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Synthesis and Processing of Glycoprotein gG of Herpes Simplex Virus Type 2

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Monoclonal antibody 13αC5-1-A11 immunoprecipitated two major polypeptides of molecular weights 108,000 and 120,000 from extracts of herpes simplex virus type 2-infected BHK-21 cells labeled with [^{35}S]methionine or [^{3}H]glucosamine. In pulse-chase experiments, both labels were chased from the 120,000-molecular-weight peptide (120K peptide) into the 108K molecule. Endoglycosidase H (endo H) reduced the 120K peptide to a 112K peptide but did not affect the 108K peptide. Similar profiles were obtained with monoclonal antibody AP-1 which reacts with a 92K glycoprotein, gG, which maps to the short unique region of the genome. Cross-absorption experiments indicated that both antibodies reacted with the same peptides, suggesting that the 120K peptide is a partially glycosylated high-mannose-type precursor of gG (pgG$_1$). Immunoprecipitation from monensin-treated cells indicated that pgG$_1$(120K) may undergo peptide cleavage to form a 74K high-mannose-type peptide (pgG$_2$) and that this 74K peptide may be further processed into an endo H-resistant 110K to 116K peptide. In the presence of tunicamycin, gG(108K) was replaced by 110K and 105K peptides which were resistant to both endo H and endoglycosidase F. The 105K peptide was the only molecule labeled by [^{3}H]galactose or [^{3}H]glucosamine in the presence of tunicamycin, and none of the peptides were labeled with [^{3}H]mannose, indicating the probable presence of O-linked sugars in the 105K peptide. Our results imply that cotranslational glycosylation of the unglycosylated precursor 110K peptide results in the high-mannose-type pgG$_1$(120K), which probably undergoes peptide cleavage. This putative cleavage product may then mature into gG(108K) by the trimming of sugars and the addition of complex and probably O-linked sugars; the high-mannose-type pgG$_2$(74K) is probably an intermediate peptide formed in this process.

In the course of productive infection with herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), at least four virus-specified glycoproteins, designated gC, gB, gD, and gE, are synthesized and become incorporated into both the infected cell membrane and the virion envelope (19). The glycoproteins gB, gE, and gD possess both type-specific and type-common antigenic sites (7, 8, 14), and the genes encoding them map colinearly in HSV-1 and HSV-2 genomes (12–14). The gC of HSV-1 was originally thought to contain exclusively type-specific sites, and a counterpart in HSV-2 was not known (7). However, using monoclonal antibodies we described two additional antigenically and structurally distinct HSV-2-specific glycoproteins (3). One of these new glycoproteins was originally designated gF (2, 3) but has since been shown to be encoded by an HSV-2 gene mapping colinearly with the gC gene of HSV-1. It shares antigenic determinants with gC of HSV-1 (15, 25, 26, 27, 28) and is now referred to as gC2. The second apparently type-specific glycoprotein is immunoprecipitated by monoclonal antibody 13αC5-1-A11 (13αC5). We here identify this glycoprotein as gG, the previously described 92K glycoprotein of HSV-2 which maps in the unique short region of HSV-2 DNA (11). We describe the biosynthesis of the mature glycosylated molecule and present evidence for probable major proteolytic cleavage of a partially glycosylated precursor during its maturation.

MATERIALS AND METHODS

Cells and virus. Monolayers of baby hamster kidney (BHK-21) and Vero cells were grown in modified Dulbecco medium (GIBCO Laboratories) supplemented with antibiotics and heat-inactivated fetal bovine serum. Stock virus preparation and plaque assay of HSV-2 (strain 333) were done in Vero cells (18).

Monoclonal antibodies. Preparation and characterization of ascitic fluid of monoclone 13αC5 have been described previously (1–3). Monoclonal antibody AP-1 (ascitic fluid) was a gift from A. C. Minson, University of Cambridge, England (11).

Radiolabeling procedures. Confluent monolayers of BHK-21 cells were infected with HSV-2 at a multiplicity of infection of 10 PFU per cell and allowed to adsorb for 2 to 3 h. For long-term labeling (3 to 20 h), cells were incubated in Hanks balanced salt solution containing 20 μCi per ml of L-[^{35}S]methionine (specific activity, 1,380 μCi/mmol; Amersham Corp.), 5% dialyzed fetal bovine serum, and 1/10 the normal concentration of methionine. Cells were also labeled for 3 to 20 h with [^{3}H]mannose (specific activity, 27.2 μCi/mmol; New England Nuclear Corp.), [^{3}H]galactose (specific activity, 51.7 μCi/mmol; New England Nuclear), and [^{3}H]glucosamine (specific activity, 15 μCi/mmol; ICN Pharmaceuticals Inc.) at concentrations of 100 μCi/ml in medium with 1/10 the normal concentration of glucose. In pulse-chase experiments, cells were labeled for 10 min with [^{35}S]methionine (100 μCi/ml) or for 30 min with [^{3}H]glucosamine (100 μCi/ml) at 5 h postinfection (p.i.) and chased in medium containing 100 times the normal concentration of unlabeled methionine or glucose and cycloheximide (50 μg/ml).

In experiments with tunicamycin or monensin, cells were washed and preincubated for 3 h with medium containing 3 μg of tunicamycin (Sigma Chemical Co.) per ml or 1 μM monensin (Sigma). Both drugs were then present throughout the remaining course of each experiment.

