12, 16), and the vaccinia hemagglutinin glycoprotein has been reported to contain both *N*- and *O*-linked oligosaccharides (24). The addition of *O*-linked oligosaccharides to these glycoproteins is accomplished in the presence of tunicamycin (12, 24). Tunicamycin (27) in an antibiotic which inhibits the production of *N*-acetylglucosamine-pyrophosphoryldolichol (9), the first step in the synthesis of lipid-linked oligosaccharides (15). It is possible to use the antibiotic to specifically inhibit the synthesis of *N*-linked oligosaccharides without inhibiting the attachment of *O*-linked oligosaccharides to the glycoprotein and thus tentatively identify glycoproteins which have *O*-linked oligosaccharides (7, 12, 24).

The effect of tunicamycin on the synthesis of herpes simplex virus type 1 (HSV-1) KOS glycoproteins was examined by analysis of the proteins synthesized in infected MRC-5 cell cultures maintained in the presence or absence of 1 μg of tunicamycin per ml. HSV-1-infected MRC-5 cells were isotopically labeled with [^{35}S]methionine (2 to 3 μCi/ml) 4 to 18 h postinfection, and the whole cell fraction was harvested at 18 h postinfection.

Cell extracts were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1) as described previously (3, 8, 22, 31). In agreement with other studies (14, 18, 21), little effect on viral polypeptide synthesis was observed except for the

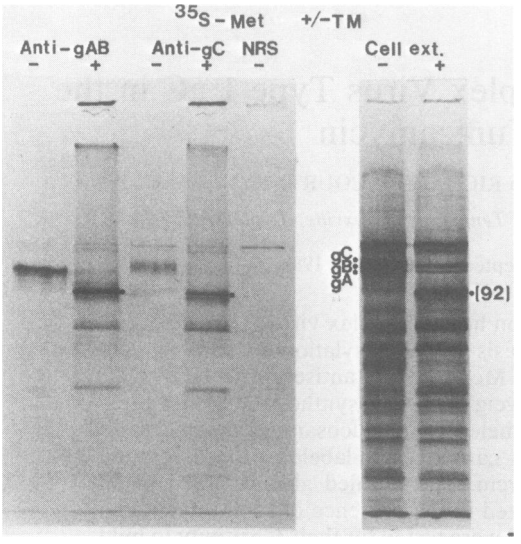

FIG. 1. Identification of HSV-1 [^{35}S]methionine-labeled 92,000-molecular-weight proteins synthesized in the presence of tunicamycin (TM). HSV-1-infected MRC-5 cells maintained in the absence (−) or presence (+) of tunicamycin (1 μg/ml) were labeled with [^{35}S]methionine (2 μCi/ml) from 4 to 18 h postinfection. At 18 h postinfection, the cells were harvested and solubilized, and HSV-1 glycoproteins were immunoprecipitated from cell extracts (Cell ext.) with monospecific antisera, anti-gAB and anti-gC. Immunoprecipitates and cell extracts were analyzed by SDS-PAGE. Normal rabbit serum (NRS) was included as a control. The position of the 92,000-molecular-weight protein is indicated at right by (92).

appearance of a new lower-molecular-weight polypeptide. The synthesis of a viral 92,000-molecular-weight polypeptide was observed in tunicamycin-treated cells along with the concomitant disappearance of the high-molecular-weight glycoproteins (Fig. 1, lanes 6 and 7).

The [^{35}S]methionine isotopically labeled cell extracts were immunoprecipitated with monospecific anti-gAB and anti-gC sera (4, 5) to identify the polypeptides in the 92,000-molecular-weight region. The immunoprecipitates were washed, solubilized, and then analyzed by SDS-PAGE. Normal rabbit serum was included in the immunoprecipitation assays as a control. The SDS-PAGE pofiles of these samples are shown in Fig. 1. In the absence of tunicamycin, the glycoprotein gB migrated to a position in the gel corresponding to an apparent molecular weight of 120,000 (Fig. 1, lane 1), and the fully glycosylated gC migrated to a position in the gel corresponding to an apparent molecular weight of 130,000 (Fig. 1, lane 3).

Immunoprecipitates from HSV-1-infected cells maintained in the presence of tunicamycin are also presented in Fig. 1. Anti-gAB did not immunopreciptiate significant amounts of gB from tunicamycin-treated cells; however, a protein with an apparent molecular weight of 92,000 was detected (Fig. 1, lane 2). Tunicamycin also inhibited the synthesis of gC, and a protein with an apparent molecular weight of 92,000 was also immunoprecipitated with anti-gC (Fig. 1, lane 4). The [^{35}S]methionine-labeled proteins, which migrated with a greater electrophoretic mobility than the 92,000-molecular-weight proteins, were detectable in the immunoprecipitates with the monospecific antisera and may be due to degradation of the 92,000-molecular-weight proteins (Fig. 1, lanes 2 and 4). The specificity of the antisera was demonstrated by the inability of the anti-gC serum to immunoprecipitate a 92,000-molecular-weight protein from tunicamycin-treated cells infected with the MP (10, 11) strain of HSV-1 which does not synthesize gC (10). In contrast, the anti-gAB serum did immunoprecipitate a 92,000-molecular-weight protein from the tunicamycin-treated cells infected with HSV-1 strain MP (data not shown).

