Glycopeptides of the Type-Common Glycoprotein gD of Herpes Simplex Virus Types 1 and 2

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We have carried out detailed structural studies of the glycopeptides of glycoprotein gD of herpes simplex virus types 1 and 2. We first examined and compared the number of *N*-asparagine-linked oligosaccharides present in each glycoprotein. We found that treatment of either pgD-1 or pgD-2 with endo- β -*N*-acetylglucosaminidase H (Endo H) generated three polypeptides which migrated more rapidly than pgD on gradient sodium dodecyl sulfate-polyacrylamide gels. Two of the faster-migrating polypeptides were labeled with [³H]mannose, suggesting that both pgD-1 and pgD-2 contained three *N*-asparagine-linked oligosaccharides. Second, we characterized the [³H]mannose-labeled tryptic peptides of pgD-1 and pgD-2. We found that both glycoproteins contained three tryptic glycopeptides, termed glycopeptides 1, 2, and 3. Gel filtration studies indicated that the molecular weights of these three peptides were approximately 10,000, 3,900, and 1,800, respectively, for both pgD-1 and pgD-2. Three methods were employed to determine the size of the attached oligosaccharides. First, the [³H]mannose-labeled glycopeptides were treated with Endo H, and the released oligosaccharide was chromatographed on Bio-Gel P6. The size of this molecule was estimated to be approximately 1,200 daltons. Second, Endo H treatment of [³⁵S]methionine-labeled glycopeptide 2 reduced the molecular size of this peptide from approximately 3,900 to approximately 2,400 daltons. Third, glycopeptide 2 isolated from the gD-like molecule formed in the presence of tunicamycin was approximately 2,200 daltons. From these experiments, the size of each *N*-asparagine-linked oligosaccharide was estimated to be approximately 1,400 to 1,600 daltons. Our experiments indicated that glycopeptides 2 and 3 each contained one *N*-asparagine-linked oligosaccharide chain. Although glycopeptide 1 was large enough to accommodate more than one oligosaccharide chain, the experiments with Endo H treatment of the glycoprotein indicated that there were only three *N*-asparagine-linked oligosaccharides present in pgD-1 and pgD-2. Further studies of the tryptic glycopeptides by reverse-phase high-performance liquid chromatography indicated that all of the glycopeptides were hydrophobic in nature. In the case of glycopeptide 2, we observed that when the carbohydrate was not present, the hydrophobicity of the peptide increased. The properties of the tryptic glycopeptides of pgD-1 were compared with the properties predicted from the deduced amino acid sequence of gD-1. The size and amino acid composition compared favorably for glycopeptides 1 and 2. Glycopeptide 3 appeared to be somewhat smaller than would be predicted from the deduced sequence of gD-1. It appears that all three potential glycosylation sites predicted by the amino acid sequence are utilized in gD-1 and that a similar number of glycosylation sites are present in gD-2.

Glycoprotein D (gD), a structural virion component of herpes simplex virus type 1 (HSV-1) (gD-1) and HSV-2 (gD-2) (5, 31), is important in the initial phases of infection, although its precise role remains obscure. In addition, this glycoprotein is incorporated into the plasma mem-

brane of infected cells (12, 23, 24, 32) and functions as a major target antigen recognized by both the cellular and the humoral immune response of the host to HSV infections (1, 4, 5, 7, 11, 22, 27, 32).

Recently, we have carried out detailed struc-

tural and immunological studies of glycoprotein gD (5, 6, 8–11, 26) in an effort to map and characterize the domains of the protein which are involved in its biological and immunological activities. Inherent in this type of study is an understanding of the contribution of the carbohydrate moiety to the structural, biological, and immunological properties of the glycoprotein. Our earlier studies (5, 6, 26), as well as those of others (13, 14, 16, 25, 31), suggested that the processing of the oligosaccharides of gD-1 occurred in a fashion similar to that reported for other glycoproteins (reviewed in reference 29). We showed that glycosylation of pgD-1 was inhibited by tunicamycin (TM), suggesting that oligosaccharide(s) were bound to the protein via an *N*-asparagine linkage (26). By using the enzyme endo- β -*N*-acetylglucosaminidase (endo-glycosidase H [Endo H]), which cleaves *N*-asparagine-linked oligosaccharides of the high-mannose type (33), two groups (25, 30) found that the mobility of pgD-1 on sodium dodecyl sulfate (SDS)-polyacrylamide gels was significantly increased. This was further evidence that pgD-1 contained *N*-asparagine-linked oligosaccharides. From studies of the nature and size of the oligosaccharides found in the precursor and product forms of gD-1, we previously proposed that this glycoprotein contained from one to three *N*-asparagine-linked oligosaccharides (9, 26). By examining similar details in gD-2 (10) and comparing it with gD-1, we further speculated that gD-2 might contain fewer oligosaccharide residues than does gD-1. Recently, the complete nucleotide sequence for the gene encoding gD-1 was determined (35). The deduced amino acid sequence indicates that the molecule contains three potential glycosylation sites. Moreover, the sequence allows one to speculate about the biochemical nature of the tryptic peptides bearing the oligosaccharide chains.