Immunoprecipitation and electrophoresis. Immunoprecipitation was carried out as previously described (2). Briefly,

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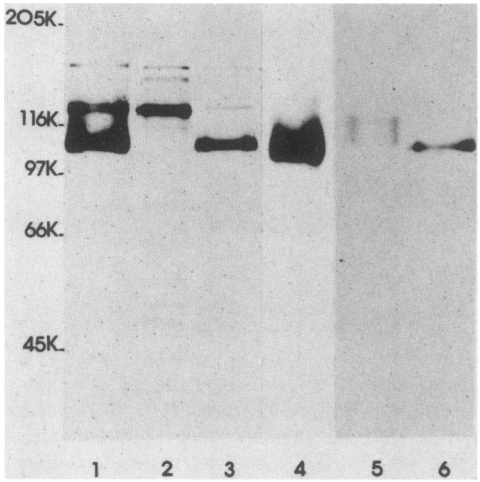

FIG. 1. Autoradiograms of peptides immunoprecipitated from HSV-2-infected BHK-21 cells by monoclonal antibody 13αC5. Lanes show infected cells labeled with: 1, [^{35}S]methionine between 3 to 20 h p.i.; 2, [^{35}S]methionine pulse for 10 min at 5 h p.i.; 3, [^{35}S]methionine pulse for 10 min at 5 h p.i. followed by chase for 5 h in nonradioactive medium–cycloheximide–100× cold methionine; 4, [^{3}H]glucosamine between 3 to 20 h p.i.; 5, [^{3}H]glucosamine pulse for 30 min at 5 h p.i.; 6, [^{3}H]glucosamine pulse for 30 min at 5 h p.i. followed by chase for 5 h in nonradioactive medium–cycloheximide–cold glucose. SDS-PAGE was carried out on 9% acrylamide cross-linked with *N,N'*-diallyltartardiamide.

cells were solubilized with RIPA buffer containing 0.1 mM phenylmethylsulfonyl fluoride and 100 U of aprotinin per ml (2) and mixed with antibody and protein A-Sepharose CL-4B beads (Sigma). The precipitates were washed, dissociated by boiling, and analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in 9% acrylamide cross-linked with 0.24% *N,N'*-diallyltartardiamide. Molecular weight markers (Sigma) were electrophoresed in parallel channels. Gels were stained, destained, infused with 2,5-diphenyloxazole, dried on filter paper, and placed in contact with Kodak XAR-5 film at −70°C for fluorography (4).

Enzyme digestion. Immunoprecipitated proteins bound to the protein A-Sepharose beads were eluted by incubating the pellets for 20 min at 37°C in 50 μl of sodium citrate buffer (50 mM, pH 5.5) containing 0.2% SDS and then boiling for 30 s. Endoglycosidase H (50 μl, endo H) in citrate buffer (25 mU; Miles Laboratories, Inc.) was added to the samples and incubated for 24 h at 37°C. For digestion by endo F, beads were suspended in 100 μl of sodium phosphate buffer (100 mM, pH 6.1) containing 0.1% SDS, 0.5% Nonidet P-40, and 50 mM EDTA. Five microliters of endo F (440 U/ml; New England Nuclear) was added to the sample and incubated for 24 h at 37°C. Control samples without additional endo H or endo F were treated in the same way, and after incubation 100 μl of sample buffer (2×) containing 10% SDS and 5% mercaptoethanol was added and boiled for 3 min. Samples containing equal amounts of radioactivity were analyzed by SDS-PAGE and autoradiography.

RESULTS

Characterization of glycoprotein immunoprecipitated by monoclonal antibody 13αC5. BHK-21 cells were infected with HSV-2 and labeled with [^{35}S]methionine for 3 to 20 h, and the RIPA-solubilized lysate was immunoprecipitated. Autoradiograms of the electrophoresed immunoprecipitate showed two major bands, one an intensely labeled peptide of approximate molecular weight 108,000 and the other a peptide of about molecular weight 120,000 (Fig. 1, lane 1). Similar size peptides were also precipitated from infected Vero cells and from BHK-21 cells in the absence of proteolytic inhibitors, and no peptides were precipitated from uninfected cells (data not shown). In addition to the two major peptides, two to three high-molecular-weight peptides were also seen in the majority of the immunoprecipitates; these were probably nonspecific contaminants as they were precipitated by several other monoclonal antibodies. Both the 120K and 108K peptides could be labeled with [^{3}H]glucosamine (Fig. 1, lane 4) and with [^{3}H]mannose (see Fig. 5, lane 6), while the high-molecular-weight forms could not; most of the label was seen in the 108K peptide.

Previous studies by us and others (2, 3, 15, 16) have shown that multiple glycosylated peptides precipitated by monoclonal antibodies show precursor product relationships and that different molecular weight species represent different levels of glycosylation. To determine the precursor molecule of the glycoprotein immunoprecipitated by 13αC5, HSV-2-infected cells were pulsed for 10 min at 5 h p.i. with [^{35}S]methionine and chased for 5 h in the presence of cold methionine and cycloheximide. The major labeled peptide precipitated from cell lysates made during the pulse period was a 120K peptide. In the chase period, the label was found in the 108K peptide (Fig. 1, lanes 2 and 3). After long exposure of the autoradiographs, some label was also seen in a 74K peptide in the lysates from the pulse but not from the chase period. The conversion of the 120K peptide into a 108K peptide was very rapid. In a short pulse-chase experiment, the 108K peptide was labeled after only a 15-min chase, and chase of the label into this molecule from the

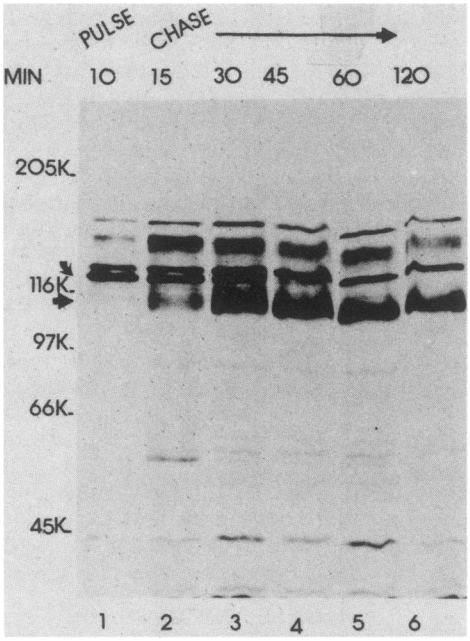

FIG. 2. SDS-PAGE analysis of peptides immunoprecipitated by monoclonal antibody 13αC5. Lanes show infected cells labeled with: 1, [^{35}S]methionine for 10 min at 5 h p.i.; 2 to 6, [^{35}S]methionine for 10 min at 5 h p.i. followed by chase for 15, 30, 45, 60, and 120 min, respectively, in nonradioactive medium–cycloheximide–100× cold methionine. Short arrows indicate the position of 120K and 108K peptides.

