JOURNAL OF VIROLOGY, Sept. 1979, p. 608-620
0022-538X/79/09-0608/13$02.00

Vol. 31, No. 3

Structural Analysis of Precursor and Product Forms of Type-Common Envelope Glycoprotein D (CP-1 Antigen) of Herpes Simplex Virus Type 1

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Received for publication 16 February 1979

The type-common CP-1 antigen of herpes simplex virus type 1 (HSV-1) is associated in the infected cell with two components, a 52,000-molecular-weight glycoprotein (gp52 or pD) and a 59,000-molecular-weight glycoprotein (gp59 or D). The larger form (D) is also found in the virion envelope. It was postulated that pD is a precursor of D. We found that pD shared methionine and arginine tryptic peptides with D isolated from infected cell extracts. D isolated from infected extracts had the same tryptic methionine peptide profile as D isolated from the virion envelope. Thus, processing of pD to D does not involve any major alterations in polypeptide structure. Furthermore, D did not share tryptic methionine peptides with the other major glycoproteins of HSV-1. Using [2-^{3}H]mannose as a specific glycoprotein label, we found that pD, which is a basic protein (isoelectric point = 8.0) contained a 1,800-molecular-weight oligomannosyl core moiety and was processed by further glycosylation and sialyation to a more acidic and heterogeneous molecule D, which has a molecular weight of at least 59,000.

Herpes simplex virus type 1 (HSV-1) glycoproteins are present in infected cells in both a precursor and product form (2, 7, 19). The major glycosylated proteins are derived from four antigenically distinct polypeptides (19). The mature glycoproteins have been designated A, B, C, and D, and the precursors have been designated pA, pB, pC, and pD by Spear (19). This terminology was accepted provisionally at the 1978 Herpes Workshop at Cambridge, England. Processing of these glycoproteins from a lower-molecular-weight precursor to the higher-molecular-weight product does not require protein synthesis (19), and the modification of each molecule apparently involves several glycosylation steps (2, 19).

The virion envelope contains several glycoproteins (2, 7, 19), one component at approximately 59,000 daltons, and a 130,000-molecular-weight complex (gp130) containing two or three components. Previously, we described an HSV-specific antigen, CP-1 (2, 3), purified from HSV-1-infected cells and having the properties of a glycoprotein. This glycoprotein stimulated the production of high titers of type-common neutralizing antibody. Our recent study (2) showed that the type-common CP-1 antigenic activity was associated in infected cells with two molecular weight components, a 52,000-molecular-weight glycoprotein (gp52) and a 59,000-molecular-weight glycoprotein (gp59). We presented evidence that gp52 is the precursor of gp59, the latter being found in the infected cell extract as well as in the purified virion. We believe that the precursor, gp52, corresponds to pD and that the product, gp59, corresponds to D. In this paper, we shall adopt the Cambridge terminology and refer to the two forms of CP-1 as pD and D.

The present study was undertaken to document the synthesis and processing of the HSV type-common glycoprotein D. The evidence presented here demonstrates that pD shares methionine and arginine tryptic peptides with D. The first detectable precursor, pD, contains an 1,800-molecular-weight oligomannosyl core moiety and is basic in nature (isoelectric point = 8.0). Furthermore, pD is processed by further glycosylation and sialyation to a more acidic and heterogeneous molecule, D, which has a molecular weight of at least 59,000. Finally, D does not appear to share any methionine peptides with the gp130 complex extracted from the virion.

MATERIALS AND METHODS

Cell cultures. Conditions for the growth and maintenance of KB cells and baby hamster kidney cells have been previously described (3).

Virus preparation and titration. The procedures used for the preparation of virus stocks of HSV-1 (strain HF), as well as the plaque assay, were described previously (3). For infection, unless otherwise noted, an input multiplicity of 20 PFU of HSV per cell was employed.

Virus purification. HSV-1 was purified by the method of Spear and Roizman (20). For radioactive labeling of virus 10 μCi of [^{35}S]methionine (specific activity, >200 Ci/mmol) per ml, 30 μCi of [*methyl*-^{3}H]methionine (specific activity, 9 Ci/mmol) per ml, or 10 μCi of [2-^{3}H]mannose (specific activity, 18 Ci/mmol) per ml was added at 5 h postinfection (p.i.). Cells were harvested for virus purification at 18 h p.i. To solubilize the HSV envelope, purified virions were suspended in 0.02 M Tris buffer (pH 7.5) containing 0.05 M NaCl and 0.5% Nonidet P-40 (NP40) and incubated at 37°C for 45 min. TPCK (L-1-tosylamide-2-phenethyl chloromethyl ketone) and TLCK (*N*-α-*p*-tosyl-L-lysine chloromethyl ketone hydrochloride) were added, each at a concentration of 0.1 mM, to inhibit proteolytic activity. Nucleocapsids were removed by centrifugation at 100,000 × *g* for 1 h. The 100,000-×-*g* pellet was reextracted with NP-40 and recentrifuged. The supernatants from both extractions were pooled and frozen at −70°C.