In the present study, our first goal was to determine the number of *N*-asparagine-linked oligosaccharide residues which are contained in both gD-1 and gD-2. A further goal was to compare the biochemical properties of the tryptic glycopeptides of gD-1 and gD-2 and to compare these data with those predicted from the nucleotide sequence.

MATERIALS AND METHODS

Cells and virus. Conditions for the growth and maintenance of BHK and KB cells and the propagation of virus have been previously described (11). For infection, an input multiplicity of 20 PFU of HSV-1 (strain HF) and 10 PFU of HSV-2 (strain SAVAGE) per cell was used.

Labeling procedures. Experiments were performed by the same methods as previously described (5, 6, 10). Monolayers (75-cm² flask) were pulse-labeled for 10 min at 5 h postinfection in Hanks salts with one of

the following radioisotopes and amounts: [³⁵S]methionine (specific activity, 600 Ci/mmol), 1 mCi; [2,3-³H]arginine (specific activity, 15 Ci/mmol), 1 mCi; [4,5-³H]lysine (specific activity, 60 Ci/mmol), 1 mCi; [2-³H]mannose, (specific activity, 10 Ci/mmol), 1 mCi; [³⁵S]cysteine (specific activity, 933 Ci/mmol), 1 mCi, or [2,3,4,5-³H]proline (specific activity, 102 Ci/mmol), 1 mCi; or [³H]isoleucine (specific activity, 110 Ci/mmol), 1 mCi. All isotopes were purchased from New England Nuclear Corp. After the pulse-labeling, cytoplasmic extracts were prepared from the monolayers as previously described (11).

For experiments in which TM (Calbiochem Behring Corp.) was employed, the following procedures were used. BHK cells were infected, and at 2 h postinfection, the cells were overlaid with medium containing 1/10 the normal concentration of methionine, 1% fetal calf serum, and TM at a final concentration of 5 μ g/ml. At 6 h postinfection, the cells were washed with fresh medium containing no methionine but with TM and then were labeled with [³⁵S]methionine for 30 min. The cells were treated as described previously (26).

Immunoprecipitation. Glycoprotein gD was immunoprecipitated with anti-CP-1 serum (5) plus *Staphylococcus aureus* protein A (IgG Sorb, New England Enzyme Center) by a previously described procedure (26).

SDS-PAGE. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in slabs of a 9 to 12% linear gradient of polyacrylamide cross-linked with 0.4% *N,N'*-diallyltartardiamide (8, 31). After electrophoresis, the gels were stained with Coomassie brilliant blue. For fluorography, the gels were impregnated with 2,5-diphenyloxazole, dried on filter paper, placed in contact with Kodak X-Omat AR5 film, and stored at -70°C (2).

Preparation of samples for tryptic peptide analysis. Glycoprotein gD of HSV-1 and HSV-2 was prepared for trypsinization by previously described methods (11). Briefly, pgD was immunoprecipitated and then suspended in SDS-disrupting buffer containing 10% β -mercaptoethanol. Bovine serum albumin (1 mg) was added as a protein carrier, and the protein was precipitated with 25% trichloroacetic acid at 4°C for 18 h. The samples were centrifuged, dissolved in 0.1 N NaOH, and precipitated with 25% trichloroacetic acid. Performic acid oxidation, trypsinization, and ion-exchange chromatography on Chromabeads P resin (Technicon Corp., Inc.) were carried out as previously described (6, 8, 11, 34). A sample of each of the column fractions was assayed for radioactivity, and the peak(s) was pooled and lyophilized. The sample was resuspended in the appropriate buffer for analysis by high-performance liquid chromatography (HPLC) or gel filtration.

Gel filtration. The tryptic digests of glycoprotein gD from HSV-1 and HSV-2 were chromatographed on a column (1.8 by 75 cm) of Bio-Gel P6 (200 to 400 mesh; Bio-Rad Laboratories). The methods used were essentially the same as previously described (8, 20). The column was eluted with 0.1 M ammonium acetate containing 0.02% sodium azide at a flow rate of 12 ml/h. ¹²⁵I-immunoglobulin G was used to define the void volume, and the total volume was determined by using [³H]mannose. To calibrate the column, [¹⁴C]glucosamine-labeled glycopeptide markers (generously donated by Clayton Buck, Wistar Institute, Philadel-

phia, Pa.) were used (4,300 daltons; 3,600 daltons; 1,800 daltons). Fractions of 2 ml were collected and assayed for radioactivity.

HPLC. HPLC experiments were performed on a Varian 5000 HPLC system. Reverse-phase partition chromatography with an MCH 10 column was performed as described by McMillan et al. (19). The samples were injected in 0.1 M phosphate buffer (pH 2.1) at 49°C, and a gradient of 100% 0.1 M phosphate to 100% acetone at a flow rate of 1 ml/min was used to elute the peptides.

Endo H treatment. (i) **Treatment of tryptic peptides.** The Endo H procedure of Kornfeld and Wold (18) was followed. Briefly, pgD-1 and pgD-2 were trypsinized by previously described procedures (11), lyophilized, and dissolved in buffer A (11); they were then lyophilized again and dissolved in 2 ml of water. The samples were split in half, lyophilized, and suspended in 100 μ l of 50 mM citrate-phosphate buffer (pH 6.5). Endo H (Miles Laboratories, Inc.) was added to one sample at a final concentration of 2 mU. The other sample received no enzyme. Both samples were incubated for 18 h at 37°C and then diluted to 1 ml with water, boiled for 3 min, lyophilized, suspended in 0.1 M ammonium acetate, and subjected to gel filtration on Bio-Gel P6.