120K peptide was complete after 60 min (Fig. 2, lanes 1 to 6). In the pulse-chase experiment with [^{3}H]glucosamine (30-min pulse, 5-h chase), the label was initially associated with a diffuse band of a 120K to 112K peptide and later with the 108K peptide (Fig. 1, lanes 5 and 6). These results suggested that the 120K peptide is the glycosylated precursor for the fully glycosylated 108K peptide.

Endo H digestion. The enzyme endo H has been shown to cleave the two proximal *N*-acetylglucosamine residues of the high-mannose-type oligosaccharides but not those of the complex type (22). To identify the primary precursor of the 108K peptide and to characterize its oligosaccharide side chains, immunoprecipitates from [^{35}S]methionine pulse-chase experiments were treated with endo H as described above.

Endo H treatment of the immunoprecipitate from the pulse period resulted in the complete disappearance of the 120K peptide and the appearance of a new species with a molecular weight of approximately 112,000 (Fig. 3, lanes 1 and 2). In contrast, treatment of immunoprecipitate from the chase period did not alter the 108K peptide (Fig. 3, lanes 3 and 4). Long-term exposure of the autoradiographs revealed the presence of a 74K peptide in the pulse period, and upon treatment with endo H, a 66K molecule appeared in its place. Neither of these were detectable in the chase period. The other higher-molecular-weight proteins which were non-specifically precipitated by this and other antibodies served as a good control for demonstrating the lack of detectable protease activity in the endo H preparation.

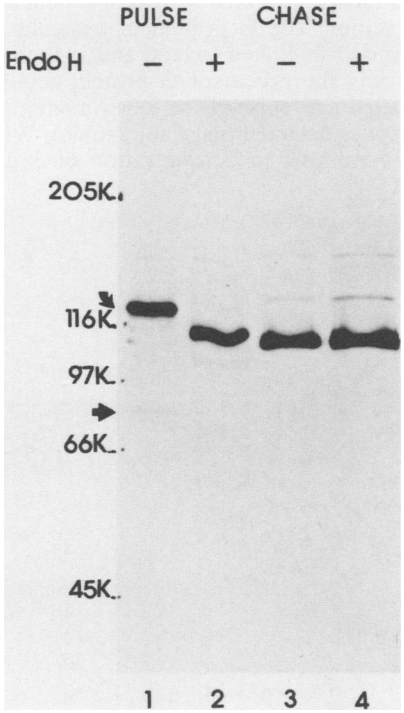

FIG. 3. SDS-PAGE analysis of endo H susceptibility of peptides immunoprecipitated by monoclonal antibody 13αC5. Lanes show infected cells pulsed with: 1 and 2, [^{35}S]methionine for 10 min at 5 h p.i.; 3 and 4, [^{35}S]methionine for 10 min at 5 h p.i. followed by a 5-h chase as described in the legend to Fig. 1. Immunoprecipitates were eluted and incubated in the presence (+) or absence (–) of endo H, and samples were analyzed. Arrows indicate the position of 120K and 74K peptides.

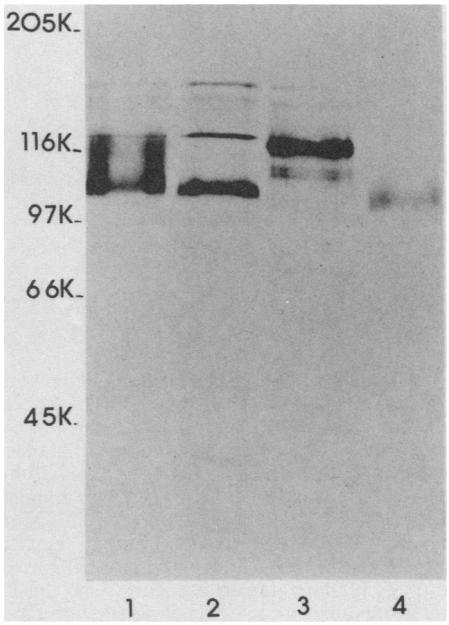

FIG. 4. SDS-PAGE analysis of gG immunoprecipitated by monoclonal antibody AP-1 from HSV-2-infected BHK-21 cells. Lanes show infected cells labeled with: 1, [^{35}S]methionine between 3 and 20 h p.i.; 2, [^{35}S]methionine pulse for 10 min at 5 h p.i. followed by a 5-h chase as described in the legend to Fig. 1; 3, [^{35}S]methionine pulse for 10 min at 5 h p.i.; 4, [^{3}H]glucosamine between 3 and 20 h p.i.

These results demonstrated that (i) the 120K peptide has N-linked oligosaccharides of the high-mannose type, (ii) the 108K peptide lacks the high-mannose-type sugars and probably has oligosaccharides of the complex type, (iii) the 112K peptide is probably the primary precursor with the proximal glucosamine residue attached to it, and (iv) the cotranslational addition of sugars by N-linkage converts it to the partially glycosylated precursor gp120K. The apparent smaller size and the endo H-resistant nature of gp108K imply that gp120K undergoes peptide cleavage before it matures into the mature gp108K by trimming and addition of further sugar residues. The size and the fate of the putative cleaved peptide cannot be determined by immunoprecipitation with 13αC5, which evidently reacts only with a site on the uncleaved portion of the molecule.