Pulse-chase experiments. Pulse-chase experiments were performed on KB cell cultures (60-mm plates containing label in 1.5 ml of Hanks salts) essentially by the same method as described previously (2). The following labels and total amounts were used: [^{35}S]methionine (specific activity, 503 Ci/mmol), 200 μCi; [^{3}H]methionine (specific activity, 9 Ci/mmol), 1.0 mCi; [*U*-^{14}C]arginine (specific activity, 340 mCi/mmol), 125 μCi; [^{3}H]arginine (specific activity, 22 Ci/mmol), 1 mCi; [2-^{3}H]mannose (specific activity, 18 Ci/mmol), 1.0 mCi; and [*U*-^{14}C]glucosamine (specific activity, 238 mCi/mmol), 100 μCi. The pulse period for mannose and glucosamine labeling was 30 min, and that for methionine and arginine was 15 min. After the pulse and chase, the cells were lysed as described previously (2), except that TPCK and TLCK (0.1 mM) were added to all solutions and all glassware was sterilized to minimize proteolysis.

Immunological procedures. The antisera used in this study have been described previously (2, 3). Anti-CP-1 serum was employed as a monoprecipitin antibody against pD and D components. Anti-ENV-1 serum (previously designated anti-HSV-ENV serum [2]) prepared against the NP40 extract of the virion envelope of HSV-1 was employed to precipitate the envelope glycoproteins.

Immune precipitations were carried out as previously described (2), except that: (i) 0.1 mM TPCK and 0.1 mM TLCK were added to the mixtures, and (ii) the mixtures were maintained at 4°C for 2 days.

Electrophoresis on SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in slabs of 10% acrylamide cross-linked with 0.26% *N,N'*-methylenebisacrylamide (BIS) as described previously (2) or in slabs of 10% acrylamide cross-linked with 0.4% *N-N'*-diallyltartardiamide (DATD) essentially as described by Spear (19). After electrophoresis, the gels were stained with Coomassie brilliant blue as described previously (2). For autoradiography, the gels were dried on filter paper and placed in contact with Kodak X-Omat R (XR-5) film. For fluorography, the gels were impregnated with 2,5-diphenyloxazole (1) placed in contact with Kodak X-Omat R film and stored at −70°C. Protein standards ranging from 15,000 to 130,000 daltons were run on each gel (2). Two-dimensional gel electrophoresis was performed by the technique of O'Farrell (15).

Preparation of samples for tryptic peptide analysis. Proteins were eluted from BIS cross-linked gels and prepared for trypsinization by the procedure of Vogt et al. (23). DATD cross-linked gel slices were dissolved in 2 ml of 2% periodic acid per four to five slices by the procedure of Gibson (6). Both methods gave identical tryptic methionine peptide profiles for the major capsid protein of HSV-1 (M. Ponce de Leon, G. H. Cohen, and R. J. Eisenberg, unpublished data). For DATD gels, bovine serum albumin was added as a protein carrier (1 mg per four to six gel slices), and the proteins were precipitated with 25% trichloroacetic acid at 4°C for 12 to 18 h. Precipitates were collected by centrifugation at 100,000 × *g* for 2 h (W. Gibson, personal communication), dissolved in 0.1 N NaOH, and precipitated twice more with trichloroacetic acid. Trypsinization and ion-exchange chromatography on Chromobeads P (Technicon) were carried out as described by Vogt et al. (23). Briefly, the pellet was oxidized for 1 h at 0°C with 0.1 ml of freshly made performic acid (0.1 ml of H_2O_2 + 1.9 ml of formic acid incubated for 1 h at 25°C). A 1-ml amount of water was added to stop the reaction, and the sample was lyophilized. The dried protein was dissolved in 0.5 ml of 0.05 M ammonium carbonate and lyophilized a second time. The protein was dissolved in 0.2 ml of 0.05 M ammonium carbonate containing TPCK-trypsin (Worthington Biochemicals Corp.) in a 10:1 ratio of bovine serum albumin to trypsin and incubated for 1 h at 37°C. A second equal amount of trypsin was added, and the reaction was allowed to proceed for 1 h more. The trypsinized protein was lyophilized, suspended in buffer A (280 ml of acetic acid, 4 ml of pyridine, and 716 ml of water, pH 2.45), and stored at −20°C.

Cation-exchange chromatography on a jacketed Chromobeads P column (1 by 21 cm) was carried out essentially by the procedure of Vogt et al. (23). The column temperature was maintained at 50°C. Peptides were eluted in 2.2-ml fractions, with a pH gradient of 2.45 to 3.8. Three mixing chambers were used to generate this gradient. Mixing chambers 1 and 2 each contained 180 ml of buffer A, and chamber 3 contained 60 ml of buffer A and 120 ml of buffer B (143 ml of acetic acid, 161 ml of pyridine, and 696 ml of water, pH 4.9). At the end of the gradient elution (pH 3.8), the column was washed with buffer B.