To determine the effect of tunicamycin on the glycosylation of gB and gC, immunoprecipitates of tunicamycin-treated infected cell extracts isotopically labeled with [^{14}C]glucosamine (5 μCi/ml) or [^{3}H]galactose (20 μCi/ml) were analyzed by SDS-PAGE. The SDS-PAGE profiles of [^{14}C]glucosamine-labeled cell extracts from infected cells cultured in the presence or absence of tunicamycin are shown in Fig. 2. In the absence of tunicamycin, the high-molecular-weight glycoproteins were synthesized (Fig. 2, lane 8). In contrast, HSV-1-infected cells cultured in the presence of tunicamycin exhibited a significant inhibition of isotopically labeled proteins; however, a [^{14}C]glucosamine-labeled protein was resolved in the 92,000-molecular-weight region (Fig. 2, lane 7).

To determine whether the [^{14}C]glucosamine-labeled protein (92,000 molecular weight) was related to gB or gC, anti-gAB and anti-gC were used, and the resulting immunoprecipitates were analyzed (Fig. 2). The monospecific anti-gC immunoprecipitated the fully glycosylated gC (130,000 molecular weight) and the precursor, pgC(105) (105,000 molecular weight), in the absence of tunicamycin (Fig. 2, lane 4). In tunicamycin-treated cell extracts, the anti-gC immunoprecipitated the 92,000-molecular-weight protein (Fig. 2, lane 3). The glycoproteins gA and gB were immunoprecipitated by anti-gAB from cells infected in the absence of tunicamycin (Fig. 2, lane 2). In contrast to anti-gC, anti-gAB did not immunoprecipitate detectable glucosamine-labeled proteins from tunicamycin-treated cell extracts (Fig. 2, lane 1).

The initial carbohydrate added to threonine or

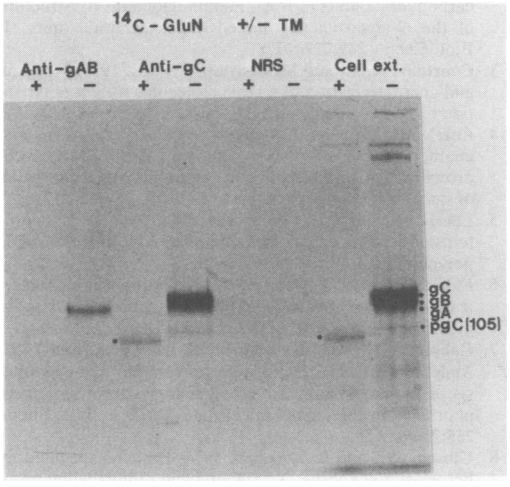

FIG. 2. Effect of tunicamycin (TM) on the glycosylation of HSV-1 glycoproteins. HSV-1-infected MRC-5 cells maintained in the absence (−) or presence (+) of tunicamycin (1 μg/ml) were labeled with [^{14}C]glucosamine (^{14}C-GluN; 5 μCi/ml) from 4 to 24 h postinfection. Glucosamine-labeled proteins were immunoprecipitated from solubilized cell extracts with monospecific antisera, anti-gAB and anti-gC. Immunoprecipitates and cell extracts were analyzed by SDS-PAGE. Normal rabbit serum (NRS) was included as a control. The positions of gC, gB, gA, and pgC (105) are indicated at right.

serine during the synthesis of *O*-linked oligosaccharides is *N*-acetylgalactosamine (2). Since tunicamycin does not inhibit the synthesis of *O*-linked oligosaccharides, the experiment was repeated to determine whether the 92,000-molecular-weight protein could also be isotopically labeled with galactose (Fig. 3). Only anti-gC immunoprecipitated a 92,000-molecular-weight protein from tunicamycin-treated cells infected with HSV-1. Purified glycoproteins isotopically labeled with [^{3}H]glucosamine were tested for their sensitivity to alkaline hyrolysis. The fully glycosylated gC glycoprotein (130,000 molecular weight) and the 92,000-molecular-weight gC synthesized in the presence of tunicamycin were both sensitive to alkaline hydrolysis (25), whereas purified gAB and the purified G glycoprotein of vesicular stomatitis virus, which contains only *N*-glycosidic linkages (23), appeared to be resistant to alkaline hydrolysis (data not shown).