Identity of the gp108K. Recently Marsden et al. (11) reported a 92K glycoprotein immunoprecipitated by monoclonal antibodies AP-1 and LP-5 from HSV-2-infected cell extracts which has been shown to map in the short unique region of the genome; they called it gG. Similarly, Roizman et al. (17) have mapped gG(125K) to the short unique region of the genome. Although of different apparent molecular weight (probably due to a different gel system and virus strain), the 92K peptide had characteristics common to gp108K (A. C. Minson, personal communication). To determine the exact relationship of gp108K and the 92K gG, we compared the immunoprecipitation properties of 13αC5 with those of the AP-1 antibody kindly donated by A. C. Minson. Peptides immunoprecipitated by AP-1 antibody both from [^{3}H]glucosamine- (Fig. 4, lane 4) and [^{35}S]methionine- (Fig. 4, lane 1) labeled extracts were similar to those precipitated by 13αC5. In addition, AP-1 antibody immunoprecipitated 120K and 112K peptides in the pulse period and a 108K peptide in the chase period (Fig. 4, lanes 2 and 3), clearly indicating that AP-1 and 13αC5 react with the same gp108K.

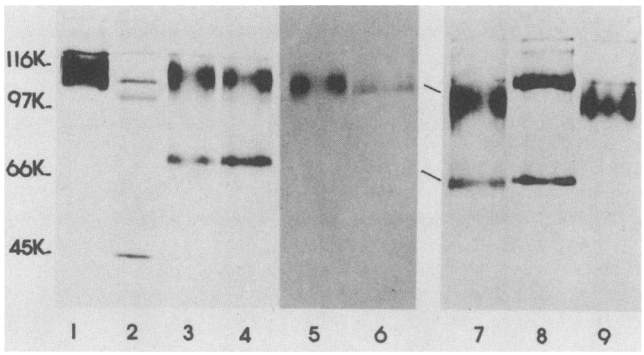

FIG. 5. SDS-PAGE analysis of gG immunoprecipitated by monoclonal antibody (13αC5) from HSV-2-infected BHK-21 cells untreated or treated with monensin or tunicamycin. Lanes show infected cells labeled with: 1, [^{35}S]methionine between 3 and 20 h p.i. (untreated cells); 2, [^{35}S]methionine between 3 and 20 h p.i. (cells treated with 3 μg of tunicamycin); 3 and 4, [^{35}S]methionine between 3 and 20 h p.i. (cells treated with 0.5 and 1 μM monensin, respectively); 5, [^{3}H]mannose between 3 and 20 h p.i. (untreated cells); 6, [^{3}H]mannose between 3 and 20 h p.i. (cells treated with 1 μM monensin); 7, [^{35}S]methionine between 3 and 20 h p.i. (cells treated with 1 μM monensin); 8, [^{35}S]methionine pulse for 10 min at 5 h p.i. (cells treated with 1 μM monensin); 9, [^{35}S]methionine pulse for 10 min at 5 h p.i. followed by a 120-min chase as described in the legend to Fig. 1 (cells treated with 1 μM monensin). Drug treatment was begun 3 h before infection and continued for the remainder of the experiment.

This was confirmed by cross-adsorption tests. Removal of gP108K by successive immunoprecipitation with either of the monoclonal antibodies depleted the molecules precipitable by the other antibody (data not shown). These experiments demonstrated that the 108K peptide is the mature form of glycoprotein gG [designated gG(108K)]; the precursor 120K peptide was designated pgG_1(120K).

Glycosylation in the presence of monensin. To examine further the processing of gG, lysates of HSV-2-infected cells treated with the ionophore monensin were immunoprecipitated and analyzed by SDS-PAGE. Monensin disrupts ion gradients across membranes and has been shown to block the processing of HSV-1 glycoproteins at the addition of O-linked sugars; it also probably inhibits some processing of N-linked sugars (10). Immunoprecipitates from cells treated with 0.5 or 1 μM monensin gave identical results (Fig. 5, lanes 3 and 4), and subsequent experiments were carried out with 1 μM monensin. In contrast to the pgG_1(120K) and gG(108K) peptides immunoprecipitated from untreated cells (Fig. 5, lane 1), immunoprecipitates from monensin-treated cells contained an intermediate peptide of about 110,000 to 116,000 molecular weight and more of the faster-migrating 74K peptide (Fig. 5, lanes 3, 4, and 7); both could be labeled with [^{3}H]mannose (Fig. 5, lane 5). In a pulse-chase experiment with [^{35}S]methionine, the 120K and 74K peptides were present in a 10-min pulse, but label was chased within 120 min into the 110K to 116K peptide (Fig. 5, lanes 8 and 9), indicating a precursor-product relationship between these molecules.

The 110K to 116K peptide was not affected by endo H, while the 74K peptide was partially susceptible, resulting in faster-migrating peptides of about 66,000 to 69,000 molecular weight (Fig. 6, lanes 1 and 2). The 120K and 74K peptides present in the pulse period were completely susceptible to endo H, being reduced to 112K and 66K peptides, respectively (Fig. 6, lanes 3 and 4); thus, these contain high-mannose-type sugars. In contrast, the 110K to 116K peptide present after the 120-min chase was resistant to endo H (Fig. 6, lanes 5 and 6). The results suggest that the high-mannose-type precursor pgG_1(120K) undergoes a proteolytic cleavage to become a 74K peptide (pgG_2); further processing of sugars took place, resulting in the endo H-resistant 110K to 116K peptide.