Preparation of pronase digests for Bio-Gel P6 chromatography. Cytoplasmic extracts were precipitated with 20% trichloroacetic acid at 4°C. The precipitates were centrifuged at 10,000 rpm for 30 min, suspended in a minimal volume of 0.1 N NaOH, and dialyzed against several changes of distilled water for 16 h at 4°C. The dialysates were brought to a concentration of 0.1 M Tris, pH 8.0, containing 1 mM $CaCl_2$ (T-C buffer) (13). Pronase (10 mg/ml, autodigested in

T-C buffer for 2 h at 37°C) was added to the protein sample in three portions over a period of 72 h to achieve a final concentration of 1 mg of pronase per mg of protein. Toluene was added to retard bacterial growth, and incubation was carried out at 37°C. Pronase digests were centrifuged at 10,000 × *g* for 30 min, and the supernatants were frozen at −20°C. The following procedure was employed to extract proteins from BIS-linked polyacrylamide gels. The appropriate bands were excised from the dried gel and dissolved in 5 ml of T-C buffer containing 0.5 mg of pronase. The gel pieces were incubated in this solution for 3 days, and additional pronase (0.5 mg at a time) was added each day. The extract was centrifuged at 10,000 × *g*, and the supernatant was frozen at −20°C (R. Klemenz, personal communication).

To remove sialic acid, samples were adjusted to a pH of 1.5 to 1.6 with H_2SO_4 (final concentration, 0.05 M) and heated in tubes fitted with Teflon-lined caps at 90°C for 1 h (5). The samples were immediately chilled, then adjusted to pH 7.0 with 1 M Tris.

Gel filtration. The pronase-digested glycopeptides were subjected to gel filtration on a Bio-Gel P6 column (1.8 by 75 cm; 200 to 400 mesh; Bio-Rad Laboratories) equilibrated with 0.1 M ammonium acetate containing 0.01% sodium azide. The flow rate of the column was 16 ml/h. Fractions were collected and assayed for radioactivity. Bovine serum albumin was used to define the void volume (V_0). The [^{14}C]glucosamine-labeled glycopeptide markers (A = 4,300; B = 3,600; C = 1,800 [25]), graciously supplied by Clayton Buck of the Wistar Institute, Philadelphia, Pa., were used to calibrate the column. At least one marker was included when [^{3}H]mannose-labeled glycopeptides were chromatographed (Fig. 6 through 8). The total volume of the column (V_t) was determined by using [^{3}H]mannose or [^{35}S]methionine. The position of sialic acid was determined by gel filtration of [^{14}C]sialic acid (all isotopes were obtained from New England Nuclear Corp.).

RESULTS

SDS-PAGE of HSV glycoproteins. Figure 1A shows the labeling pattern of [^{3}H]methionine-labeled proteins (tracks 1 through 4) and [^{35}S]methionine-labeled proteins (tracks 5 through 8) immunoprecipitated from cytoplasmic extracts after a 15-min pulse at 5 h p.i. or after a 15-min pulse at 5 h p.i. followed by a 5-h chase. Two antisera were used: anti-CP-1 serum, a monoprecipitin serum directed against pD, and anti-ENV-1 serum, prepared against NP40 extracts of virion envelope from HSV type 1 (2). Tracks 1 and 5 show that pD was immunoprecipitated by anti-CP-1 serum from the pulse-labeled cytoplasmic extract. Tracks 3 and 7 show that the same serum precipitated D from the cytoplasmic extract of 5-h chase. Anti-ENV-1 serum precipitated pD from pulse-labeled extracts (tracks 2 and 6) as well as additional bands at 110,000 molecular weight (110K) and 130K. This serum precipitated D from the chase