(ii) **Treatment of pgD-1 and pgD-2.** The Endo H procedure of Braell and Lodish (3) was followed. Briefly, pgD-1 or pgD-2 was eluted from the *S. aureus* protein A immunosorbent by boiling for 5 min in 1% SDS-1% 2-mercaptoethanol. Samples were brought to 250 mM citrate (pH 6.0) containing 1 mM phenylmethylsulfonyl fluoride and digested for 20 h at 37°C with 3 mU of Endo H. Carrier bovine serum albumin was added, and the proteins were precipitated with trichloroacetic acid. The pellets were washed with ethanol-ether and dried. The pellets were redissolved in SDS-disrupting buffer containing mercaptoethanol and evaluated by SDS-PAGE.

RESULTS

Number of *N*-asparagine-linked oligosaccharides in pgD-1 and pgD-2. (i) **Endo H treatment of the glycoproteins.** Endo H cleaves high-mannose-type *N*-asparagine-linked oligosaccharides on proteins and peptides. The site of cleavage is between the first and second *N*-acetylglucosamine residues of the oligosaccharide (33). Experiments were undertaken to determine the effect of Endo H treatment on the electrophoretic mobilities of pgD-1 and pgD-2. HSV-1- or HSV-2-infected cells were pulse-labeled with [³⁵S]methionine or [³H]mannose and immunoprecipitated with anti-CP-1 serum (5). The glycoproteins were treated with Endo H for 20 h at 37°C, and the products were resolved by SDS-PAGE. For pgD-1 (Fig. 1, lane 2) or pgD-2 (Fig. 1, lane 6), Endo H treatment resulted in the generation of four [³⁵S]methionine-labeled polypeptide bands, three of which were [³H]mannose labeled (Fig. 1, lane 4). It appears from this gel that the lowest-molecular-weight form of pgD-1 generated by Endo H treatment contained no [³H]mannose. A similar result was obtained for mannose-labeled pgD-2 (data not shown).

FIG. 1. SDS-PAGE analysis of HSV-1 and HSV-2 gD treated with Endo H: fluorogram of a 9 to 12% gradient *N,N'*-diallyltartardiamide-cross-linked polyacrylamide gel of immune precipitates obtained from lysates of pulse-labeled HSV-1 or HSV-2-infected cells. Track 1, [³⁵S]methionine-labeled pgD-1; track 2, pgD-1 labeled with [³⁵S]methionine and treated with Endo H; tracks 3 and 4, identical to tracks 1 and 2 except that pgD-1 was labeled with [³H]mannose; track 5, pgD-2 labeled with [³⁵S]methionine; track 6, pgD-2 labeled with [³⁵S]methionine and treated with Endo H. The exposure time of the film was 10 days. Molecular size estimates (made from other exposures and gels) are as follows: pgD-1 (track 1), 53,000 daltons; the fastest-migrating polypeptide in track 2, 48,000 daltons; pgD-2 (track 5), 52,000 daltons; and the fastest-migrating polypeptide in track 6, approximately 47,500 daltons. All of these values are approximate and may vary by $\pm$ 500 daltons.

These data are consistent with the ideas that both gD-1 and gD-2 contain a total of three *N*-asparagine-linked oligosaccharides and that the four bands seen on the gel represent various extents of Endo H digestion (28). Each of the four Endo H-generated polypeptides differed in size by approximately 1,200 daltons.

(ii) **Gel filtration of [³H]mannose-labeled tryptic glycopeptides of pgD-1 and pgD-2.** In the next experiment, we employed tryptic peptide analysis as another approach for determining the number of oligosaccharides in pgD-1 and pgD-2. Cells infected with HSV-1 or HSV-2 were pulse-labeled for 10 min with [³H]mannose at 5 h postinfection. pgD-1 and pgD-2 were immunoprecipitated, and tryptic peptides were prepared and chromatographed on Bio-Gel P6. The radioactive elution profiles of [³H]mannose-labeled glycopeptides of pgD-1 and pgD-2 were virtually identical (Fig. 2A and B). For both proteins, three glycopeptides were resolved on Bio-Gel P6. The largest, glycopeptide 1, eluted in the flow through of the column. The size of this peptide was determined from other experiments with Bio-Gel P10 (data not shown) to be approximately 10,000 daltons. The next largest, glycopeptide 2, had an estimated molecular size of 3,900 daltons, and the smallest, glycopeptide 3,

FIG. 2. Gel filtration chromatography on Bio-Gel P6 of [^3H]mannose-labeled tryptic glycopeptides of pgD-1 and pgD-2. Glycoproteins of pgD-1 and pgD-2 were immunoprecipitated from [^3H]mannose-labeled HSV-1- or HSV-2-infected cells. Tryptic peptides were prepared and chromatographed on a Bio-Gel P6 column. (A) Tryptic glycopeptides of pgD-1; (B) tryptic glycopeptides of pgD-2; (C) tryptic peptides of gD-1 treated with Endo H (see text) and chromatographed on BioGel P6. Vo, void volume; Vt, total volume.