To determine whether monensin affected the other HSV-2 glycoproteins in a similar manner, a monoclonal antibody (17αA2) to gC2 shown to contain both O- and N-linked complex sugars (25) was used to immunoprecipitate the same monensin-treated lysates. As reported previously (3), in the absence of drug, fully glycosylated peptides of about 72,000 to 84,000 molecular weight were precipitated (Fig. 7, lane 1), while from monensin-treated cells only a 67K to 69K peptide was immunoprecipitated (Fig. 7, lanes 2 and 3). In pulse-chase experiments without drug, the 67K to 69K peptide was chased into the 84K peptide by 60 min (Fig. 7, lanes 4 to 6), while in the presence of monensin, the 67K to 69K peptides present in the pulse period did not undergo further alteration in size (Fig. 7, lanes 7, 9, and 10). All the forms of gC2 in the pulse and chase periods were completely susceptible to endo H (Fig. 7, lanes 8, 10, and 12), indicating that only high-mannose-type pgC2 accumulated in the presence of monensin. This is in contrast to gG, in which further processing took place in the presence of the drug.

Glycosylation in the presence of tunicamycin. The antibiotic tunicamycin inhibits the cotranslational addition of N-linked sugars but does not affect the addition of O-linked sugars (9). To verify the efficacy of tunicamycin, lysates of [^{35}S]methionine-labeled vesicular stomatitis virus-infected BHK-21 cells were immunoprecipitated with anti-vesicular stomatitis virus rabbit serum. The G protein of vesicular stomatitis virus contains only N-linked sugars, and in the presence of tunicamycin only the precursor G protein accumulated in both long-labeled and pulse-chase experiments; the mature G form was never detected (data not shown). With HSV-2-infected cells, we used a concentration of 3 μg of tuni-

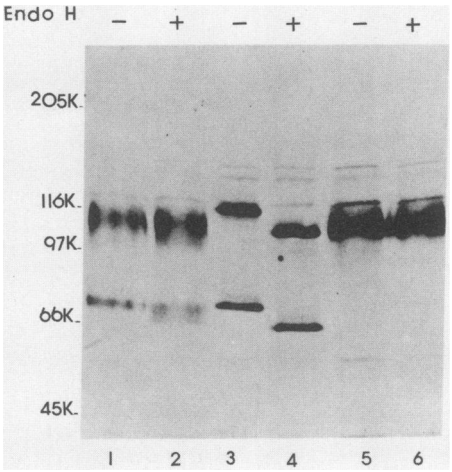

FIG. 6. SDS-PAGE analysis of endo H susceptibility of gG immunoprecipitated by monoclonal antibody (13αC5) from infected cells treated with 1 μM monensin. Lanes show infected cells labeled with: 1 and 2, [^{35}S]methionine between 3 to 20 h p.i.; 3 and 4, [^{35}S]methionine pulse for 10 min at 5 h p.i.; 5 and 6, [^{35}S]methionine pulse for 10 min at 5 h p.i. followed by a 120-min chase as described in the legend to Fig. 1. Immunoprecipitates were eluted and incubated with (+) or without (–) endo H, and samples were analyzed.

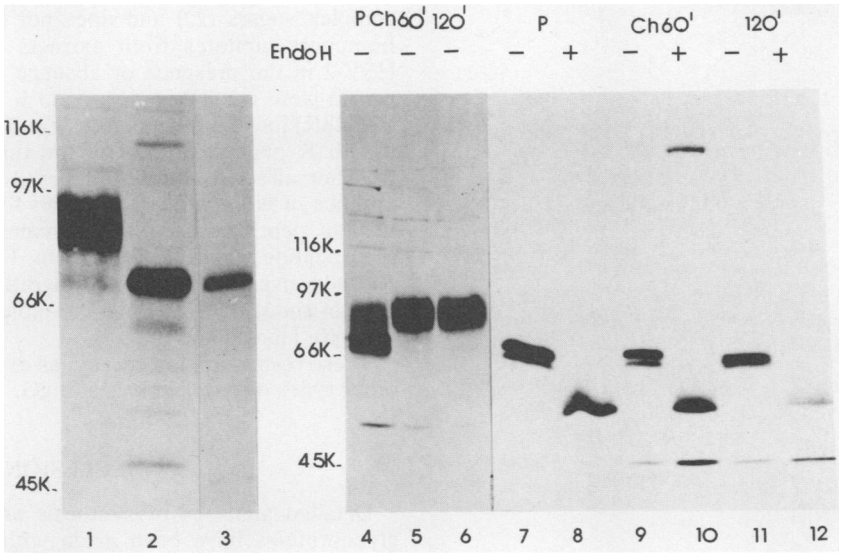

FIG. 7. SDS-PAGE analysis of gC2 immunoprecipitated by monoclonal antibody 17αA2 from HSV-2-infected BHK-21 cells. Lanes show infected cells labeled with: 1, [^{35}S]methionine between 3 and 20 h p.i.; 2, [^{35}S]methionine between 3 and 20 h p.i. (cells treated with 1 μM monensin); 3, [^{3}H]glucosamine between 3 and 20 h p.i. (cells treated with 1 μM monensin); 4, [^{35}S]methionine pulse for 10 min at 5 h p.i. (untreated cells); 5 and 6, [^{35}S]methionine pulse for 10 min at 5 h p.i. followed by a 60- and a 120-min chase, respectively, as described in the legend to Fig. 1 (untreated cells); 7 and 8, [^{35}S]methionine pulse for 10 min at 5 h p.i. (cells treated with 1 μM monensin); 9 to 12, [^{35}S]methionine pulse for 10 min at 5 h p.i. followed by a 60-min (lanes 9 and 10) or a 120-min (lanes 11 and 12) chase (cells treated with 1 μM monensin). Eluted immunoprecipitates were incubated with (+) or without (−) endo H. P, Pulse; Ch60^{1}, 60-min chase; Ch120^{1}, 120-min chase.

camycin per ml, as concentrations of 4 and 5 μg also gave similar results (data not shown). Total cell extracts were analyzed by SDS-PAGE, and, in agreement with other reports, little effect on HSV polypeptide synthesis was observed except for the appearance of new lower-molecular-weight polypeptides (data not shown).