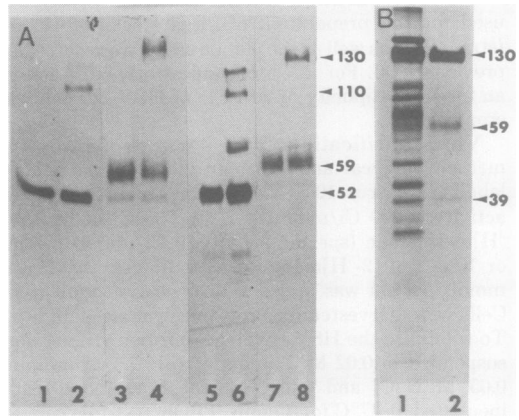

FIG. 1. *SDS-PAGE analysis. (A) Fluorogram of a 10% DATD cross-linked polyacrylamide gel of immune precipitates obtained from lysates of HSV-1-infected KB cells. Track 1, [^{3}H]methionine pulse, anti-CP-1 serum; track 2, [^{3}H]methionine pulse, anti-ENV-1 serum; track 3, [^{3}H]methionine chase, anti-CP-1 serum; track 4, [^{3}H]methionine chase, anti-ENV-1 serum; track 5, [^{35}S]methionine pulse, anti-CP-1 serum; track 6, [^{35}S]methionine pulse, anti-ENV-1 serum; track 7, [^{35}S]methionine chase, anti-CP-1 serum; track 8, [^{35}S]methionine chase, anti-ENV-1 serum. (B) Autoradiogram of electrophoretically separated (BIS-linked gels) polypeptides obtained from purified virus; track 1, purified virions, labeled with [^{35}S]methionine; track 2, [^{35}S]methionine-labeled molecules extracted by NP40 from virus particles.*

(tracks 4 and 8) and also precipitated bands at 85K and 130K. The results of this experiment are essentially the same as we previously reported (1). Figure 1B shows the labeling pattern of polypeptides extracted with NP40 from purified [^{35}S]methionine-labeled virions. As previously shown, the glycoproteins extracted from the virion have the same molecular weights in SDS-PAGE as the corresponding mature glycoproteins immunoprecipitated from infected cell extracts after the chase. The same patterns were obtained when arginine was employed as the amino acid label.

Tryptic peptide analysis of pD and D. To obtain sufficient material for tryptic peptide analysis, multiple samples of each of the immunoprecipitates shown in Fig. 1A were subjected to SDS-PAGE. The bands corresponding to pD and D were eluted, trypsinized, and co-chromatographed on a Chromobeads P column. The radioactive elution profiles obtained for pD (52K) and D (59K) were quite similar (Fig. 2A). For both proteins, two major methionine peptides were resolved, one eluting from the column with the flow-through and the second eluting at pH 3.33 ± 0.04 (mean of six determinations).

This experiment indicates that pD and D are structurally related. The tryptic fingerprint profile of methionine-labeled D extracted from purified virions was identical to that of methionine-labeled D obtained from the chase (Fig. 2B). These studies show that processing of pD to the mature form of D found in the virion does not involve any detectable change in methionine peptides.

The fingerprint profile for the methionine-labeled peptides of D is unusual in two respects. First, the flow-through represents at least two-thirds of the radioactive label. Second, the pattern is remarkably simple, in that only one major peptide is actually resolved by being retained on the column. We considered the possibility that the flow-through may contain a peptide(s) possessing trypsin-insensitive sites. In the light of reports that carbohydrate and especially sialic acid residues block trypsin action (9), we considered the possibility that the flow-through may contain a peptide(s) possessing sites that are blocked to trypsin action. However, removal of sialic acid from D by mild acid hydrolysis before trypsinization had no effect on the methionine profile (data not shown). Alternatively, as suggested by H. Diggelmann (personal communication), carbohydrate-containing peptides may not bind to Chromobeads P. If the methionine label in the flow-through represents a large fraction of the total peptides, then it is possible that pD and D contain some peptides which are different.

For this reason, we compared the tryptic fingerprint profiles of arginine-labeled pD and D. Both molecules share all arginine peptides (Fig. 3). Moreover, a large number of peptides were resolved, indicating that the trypsinization procedure was effective and that pD and D both contain a large number of arginine residues.

We conclude that the conversion of pD to D does not entail any major alteration in the peptide portion of the molecule and that glycoprotein D from the chase is apparently identical to