had a molecular size of 1,800 daltons. The proportion of mannose label in the three glycopeptides in several experiments was approximately 1:2:1, suggesting that (i) glycopeptide 2 may contain more oligosaccharide chains than does glycopeptide 1 or 3; (ii) there is a selective

glycosylation of certain sites in preference to others; or (iii) recoveries of the three peptides varied in a relatively consistent manner. The data in Fig. 2 suggest that both gD-1 and gD-2 contain three glycosylated tryptic peptides. However, it is possible that glycopeptide 1 represents an incomplete digestion of glycopeptide 2 or 3. We carried out additional experiments (not shown) and found that glycopeptides 1 and 3 are arginine peptides and that glycopeptide 2 is a lysine peptide. Experiments in Fig. 4 will show that glycopeptide 2 of gD-1 and gD-2 contains methionine and that glycopeptide 3 contains no methionine. Thus, glycopeptide 2 is clearly distinct from the other two. The remaining possibility is that glycopeptide 3 represents a portion of glycopeptide 1. We have isolated glycopeptide 1 by Bio-Gel P6 chromatography and attempted unsuccessfully to generate the smaller component with extensive trypsinization. This suggests that glycopeptide 1 does not represent an incomplete digestion product. Thus, the data appear to be consistent with the idea that gD-1 and gD-2 contain three distinct tryptic glycopeptides.

Figure 2C shows that all three tryptic glycopeptides for gD-1 contained Endo H-sensitive oligosaccharides and that the size of the cleaved oligosaccharide was approximately 1,200 daltons. Glycopeptide 3 could only accommodate one oligosaccharide chain of this size. However, glycopeptides 1 and 2 could each accommodate more than one. Thus, this experiment suggests that pgD-1 and pgD-2 contain at least three *N*-asparagine-linked oligosaccharides.

Analysis of the glycopeptides of gD-1 and gD-2 by cation-exchange chromatography. We previously characterized the tryptic peptides of gD-1 and gD-2 labeled with either [^{35}S]methionine or [^3H]arginine by cation-exchange chromatography on Chromabeads P (6, 8–11, 26). Figure 3 compares the radioactive elution profiles of [^3H]mannose- and [^{35}S]methionine-labeled tryptic peptides of pgD-1 (Fig. 2A) and pgD-2 (Fig. 2B). The profiles of the [^{35}S]methionine-labeled tryptic peptides eluted were similar to those previously described (6, 8–11, 26). Approximately 95% of the [^3H]mannose label incorporated into either pgD-1 or pgD-2 eluted in the Chromabeads P flow-through (CBFT) fraction. The remainder of the label was distributed throughout the resolved area. These results indicate that most of the tryptic peptides bearing *N*-asparagine-linked oligosaccharides were not resolved by cation-exchange chromatography.

Gel filtration studies of tryptic peptides eluting in the CBFT fraction. The next series of experiments was undertaken to examine the [^{35}S]methionine-labeled tryptic peptides of gD-1 and gD-2 that eluted in the CBFT fraction and to determine whether any of these peptides were

FIG. 3. Tryptic fingerprint analysis of HSV-1 and HSV-2 pgD labeled with [^{35}S]methionine or [^3H]mannose. pgD labeled with either radioisotope was isolated by immunoprecipitation, trypsinized, and chromatographed on Chromabeads P. (A) [^{35}S]methionine-labeled pgD-1 (-----) cochromatographed with [^3H]mannose-labeled pgD-1 (—); (B) [^{35}S]methionine-labeled pgD-2 (-----) cochromatographed with [^3H]mannose-labeled pgD-2 (—).

glycosylated. For these experiments, HSV-1- and HSV-2-infected cells were pulse-labeled with [^{35}S]methionine or [^3H]mannose, and the tryptic peptides were cochromatographed on a Chromabeads P column. The CBFT fractions were pooled, lyophilized, redissolved in ammonium acetate, and cochromatographed on Bio-Gel P6. The CBFT fraction of gD-1 contained two [^{35}S]methionine-labeled peptides, one of which comigrated with glycopeptide 2 (Fig. 4). The CBFT fraction of gD-2 contained only one [^{35}S]methionine-labeled peptide which coeluted with glycopeptide 2 (Fig. 4B). Glycopeptide 2 of pgD-1 comigrated with glycopeptide 2 of pgD-2 (Fig. 4C). These results indicate that this peptide is very similar or identical in amino acid compo-

sition in the two glycoproteins. In separate experiments, as mentioned previously, glycopeptide 2 was found to be a lysine peptide and was, in fact, the only lysine peptide found in the CBFT fraction of pgD-1 or pgD-2.