Immunoprecipitation by 13αC5 antibody of tunicamycin-treated HSV-2-infected cells indicated the complete absence of pgG$_1$(120K) and gG(108K) peptides; instead, three major peptides of 110,000, 105,000, and 100,000 molecular weight and four to five minor faster-migrating forms (Fig. 8, lane 3) were seen. Since our experiments with endo H showed that the 112K molecule is the precursor gG molecule with proximal glucosamine residues, the 110K peptide is probably the unglycosylated precursor. In pulse-chase experiments, the 110K peptide and other faster-migrating species were precipitated in the pulse period, and in the chase period the 105K peptide appeared (Fig. 8, lanes 4 and 5). The appearance and relative mobility of the three higher-molecular-weight nonspecific peptides were very consistent and aided us in positioning the specific peptides immunoprecipitated by the monoclonal antibodies.

To confirm the effect of tunicamycin on glycosylation, two sets of experiments were carried out. First, immunoprecipitates from pulse-chase experiments were treated with endo H. Neither the 110K peptide seen in the pulse period nor the 105K peptide seen in the chase period were affected by endo H (data not shown), demonstrating the absence of N-linked sugars in these molecules. Second, we labeled tunicamycin-treated cells with [^{3}H]glucosamine and [^{3}H]galactose. *N*-Acetylgalactosamine is the carbohydrate most commonly added first to serine or threonine during the synthesis of O-linked oligosaccharide; glucosamine and other sugars are added subsequently. Total cell extracts of infected cells showed that tunicamycin treatment dramatically reduced the amount of [^{3}H]galactose-labeled peptides. Instead of mature, fully glycosylated, higher-molecular-weight peptides, only faster-migrating peptides were seen (Fig. 8, lanes 1 and 2). Immunoprecipitation revealed that in the absence of tunicamycin, the pgG$_1$(120K) and the gG(108K) were labeled

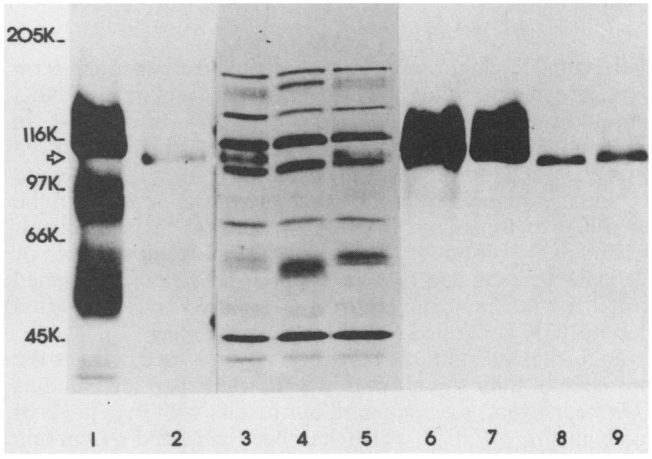

FIG. 8. SDS-PAGE analysis of gG immunoprecipitated (lanes 3 to 9) by 13αC5 from untreated or tunicamycin- (3 μg/ml) treated HSV-2-infected BHK-21 cells. Lanes: 1, whole cell extracts of untreated cells labeled with [^{3}H]galactose between 3 and 20 h p.i.; 2, whole cell extracts of tunicamycin-treated cells labeled with [^{3}H]galactose between 3 and 20 h p.i.; 3, tunicamycin-treated cells labeled with [^{35}S]methionine between 3 and 20 h p.i.; 4, tunicamycin-treated cells labeled with [^{35}S]methionine pulse for 10 min at 5 h p.i.; 5, tunicamycin-treated cells labeled with [^{35}S]methionine pulse for 10 min at 5 h p.i. followed by a 5-h chase as described in the legend to Fig. 1; 6 and 7, untreated cells labeled with [^{3}H]glucosamine and [^{3}H]galactose, respectively, between 3 and 20 h; 8 and 9, tunicamycin-treated cells labeled with [^{3}H]galactose and [^{3}H]glucosamine, respectively, between 3 and 20 h.

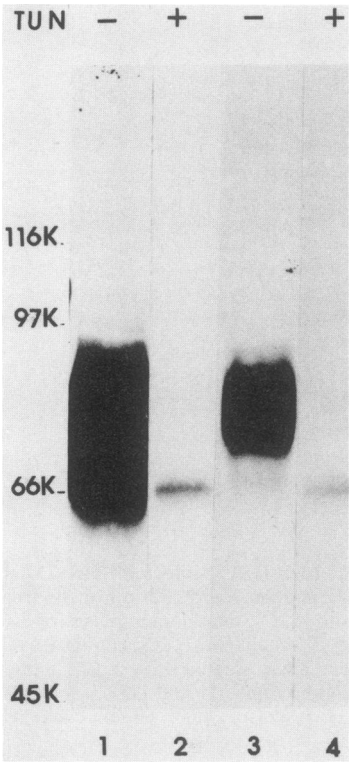

FIG. 9. SDS-PAGE of gC2 immunoprecipitated by 17αA2 from HSV-2-infected BHK-21 cells untreated or treated with tunicamycin. Lanes show infected cells labeled with: 1, [^{3}H]glucosamine between 3 and 20 h p.i. (untreated cells); 2, [^{3}H]glucosamine between 3 and 20 h p.i. (tunicamycin-treated cells); 3, [^{3}H]galactose between 3 and 20 h p.i. (untreated cells); 4, [^{3}H]galactose between 3 and 20 h p.i. (tunicamycin-treated cells). TUN, Tunicamaycin.

with both [^{3}H]glucosamine and [^{3}H]galactose, and long-term exposure of autoradiographs revealed the label in 80K, 90K, and 100K peptides (Fig. 8, lanes 6 and 7). In contrast, in tunicamycin-treated cells, only the 105K peptide was labeled with [^{3}H]galactose and [^{3}H]glucosamine (Fig. 8, lanes 8 and 9), and [^{3}H]mannose did not label any of the peptides in the presence of tunicamycin (data not shown). The ability of monoclonal antibody to precipitate [^{3}H]glucosamine or [^{3}H]galactose-labeled proteins but not [^{3}H]mannose-labeled proteins from tunicamycin-treated infected cells suggested that gp105K contained O-linked sugars.