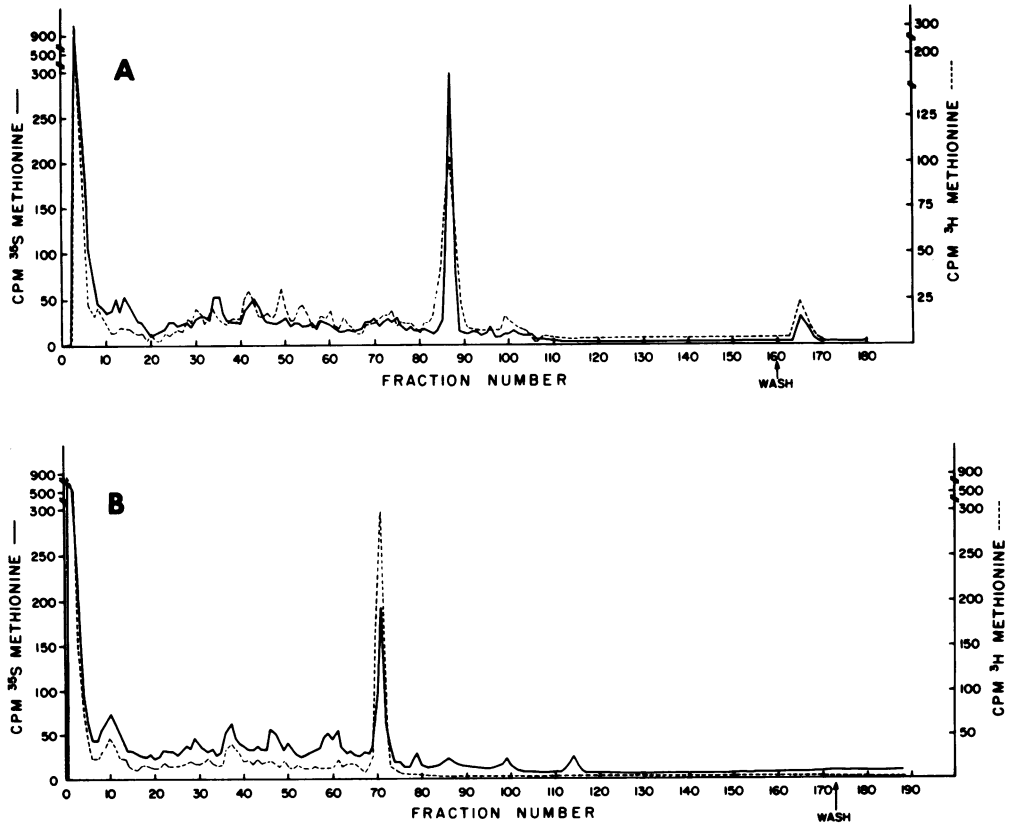

FIG. 2. *Tryptic fingerprint analysis of the type-common (CP-1) glycoprotein of HSV. (A) pD (——) isolated by immunoprecipitation and SDS-PAGE from [35S]methionine pulse-labeled extracts (15 min at 5 h p.i.) was co-chromatographed on a column of Chromobeads P with [3H]methionine-labeled D (- - -) isolated in a similar fashion from the chase (15-min pulse at 5 h p.i., followed by a 5-h chase).*

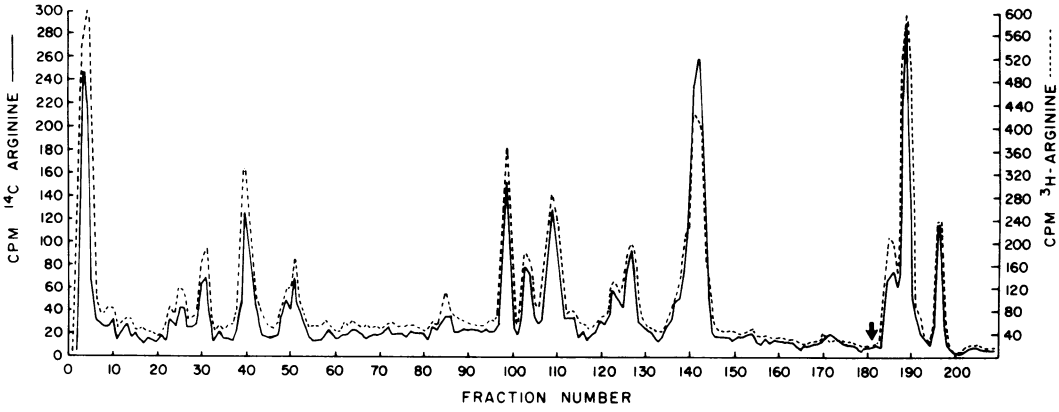

FIG. 3. *Tryptic fingerprint analysis of the type-common (CP-1) glycoprotein of HSV. pD (——) isolated by immunoprecipitation and SDS-PAGE from [^{14}C]arginine pulse-labeled extracts (15 min at 5 h p.i.) was co-chromatographed on a column of Chromobeads P with [^{3}H]arginine-labeled D (- - -) isolated in a similar fashion from the chase (15-min pulse at 5 h p.i., followed by a 5-h chase). Free arginine elutes at pH 3.63 (fraction 142).*

glycoprotein D found in the envelope of the virion.

Is D derived from one of the glycoproteins in the gp130 complex? It is possible that D is derived from one of the glycoproteins that make up the gp130 complex analogous to the situation for Rauscher leukemia virus (10, 11). If this were the case, D should contain a subset of tryptic peptides found in the gp130 complex. The profile for gp130 extracted from the virion with NP40 (Fig. 4A) consists of a flow-through and three major methionine peptides eluting at pH 3.11, 3.23, and 3.74. An identical profile was obtained for the gp130 complex isolated from the chase by immunoprecipitation. Figure 4B compares the elution profile of D with the gp130 complex. In this experiment, both proteins were extracted from the virion. The three methionine peptides of the gp130 complex eluted at pH 3.12, 3.20, and 3.76, whereas the methionine peptide of D eluted at pH 3.36. It is clear from this experiment that D does not share methionine peptides with the gp130 complex. It should be noted that there are unresolved methionine-containing peptides that appear in the column flow-through for each protein. Although it is possible that some of the unresolved peptides may be shared by D and the gp130 complex, the elution profile of the resolved peptides indicates that D is not derived from gp130.