A major point to be noted from this experiment is that glycopeptide 3 (Fig. 2A and B) was not present in the CBFT derived from pgD-1 (Fig. 4A) or pgD-2 (Fig. 4B) and that the amount of glycopeptide 1 in the CBFT fraction was markedly reduced. When the [^3H]mannose-labeled peptides of pgD-1 or pgD-2 found in the resolved area of the Chromabeads P column were pooled, lyophilized, and chromatographed on Bio-Gel P6, we found that only glycopeptides 1 and 2 were represented. These results indicate

FIG. 4. Gel filtration chromatography of [^{35}S]methionine- and [^3H]mannose-labeled pgD tryptic glycopeptides eluting in the Chromabeads P flow-through. CBFT fractions 1 to 10 (Fig. 3) were pooled, lyophilized, dissolved in 0.1 M ammonium acetate, and chromatographed on Bio-Gel P6. (A) Tryptic glycopeptides from pgD-1 labeled with [^3H]mannose (—) and [^{35}S]methionine (-----); (B) tryptic glycopeptides from pgD-2 labeled as in (A); (C) CBFT fraction of pgD-1 labeled with [^{35}S]methionine (-----) cochromatographed with the total tryptic glycopeptides from pgD-2 labeled with [^3H]mannose (—). V_o , void volume; V_t , total volume.

that properties other than size alone determined whether the glycosylated peptides were resolved on the cation-exchange column. Furthermore, one nonglycosylated methionine-containing pep-

tide of gD-1 was present in the CBFT fraction.

We conclude from these studies that for pgD-1 the CBFT fraction contained at least three tryptic peptides. For pgD-2, the CBFT fraction contained at least two tryptic peptides. One of these peptides (glycopeptide 1) appeared to partition between the flow through and the resolved portion of the Chromabeads P column. If one sums the apparent molecular weights of the three tryptic peptides of gD-1 and the two tryptic peptides of gD-2, these peptides account for at least 25 to 33% of the total protein.

Further characterization of glycopeptide 2. The presence of methionine in glycopeptide 2 and the size of the peptide made it suitable for further study. Experiments in this section were designed to examine the properties of this peptide in the presence and absence of the oligosaccharide. To simplify presentation of the data, we will show only the results for glycopeptide 2 isolated from pgD-2. However, identical results were obtained for glycopeptide 2 isolated from pgD-1.

(i) **Gel filtration studies.** Figure 5 compares the size of glycopeptide 2 synthesized in the absence (Fig. 5A) and presence (Fig. 5B) of TM. The size of the peptide was reduced from 3,900 to approximately 2,200 daltons. Figure 5C shows the size of glycopeptide 2 (from Fig. 5A) treated with Endo H. This peptide (2,400 daltons) was slightly larger than the peptide synthesized in the presence of TM (2,200 daltons). The difference in molecular size is probably due to the extra *N*-acetylglucosamine present in the Endo H-treated sample. These results show that glycopeptide 2 contains one *N*-asparagine-linked oligosaccharide chain. Thus, we have found that two of the three glycopeptides (glycopeptides 2 and 3) of pgD-1 and pgD-2 contain only a single oligosaccharide.

(ii) **HPLC analysis of glycopeptide 2 synthesized in the presence and absence of TM.** The HPLC analysis was undertaken to examine the effect of the oligosaccharide on the hydrophobicity of glycopeptide 2. Figure 6 compares the MCH-10 column elution profiles of [^{35}S]methionine-labeled glycopeptide 2 of gD-2 synthesized in the absence (Fig. 6A) and presence (Fig. 6B) of TM. The results show that glycopeptide 2 eluted at a more hydrophobic position when the oligosaccharide was absent. The results suggest that the amino acids in glycopeptide 2 were mostly non-polar or hydrophobic. In other studies (data not shown), glycopeptides 1 and 3 were isolated by Bio-Gel P6 chromatography and analyzed by HPLC. The results indicated that these peptides were also hydrophobic. Our results suggest that the presence of three *N*-asparagine-linked oligosaccharides may affect the overall hydrophobicity of glycoprotein gD. Table 1 summarizes the

properties of the three tryptic glycopeptides of gD-1 and gD-2. Included in Table 1 are data for the three peptides predicted from the deduced amino acid sequence of gD-1.

DISCUSSION

Previously, we predicted that gD-1 contained no more than two or three *N*-asparagine-linked oligosaccharide chains (9, 26). We also predicted that gD-2 might contain fewer oligosaccharide chains than does gD-1. In the present study, we found that gD-1 and gD-2 each contain at least three, and probably not more than three, *N*-asparagine-linked carbohydrate chains. This conclusion was based on results of two different kinds of experiments. First, we studied the effect of the enzyme Endo H on pgD-1 and pgD-2. We found that the enzyme generated three faster-migrating polypeptides from pgD-1 and pgD-2 which were resolvable by SDS-PAGE. The lowest-molecular-weight form could be labeled with [35 S]methionine but not with [3 H]mannose. The three larger polypeptides could be labeled with both [35 S]methionine and [3 H]mannose. These results suggested that both pgD-1 and pgD-2 contained three Endo H-sensitive sites. Our results extended the observations of other groups (25, 30) which showed that the precursor form of gD-1 contained an Endo H-sensitive oligosaccharide(s). These results also confirmed our earlier studies which used TM to show that pgD-1 contained *N*-asparagine-linked oligosaccharides (26).