To further substantiate that O-linkage was occurring in the presence of tunicamycin and that the 105K peptide contains O-linked sugars, we compared our results with the effects of tunicamycin on gC2, which has been reported to contain O-linked sugars (25). Immunoprecipitation with 17αA2 (anti-gC2) showed that in the absence of tunicamycin, the partially glycosylated precursors and final mature forms of gC2 ranging in size from 69,000 to 84,000 molecular weight were labeled with [^{3}H]glucosamine and [^{3}H]galactose (Fig. 9, lanes 1 and 3). In the presence of tunicamycin, only a 69K peptide was labeled with both sugars (Fig. 9, lanes 2 and 4). As the unglycosylated precursor for the gC2 is reported to be a 54K peptide and the high-mannose-type glycosylated precursor is a 67K to 69K peptide, the presence of [^{3}H]galactose and [^{3}H]glucosamine represents O-linked sugars and validates the tunicamycin experiment with 13αC5 antibody.

The enzyme endo F cleaves N-linked high-mannose and complex sugars (22) and does not affect O-linked sugars. Immunoprecipitates from extracts of cells infected with HSV-2 in the presence or absence of tunicamycin or monensin were incubated with endo F. Endo F converted the gG(108K) into a 105K peptide (Fig. 10, lanes 1 and 2), while the 105K peptide present in the tunicamycin-treated cells was not affected (Fig. 10, lanes 3 and 4), indicating the absence of N-linked sugars in this form. Endo F converted the 74K peptide present in the monensin-treated cells into a 66K peptide, while the 110K to 116K peptide was only partially susceptible, as faster-migrating, diffused-region peptides of about 105,000 to 108,000 molecular weight were seen (Fig. 10, lanes 5 and 6).

These results are interpreted as evidence for O-linkage or other types of sugar linkages in gG.

DISCUSSION

Detailed analyses of synthesis and processing of HSV glycoproteins have been made with polyclonal antibodies raised against the virion envelope (5, 8, 19), with monospecific antiserum against purified glycoproteins (7) and with monoclonal antibodies (2, 3, 16, 25). These studies have demonstrated that the four antigenically distinct glycoproteins of HSV-1 and -2 designated gC, gB, gD, and gE are formed by the stepwise addition of oligosaccharides to a partially glycosylated precursor. The unglycosylated precursor is not detectable under normal conditions of infection because N-linked sugars are added cotranslationally, and the primary sequences of the glycoproteins are unaltered except for the probable removal of signal peptides. The results

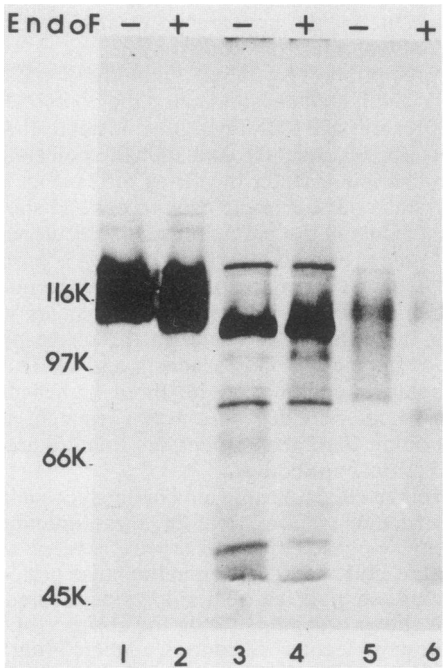

FIG. 10. Endo F susceptibility of gG immunoprecipitated by 13αC5 from HSV-2-infected cells untreated or treated with tunicamycin or monensin. Cells were labeled with [^{35}S]methionine between 3 and 20 h p.i. Lanes: 1 and 2, untreated cells; 3 and 4, cells treated with tunicamycin; 5 and 6, cells treated with monensin. Immunoprecipitated peptides were eluted and incubated with (+) or without (–) endo F, and samples were analyzed by SDS-PAGE.

presented here demonstrate that gG differs from other HSV-2 glycoproteins in several respects.

Processing of N-linked sugars from initial cotranslational glycosylation to final addition of complex sugars has been shown to consist of at least two sequential steps. First, high-mannose residues are added, at which point the glycoprotein is sensitive to endo H; second, the glycoprotein is trimmed and fucose, galactose, sialic acid, etc., are added, which renders the glycoprotein resistant to endo H. Experiments showing chase of the label from gp120K to gp108K indicate that gp120K is the precursor of the mature gp108K. This is substantiated by the fact that gp120K but not gp108K is sensitive to endo H.

There are two possible explanations for the apparent smaller size of the mature molecule. The first is that the size change can be accounted for entirely by the removal of high-mannose sugars and the addition of complex residues. This possibility seems unlikely since such changes would be expected to result in a molecule of apparent molecular weight greater than that of the unglycosylated precursor 110K peptide. The second possibility is that proteolytic cleavage occurred during the final processing stages. Although such cleavage is a novel finding for HSV, it is a step that has been shown to occur in the processing of other viral and cellular proteins. We postulate that the cleaved intermediate between gp120K and gp108K is gp74K. This molecule is seen only during very short pulse periods in the normal course of infection. It is more prominent in cells treated with monensin. We suggest that this is because monensin slows down normal functioning of the Golgi where the final processes of glycosylation occur. These conclusions are supported by the fact that larger amounts of gp74K can be found in HEp-2 cells (data not shown). Similar observations have been made by H. Su and R. Courtney (University of Tennessee, personal communication). Using our monoclonal antibody and a rabbit serum raised against gp108K, they detected a 72K intermediate in normally infected HEp-2 cells which also accumulates during monensin treatment. This further indicates differences in the efficiency and rapidity of glycosylation between cell types and gives credence to the notion that the very small amount of pgG_2(74K) found in untreated BHK-21 cells represents a cleaved intermediate in the normal processing of gG and not an artifact of monensin treatment.