Incorporation of [2-^{3}H]mannose into pD and D. We employed [2-^{3}H]mannose as the carbohydrate label to study the glycosylation steps involved in the conversion of pD into D. This precursor has been reported to be incorporated into macromolecules as mannose or other carbohydrates (17). When uninfected cells were pulse-labeled with mannose, a variety of proteins were labeled to a small extent. When infected cells were pulse-labeled with mannose (Fig. 5, track 1), most of the label was incorporated into six bands. Four of these bands at 52K, 65K, 110K, and 130K correspond in molecular weight to [^{35}S]methionine-labeled proteins immunoprecipitated by anti-ENV-1 serum (Fig. 1A, track 6). In addition, some mannose label was incorporated into a band that comigrates with the 154K capsid protein, and about 20% of the total label was associated with a diffuse band at about 17K. The fluorogram in Fig. 5 was overexposed to enhance the fainter bands and does not accurately reflect the amount of label in each band. When the gel was cut into 2-mm slices, dissolved in H_2O_2, and assayed for radioactivity, the label was distributed as follows: 3% in 154K, 18% in 130K, 20% in 110K, 11% in 65K, 25% in 52K, and 15% in 17K. The band at 17K was also seen in pulse-labeled uninfected cells and is apparently not virus specific. The other five bands did not correspond to mannose-labeled bands in uninfected cells. When infected cells were pulsed with [2-^{3}H]mannose and then chased (Fig. 5, track 2), bands appeared at 52K, 59K, and 130K, indicating that processing was occurring. However, the presence of the 52K band in the chase indicated that not all of the glycoprotein D was processed. All of the mannose-labeled viral glycoprotein precursors and products were immunoprecipitable by anti-ENV-1 serum (data not shown). However, the mannose-labeled bands at 17K and 154K were not immunoprecipitable.

Gel filtration of oligomannosyl residues on Bio-Gel P6: processing of pD into D.

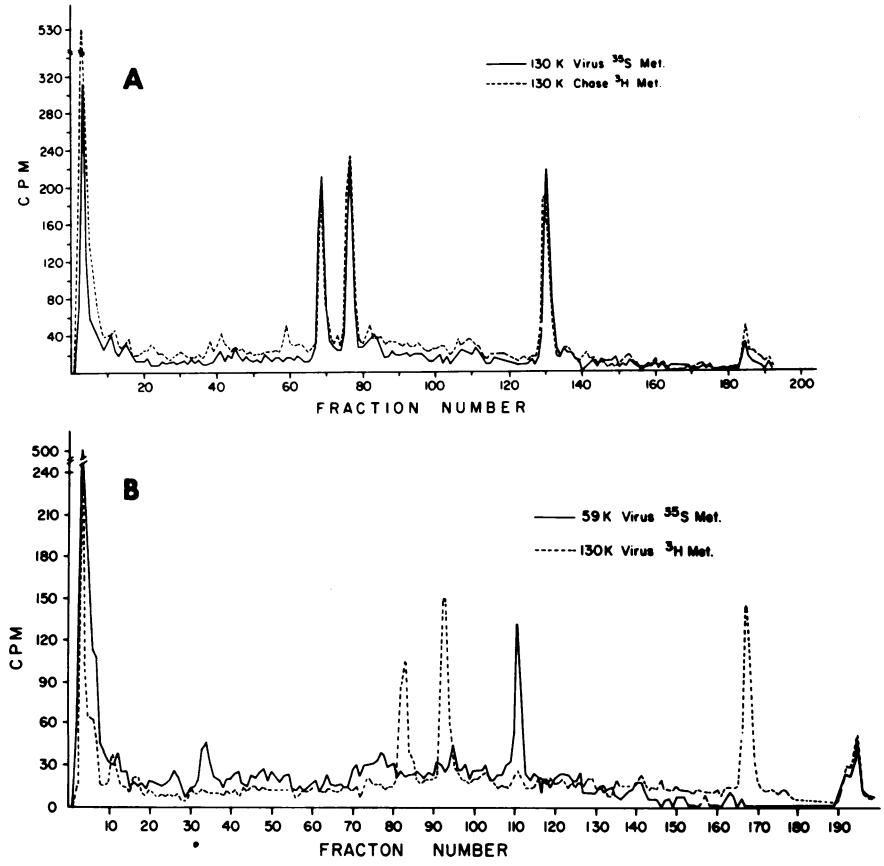

FIG. 4. *Tryptic fingerprint analysis of HSV glycoproteins. (A) gp130 complex (- - -) isolated by immunoprecipitation and SDS-PAGE from [^{3}H]methionine-labeled chase was co-chromatographed on a column of Chromobeads P with [^{35}S]methionine-labeled gp130 (——) extracted from purified virions with NP40. (B) [^{35}S]methionine-labeled D (——) extracted from purified virions with NP40 was co-chromatographed with [^{3}H]methionine-labeled gp130 complex (- - -) extracted from purified virions with NP40.*