If one includes the contribution of the uncleaved *N*-acetylglucosamine (220 daltons), the total molecular size contribution of the three high-mannose-type oligosaccharides to pgD-1 and pgD-2 appears to be approximately 4,300 daltons. This difference is consistent with the observed difference in size between pgD-1 (53,000 daltons) and the gD-1-like molecule (TM-gD-1) (49,500 daltons) formed in the presence of TM (26). This difference is also consistent for gD-2 (52,000 daltons) versus TM-gD-2 (49,000 daltons) (J. Matthews, G. H. Cohen, and R. J. Eisenberg, manuscript in preparation). As noted by Watson et al. (35), the lowest-molecular-weight form appeared to be approximately 20% larger than the molecular weight predicted from the deduced amino acid sequence (35).

The second approach used to determine the number of oligosaccharides was to identify and characterize those tryptic peptides of gD-1 and gD-2 which contained [3 H]mannose. We found that both glycoproteins contained three such tryptic peptides, called glycopeptides 1, 2, and 3, with molecular weights estimated from Bio-Gel experiments to be 10,000, 3,900, and 1,800, respectively (Table 1). Based on all of our results, we estimate that the size of each of the

FIG. 5. Gel filtration chromatography of [35 S]methionine-labeled glycopeptide 2 obtained from pgD-2 synthesized in the presence or absence of TM. Tryptic glycopeptides of pgD-2 labeled with [35 S]methionine were isolated from the CBFT fraction. The pooled sample was lyophilized, suspended in 0.1 M ammonium acetate, and chromatographed on Bio-Gel P6. (A) Glycopeptide 2 isolated from pgD-2; (B) glycopeptide 2 isolated from pgD-2 synthesized in the presence of TM; (C) glycopeptide 2 from the experiment shown in (A) treated with Endo H and rerun on Bio-Gel P6. Vo, void volume; Vt, total volume.

attached oligosaccharides in the precursor is approximately 1,400 to 1,600 daltons and that, therefore, glycopeptide 3 contains no more than one oligosaccharide. The size estimate of the

FIG. 6. HPLC of [^{35}S]methionine-labeled glycopeptide 2 isolated from pgD-2 synthesized in the presence and absence of TM. The CBFT fraction was isolated, lyophilized, suspended in 0.1 M phosphate buffer (pH 2.1), and chromatographed by HPLC. (A) Glycopeptide 2 of pgD-2; (B) glycopeptide 2 of pgD-2 synthesized in the presence of TM.

oligosaccharide was based on several different results. First, in previous experiments, we had estimated that the size of the [^3H]mannose-labeled oligosaccharide in pgD-1 after exhaustive pronase digestion was 1,800 daltons. This value was probably somewhat high due to the presence of amino acids remaining after pronase treatment (8). Second, in the present study, we estimated that each of the polypeptides generated from pgD-1 or pgD-2 after Endo H treatment differed by about 1,200 daltons, according to their migration on SDS-polyacrylamide gels. Endo H treatment of [^{35}S]methionine-labeled

glycopeptide 2 reduced its molecular size from approximately 3,900 to approximately 2,400 daltons (Table 1). Third, glycopeptide 2 derived from the gD-like molecule found in TM-treated cells was approximately 2,200 daltons. The difference in the last two values was probably due to the presence of *N*-acetylglucosamine on the glycopeptide after Endo H treatment. These results indicated that glycopeptide 2 contained one *N*-asparagine-linked oligosaccharide. Because glycopeptide 1 was large, no estimate could be made of the number of *N*-asparagine-linked oligosaccharides in this molecule. The

TABLE 1. Comparison of the properties of the three tryptic glycopeptides of gD-1 with those predicted from the deduced amino acid sequence

Tryptic glyco-peptide	Mol wt of tryptic peptide			Predicted mol wt of corresponding predicted peptide ^b	Amino acids in peptides	
	+CHO ^a	TM	Endo H		Found ^c	Predicted
1	≅10,000	ND ^d	?	10,172	Arg ^c Pro Met Ileu?	Arg Pro Met Ileu
2	3,900 (±200)	2,200 (±100)	2,400 (±100)	2,320	Lys, Pro Met, Cys Ileu?	Lys, Pro Met, Cys Ileu
3	1,800	ND	ND	1,337	Arg	Arg

^a +CHO, Containing carbohydrate.^b The locations of predicted tryptic peptide residue numbers (35) were as follows: 1, residues 271 to 367 (96 amino acids); 2, residues 127 to 147 (21 amino acids); 3, residues 116 to 126 (11 amino acids).^c Metabolically labeled. The presence in the peptide based on elution properties of the labeled tryptic peptides was resolved by at least two of the following chromatographic procedures: cation-exchange chromatography (Chromabeads P), gel filtration (Bio-Gel P6 and P4), and reverse-phase chromatography (HPLC). See the text for details.^d ND, Not determined.^e Low levels found.

overall conclusion from these results is that pgD-1 and pgD-2 each contain at least three *N*-asparagine-linked oligosaccharides. The results of the Endo H experiments (Fig. 1) favor the idea that pgD-1 and pgD-2 contain only three *N*-asparagine-linked oligosaccharides.