The partial susceptibility of accumulated pgG_2(74K) to endo H indicates that it is undergoing some modifications, and its complete absence in the chase period implies that it is not a breakdown product but the actual cleaved form of pgG_1(120K). The cleavage of pgG_1(120K) was not affected by monensin, indicating that cleavage might be occurring in sites not affected by monensin. Moreover, endo H reduces the molecular weight of both the 120K peptide and the 74K peptide by about 8,000 each, which also indicates that cleavage may be occurring before the removal of sugars, possibly in a region of the molecule where no N-linked sugars are attached.

The monensin experiments also indicate that the pathways of oligosaccharide processing for gG are different from those of other HSV-2 glycoproteins. Monensin has been shown to affect different levels of processing depending upon the cell type and glycoprotein studied. Thus, in some instances the high-mannose type accumulates, and in other systems further processing, although incomplete, is seen (6). The complete susceptibility of gC2 to endo H and the insusceptibility of gG formed in monensin-treated cells indicate that further processing of gG may occur in cellular compartments not

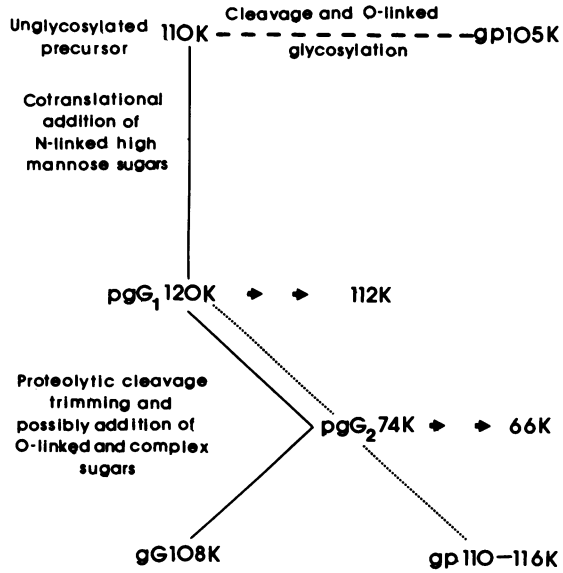

FIG. 11. Proposed pathway of processing of gG. Symbols: ——, processing in normal cells; – – –, processing in tunicamycin-treated cells; ·····, processing in monensin-treated cells; ◗ ◗, modification by endo H.

affected by monensin. However, the apparent molecular weight (110,000 to 116,000) of the peptide formed in the presence of monensin is higher than that of gG(108K) and probably reflects inhibition at some levels of removal or addition of terminal sugars or both. Similarly, the glycoprotein gC of HSV-1 was also reported to be partially processed from a high-mannose type in the presence of monensin (23). Preliminary experiments have shown that gG(108K) is susceptible to neuraminidase while the 110K to 116K peptide precipitated from monensin-treated cells remains insusceptible, possibly due to a lack of sialic acid. This further substantiates the theory that interference by monensin occurs in the final processing steps rather than in transport. We have noted that gG is the most abundant peptide present in normally infected culture supernatants (unpublished observation) and that monensin does not affect the secretion of gP110K to 116K.

The presence of glucosamine and galactose in peptides formed in the presence of tunicamycin has been considered as evidence for O-linked glycosylation, and the gC of HSV-1 has been shown to be labeled with these sugars in the presence of tunicamycin (24). The 105K peptide precipitated from tunicamycin-treated cells fits these criteria. The experiments with tunicamycin also indicated that a 110K peptide is the unglycosylated gG precursor. They imply that cotranslational addition of N-linked sugars to the 110K peptide results in pgG_1(120K) which undergoes proteolytic cleavage, trimming, and addition of complex and O-linked sugars to form the mature gG(108K). The origin of the 100K peptide and the lower-molecular-weight peptides is unclear. Since they are present even in the pulse period and do not label with sugars, they probably represent cleaved or breakdown products or both of the 110K molecule, and an investigation is under way to examine the relation between them. In the absence of tunicamycin, the label in pgG_1(120K) chases completely into the gG(108K) peptide. However, in the presence of tunicamycin, complete conversion of the 110K peptide to the 105K peptide does not occur. This is probably due either to impaired transport of the unglycosylated pre-

cursor or to interference by tunicamycin with the enzyme(s) responsible for cleavage of the precursor molecule.

During long-term labeling with [^{3}H]galactose or [^{3}H]glucosamine, these sugars can be metabolized and the ^{3}H label can be incorporated in the amino acids of a peptide. This did not happen in our experiments since there was no label in any of the other peptides precipitated from tunicamycin-treated cells, and although [^{35}S]methionine did label low-molecular-weight peptides of gB, gD, and gE, none of them contained sugar label (data not shown). Recently, lectin binding studies have suggested that gG contains O-linked sugars (21), and our data also confirm the presence of O-linked sugars in gG. However, we cannot exclude the possibility that some unusual form of sugar linkage in addition to O-linked glycosylation may occur in the presence of tunicamycin. Based on our present data, we propose a hypothetical model for the processing of gG (Fig. 11). Final proof of the hypothesis will require peptide mapping by limited proteolysis of pgG_1(120K), pgG_2(74K), and gG(108K). These experiments are in progress.

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