Figure 6 shows the elution profiles of pronase-digested glycopeptides obtained from cells pulse-labeled with [2-^{3}H]mannose. The cytoplasmic extract of both uninfected and infected KB cells (Fig. 6A and B) contained a relatively symmetrical peak of oligomannosyl residues with an estimated molecular weight of 1,800. This size corresponds to that of the core oligosaccharide which is known to be attached to cellular and viral glycoproteins by a dolichol-mediated mechanism (24). To examine the oligosaccharides of pD directly, the band corresponding to pD was excised from the SDS gel, eluted in the presence of pronase, and applied to a Bio-Gel P6 column. pD contained the same size oligomannosyl core moiety as was found in the total pulse-labeled extract (Fig. 6C). It appears that all of the pulse-labeled glycoproteins contain the same oligomannosyl core. To test for the presence of sialic acid, pronase-treated pD was subjected to mild acid hydrolysis. This treatment had no effect on the elution profile of the oligomannosyl residues isolated from pD (Fig. 6D), indicating that sialic acid was not present.

To follow processing of the carbohydrate, a similar set of experiments was performed using the [2-^{3}H]mannose-labeled cytoplasmic extract obtained from the chase. The total extract (Fig. 7A) contained the 1,800-molecular-weight glycopeptide seen in the pulse, but in addition it contained a heterogeneous set of glycopeptides that eluted in positions corresponding to larger molecules. Approximately 33% of the label eluted in a 3,600-molecular-weight fraction and approximately 10% eluted with or just after the void volume and had a molecular weight of 4,300 or greater. Mild acid treatment of the chase resulted in a dramatic alteration of the elution profile (Fig. 7B). A significant amount of the larger glycopeptide material seen in Fig. 7A was

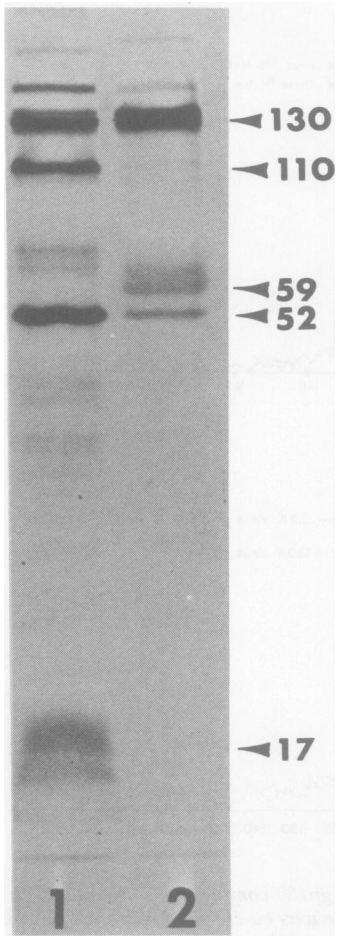

FIG. 5. *Pulse-chase experiment with [2-^{3}H]mannose as a precursor. Fluorogram of a 10% polyacrylamide gel (BIS linked) of cytoplasmic extracts from HSV-1-infected KB cells. Track 1: cells were pulse-labeled for 30 min at 5 h after infection. Track 2: cells were pulse-labeled for 30 min at 5 h after infection and then chased with nonradioactive medium for 5 h. The film was overexposed to enhance the faint bands.*

shifted to lower molecular weights. The glycopeptides obtained directly from D had a similar elution profile to those seen in the total chase (Fig. 7A). However, a somewhat greater fraction of the label (27%) was present in molecules of greater than 3,600 molecular weight. Treatment of D with mild acid (Fig. 7D) lowered the molecular weight of the larger glycopeptides. The elution profile of most of the label (83%) was shifted to a broad peak with an average molecular weight greater than 1,800. These results suggest that most of the higher-molecular-weight oligomannosyl residues found in D contain sialic acid. We conclude that processing of pD to D involves chain elongation, including extensive sialyation.

Figure 8A shows the elution profile of the oligomannosyl residues obtained by pronase digestion of the NP40 extract of purified virions. It can be seen that the elution profile is similar to that seen for the chase. However, most of the [^{3}H]mannose label was associated with molecules of 3,600 molecular weight or greater. A small fraction of the counts (15%) was found in the 1,800-molecular-weight region. Thus, there appear to be at least three different mannose-labeled oligosaccharides in the virion envelope. Furthermore, these results suggest that virion glycoproteins are heterogeneous with respect to carbohydrate content. Treatment with mild acid had precisely the same effect on the virion glycopeptides as it had on glycopeptides from D (cf. Fig. 7D and 8B). These results indicate that D found in the chase is probably the final glycosylated form of the glycoprotein found in the virion.