Recently, Watson et al. (35) localized and sequenced the gene for gD-1 (Patton strain). From the deduced amino acid sequence, they proposed that gD-1 contained three asparagine residues, with the correct adjacent sequence to be potential glycosylation sites. Our data suggest that all three potential glycosylation sites are utilized in gD-1 and predict that this should also be true for gD-2. Table 1 compares the properties of the three potential tryptic glycopeptides predicted from the sequence with the three glycopeptides characterized in the present study. We propose that glycopeptide 1 corresponds to the potential arginine tryptic peptide at residues 271 to 366 and that glycopeptide 2 corresponds to a lysine peptide at residues 127 to 147. It can be seen that there is fairly good agreement in the predicted and actual physical and chemical characterization of these two peptides. The third peptide, glycopeptide 3, at residues 116 to 126, was considerably smaller than that predicted from the amino acid sequence. Some of the size discrepancy could be attributed to inaccuracies associated with attempting to size glycopeptides by gel filtration (21). Glycopeptides 1 and 2 appear to have slightly lower molecular weights than predicted. However, in

the case of glycopeptide 3, the difference appears to be much greater. The reason for this discrepancy remains to be resolved.

It is interesting to note that the use of cation-exchange chromatography has allowed us to isolate two of the glycopeptides (glycopeptides 1 and 2) of gD and to account for the [³⁵S]methionine-containing tryptic peptides in the unresolved flow-through region of the Chromabeads P column. Thus, for gD-1, 75% of the total methionine label recovered was found in the CBFT fraction and was resolved into two peptides. For gD-2, 40% of the total methionine label was found in the CBFT fraction and was resolved into one peptide. Klemenz and Diggelmann (17) reported that the oligosaccharide-containing tryptic peptides of the glycoprotein of avian myeloblastosis virus did not bind to a similar cation-exchange resin and speculated that this might be due to the large size of these peptides. Our results suggest that the presence of specific tryptic peptides in the CBFT fraction could be due, in some cases, to their large size or to their hydrophobicity or to both. Our results also suggest that a combination of chromatographic techniques should be used to obtain a more complete tryptic peptide profile for a protein and, in particular, oligosaccharide-containing peptides.

One interesting property of all of the glycopeptides is that their elution patterns by HPLC indicated that they were hydrophobic in nature. If one assigns the hydrophobicity values of

Hopp and Woods (15) to each amino acid in the glycopeptides, the peptides should be hydrophobic in nature. In the case of glycopeptide 2, we observed that when the carbohydrate was absent, the hydrophobicity of the peptide appeared to increase. It is possible that the presence or absence of oligosaccharides could have a profound effect on the folding of gD regions and thereby alter the relation of gD to its lipid and nonlipid environment. Person et al. (25) suggested that both the quantity and the quality of a particular glycoprotein (including gB and gD) may be important in causing extensive fusion of cells. One could speculate that if certain potential sites on gD were not glycosylated, e.g., in a mutant, this region of the protein would now be hydrophobic in nature, come in contact with plasma membrane, and cause alterations and possibly fusion.