The data presented here further suggest that the HSV-1 gC contains *O*-linked oligosaccharides. This is based on (i) the ability for anti-gC to immune precipitate a [^{3}H]glucosamine- or [^{3}H]galactose- but not [^{3}H]mannose-labeled protein from tunicamycin-treated infected cells and (ii) the sensitivity of gC to mild alkaline hydrolysis (data not shown). Olofsson et al. (19) have suggested that the HSV-1 glycoprotein gC contains *O*-linked oligosaccharides, based on lectin binding studies. Recently they have also presented data demonstrating the sensitivity of HSV-1-specified glycoproteins to dilute alkali at low temperatures (18). The partial glycosylation of the gC polypeptide in the presence of tunicamycin reported in this study indicates that the addition of *O*-linked oligosaccharides to the gC glycoprotein is not dependent on the presence of *N*-linked oligosaccharides. The demonstration of both *N*- and *O*-linked oligosaccharides on the gC glycoprotein isolated from HSV-1-infected cell extracts is the first suggestion that a viral structural glycoprotein contains both types of oligosaccharide side chains.

The [^{35}S]methionine-labeled protein immunoprecipitated by anti-gC from tunicamycin-treated infected cells has an approximate molecular weight of 92,000. Other investigators have also reported the appearance of lower-molecular-weight HSV-1 proteins synthesized in the presence of tunicamycin which are antigenically

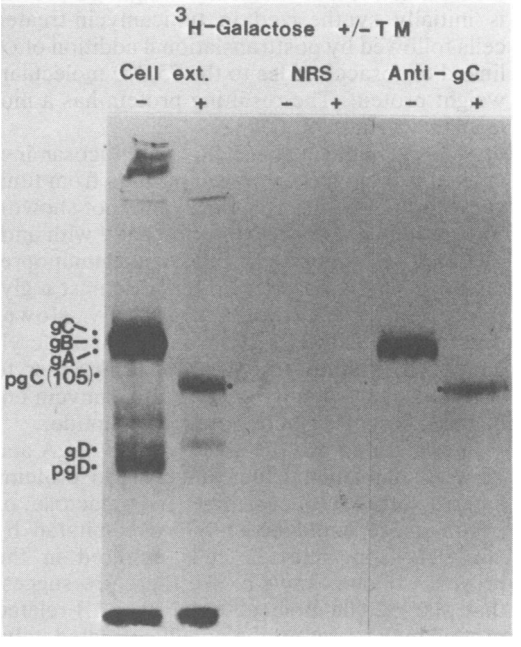

FIG. 3. Immunoprecipitation of the [^{3}H]galactose-labeled gC glycoprotein synthesized in the presence of tunicamycin (TM). HSV-1-infected MRC-5 cells maintained in the absence (−) or presence (+) of tunicamycin (1 μg/ml) were labeled with [^{3}H]galactose (20 μCi/ml) from 4 to 24 h postinfection. Galactose-labeled proteins were immunoprecipitated from solubilized cell extracts with the monospecific antiserum, anti-gC. Immunoprecipitates and cell extracts were analyzed by SDS-PAGE. Normal rabbit serum (NRS) was included as a control. The positions of gC, gB, gA, pgC(105), gD, and pgD are indicated at left.

related to the normal HSV-1 glycoproteins (20, 21). Our data suggest that this represents the molecular weight of the gC polypeptide containing *O*-linked oligosaccharides since the 92,000-molecular-weight protein can also be isotopically labeled with [^{3}H]glucosamine or [^{3}H]galactose. Digestion of pgC(105) by the endoglycosidase endo-β-N-acetylglucominidase (28) resulted in a digestion product of approximately 75,000 molecular weight, which may represent the molecular weight of the nonglycosylated form of gC (31). It is conceivable that pgC(105) does not contain *O*-linked oligosaccharides, which would explain why the majority of pgC(105) is digested by the endoglycosidase to a 75,000-molecular-weight polypeptide rather than a 92,000-molecular-weight polypeptide. These results suggest that the high-mannose oligosaccharides are added first to the growing gC polypeptide to yield pgC(105), and then the *O*-linked oligosaccharides are added to pgC(105) while the *N*-linked high-mannose oligosacchardies are being processed to complex oligosaccharides.

Preliminary data from pulse-chase studies indicate that a 75,000-molecular-weight gC protein is initially synthesized in tunicamycin-treated cells followed by posttranslational addition of *O*-linked oligosaccharides to the 75,000-molecular-weight protein. The resulting protein has a molecular weight of 92,000.

A lower-molecular-weight [^{3}H]glucosamine-labeled protein has also been purified from tunicamycin-treated infected cells (data not shown). This protein did not react significantly with anti-gAB, anti-gC, or anti-gD, but it was immunoprecipitated with an antiserum made against a glycoprotein migrating at a slightly slower migration rate than gD on an SDS-polyacrylamide gel. Studies are currently in progress to determine the identity of this tunicamycin-enhanced, partially glycosylated polypeptide.

In contrast to gC, the glycosylation of gA and gB was inhibited by tunicamycin. No proteins labeled with [^{3}H]glucosamine, [^{3}H]galactose, or [^{3}H]mannose could be immunoprecipitated by anti-gAB from infected cells cultured in the presence of tunicamycin. We therefore suggest that the 92,000-molecular-weight gAB-related protein may represent the nonglycosylated polypeptide from of gAB.

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