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LITERATURE CITED

- Balachandran, N., S. Bacchetti, and W. E. Rawls. 1982. Protection against lethal challenge of BALB/c mice by passive transfer of monoclonal antibodies to five glycoproteins of herpes simplex virus type 2. *Infect. Immun.* 37:1132-1137.
- Bonner, W. M., and R. A. Laskey. 1974. A film detection method for tritium labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* 46:83-88.
- Braell, W. A., and H. F. Lodish. 1981. Biosynthesis of the erythrocyte anion transport protein. *J. Biol. Chem.* 256:11337-11344.
- Carter, V. C., P. L. Rice, and S. S. Tevethia. 1982. Intratypic and intertypic specificity of lymphocytes involved in the recognition of herpes simplex virus glycoproteins. *Infect. Immun.* 37:116-126.
- Cohen, G. H., M. Katze, C. Hydrean-Stern, and R. J. Eisenberg. 1978. Type-common CP-1 antigen of herpes simplex virus is associated with a 59,000-molecular-weight envelope glycoprotein. *J. Virol.* 27:172-181.
- Cohen, G. H., D. Long, and R. J. Eisenberg. 1980. Synthesis and processing of glycoproteins gD and gC of herpes simplex virus type 1. *J. Virol.* 36:429-439.
- Dix, R. D., L. Pereira, and J. R. Baringer. 1981. Use of monoclonal antibody directed against herpes simplex virus glycoproteins to protect mice against acute virus-induced neurological disease. *Infect. Immun.* 29:724-732.
- Eisenberg, R. J., C. Hydrean-Stern, and G. H. Cohen. 1979. Structural analysis of precursor and product forms of type-common envelope glycoprotein D (CP-1 antigen) of herpes simplex virus type 1. *J. Virol.* 31:608-620.
- Eisenberg, R. J., D. Long, L. Pereira, B. Hampar, M. Zweig, and G. H. Cohen. 1982. Effect of monoclonal antibodies on limited proteolysis of native glycoprotein gD of herpes simplex virus type 1. *J. Virol.* 41:478-488.
- Eisenberg, R. J., M. Ponce de Leon, and G. H. Cohen. 1980. Comparative structural analysis of glycoprotein gD of herpes simplex virus types 1 and 2. *J. Virol.* 35:428-435.
- Eisenberg, R. J., M. Ponce de Leon, L. Pereira, D. Long, and G. H. Cohen. 1982. Purification of glycoprotein gD of herpes simplex virus types 1 and 2 by use of monoclonal antibody. *J. Virol.* 41:1099-1104.
- Glorioso, J. C., M. Levine, T. C. Holland, and M. S. Szczesiul. 1980. Mutant analysis of herpes simplex virus-induced cell surface antigens: resistance to complement-mediated immune cytolysis. *J. Virol.* 35:672-681.
- Haarr, L., and H. S. Marsden. 1981. Two-dimensional gel analysis of HSV type 1-induced polypeptides and glycoprotein processing. *J. Gen. Virol.* 52:77-92.
- Honess, R. W., and B. Roizman. 1975. Proteins specified by herpes simplex virus. XIII. Glycosylation of viral polypeptides. *J. Virol.* 16:1308-1326.
- Hopp, T. P., and K. R. Woods. 1981. Prediction of protein antigenic determinants from amino acid sequences. *Proc. Natl. Acad. Sci. U.S.A.* 78:3824-3828.
- Johnson, D. C., and P. G. Spear. 1982. Monensin inhibits the processing of herpes simplex virus glycoproteins, their transport to the cell surface, and the egress of virions from infected cells. *J. Virol.* 43:1102-1112.
- Klemenz, R., and H. Diggelmann. 1978. The generation of the two envelope glycoproteins of Rous sarcoma virus from a common precursor polypeptide. *Virology* 85:63-74.
- Kornfeld, R., and W. S. M. Wold. 1981. Structures of the oligosaccharides of the glycoprotein coded by early region E3 of adenovirus 2. *J. Virol.* 40:440-449.
- McMillan, M., J. M. Cecka, L. Hood, D. B. Murphy, and H. O. McDevitt. 1979. Peptide map analysis of murine Ia antigens of the I-E subregion using HPLC. *Nature (London)* 277:663-664.
- Nakamura, K., and R. W. Compans. 1977. The cellular site of sulfation of influenza virus glycoproteins. *Virology* 79:381-392.
- Nathans, S., and H. Lis. 1982. Glycoproteins, p. 1-144. *In* H. Neurath and R. L. Hill (ed.), *The proteins*, vol. 5. Academic Press, Inc., New York.
- Norrild, B. 1980. Immunochemistry of herpes simplex virus glycoproteins. *Curr. Top. Microbiol. Immunol.* 90:67-106.
- Norrild, B., O. J. Bjerrum, H. Ludwig, and B. F. Vestergaard. 1978. Analysis of herpes simplex virus type 1 antigens exposed on the surface of infected tissue culture cells. *Virology* 87:307-316.
- Peake, M. L., P. Nystrom, and L. I. Pizer. 1982. Herpesvirus glycoprotein synthesis and insertion into plasma membranes. *J. Virol.* 42:678-690.
- Person, S., K. G. Kousoulas, R. W. Knowles, G. S. Read, T. C. Holland, P. M. Keller, and S. C. Warner. 1982. Glycoprotein processing in mutants on HSV-1 that induce cell fusion. *Virology* 117:293-306.
- Pizer, L. I., G. H. Cohen, and R. J. Eisenberg. 1980. Effect of tunicamycin on herpes simplex virus glycoproteins and infectious virus production. *J. Virol.* 34:142-153.
- Rector, J. T., R. N. Lausch, and J. E. Oakes. 1982. Use of monoclonal antibodies for analysis of antibody-dependent immunity to ocular herpes simplex virus type 1 infection. *Infect. Immun.* 38:168-174.
- Rothman, J. E., F. N. Katz, and H. F. Lodish. 1978. Glycosylation of a membrane protein is restricted to the growing polypeptide chain but is not necessary for insertion as a transmembrane protein. *Cell* 15:1447-1454.
- Sabatini, D. D., G. Kreibich, T. Morimoto, and M. Adenik. 1982. Mechanisms for the incorporation of proteins into membranes and organelles. *J. Cell Biol.* 92:1-22.
- Serafini-Cessi, F., and G. Campadelli-Fiume. 1981. Studies on benzhydrazone, a specific inhibitor of herpes virus glycoprotein synthesis. Size distribution of glycopeptides and endo- β -N-acetylglucosaminidase H treatment. *Arch. Virol.* 70:331-343.
- Spear, P. G. 1976. Membrane proteins specified by herpes

- simplex viruses. I. Identification of four glycoprotein precursors and products in type 1-infected cells. *J. Virol.* 17:991-1008.
32. Spear, P. G. 1980. Herpesviruses, p. 709-750. *In* H. A. Blough and J. M. Tiffany (ed.), *Cell membranes and viral envelopes*, vol. 2. Academic Press, Inc. New York.
33. Tarentino, A. L., T. H. Plummer, Jr., and F. Maley. 1974. The release of intact oligosaccharides from specific glycoproteins by endo- β -N-acetylglucosaminidase H. *J. Biol. Chem.* 249:818-824.
34. Vogt, V. M., R. Eisenman, and H. Diggelmann. 1975. Generation of avian myeloblastosis virus structural proteins by proteolytic cleavage of a precursor polypeptide. *J. Mol. Biol.* 96:471-493.
35. Watson, R. J., J. H. Weis, J. S. Salstrom, and L. W. Enquist. 1982. Herpes simplex virus type-1 glycoprotein D gene: nucleotide sequence and expression in *Escherichia coli*. *Science* 218:381-384.