We conclude that there appear to be at least two posttranslational processing steps in production of the mature glycoprotein D. The first step involves the addition of an 1,800-molecular-weight mannose-containing oligosaccharide to the core peptide to form pD. The second step(s), which involves addition of further carbohydrate residues including sialic acid to the 1,800-molecular-weight oligosaccharide, probably accounts for the slower mobility of D in SDS-PAGE.

Incorporation of [^{14}C]glucosamine into HSV glycoproteins. HSV-infected KB cells do not incorporate significant amounts of [^{14}C]glucosamine label into trichloroacetic acid-precipitable material during a 30-min pulse. However, we found that label added during the 30-min pulse was incorporated into acid-precipitable material during the 5-h chase period. Honess and Roizman (7) observed that the rate of entry of labeled glucosamine into the acid-soluble pool of both infected and uninfected cells was much greater than its rate of withdrawal. Moreover, the fact that most of the soluble pool was UDP-*N*-acetyl glucosamine suggested that this larger pool would not exchange with the medium to permit an effective chase. Thus, we found that label added to HSV-infected cells during the pulse was present long enough to be incorporated eventually into glycoproteins during the "chase" period. Figure 9A shows the elution profile of glycopeptides obtained from the pronase-digested [^{14}C]glucosamine-labeled "chase." Approximately 23% of the glucosamine label was in the void volume, and most of the remaining labeled molecules had a molecular weight cor-

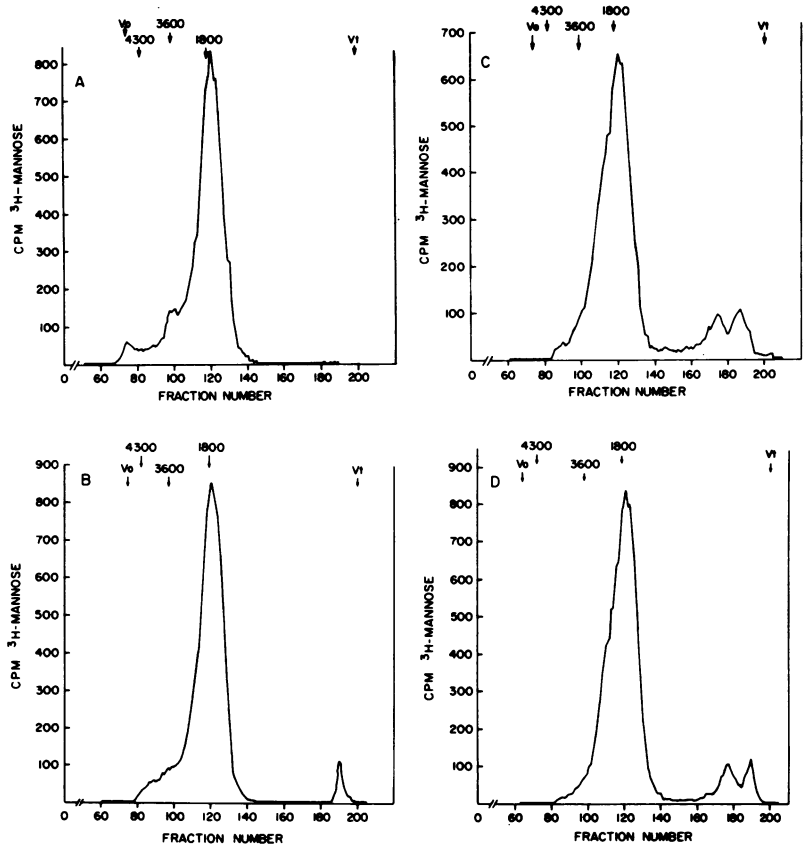

FIG. 6. *Gel filtration chromatography on Bio-Gel P6 of pronase digests obtained from HSV-1 uninfected and infected KB cells pulse-labeled with [2-^{3}H]mannose. Glycopeptides were obtained from: (A) the total cytoplasmic extract of uninfected KB cells; (B) the total cytoplasmic extract of HSV-infected cells; (C) glycoprotein pD excised from a polyacrylamide gel; (D) glycoprotein pD from the gel treated with 0.05M H_2SO_4 at 90°C for 1 h.*

responding to the 3,600-molecular-weight peak labeled with [2-^{3}H]mannose. Mild acid treatment (Fig. 9B) converted approximately 36% of the glucosamine label to a position corresponding to authentic sialic acid, and 45% of the label then eluted at a molecular weight of approximately 3,000. Little if any label was observed in the 1,800-dalton peak before or after acid treatment.

It should be noted that Honess and Roizman (7) reported that 30 to 40% of the glucosamine label present in purified virions was in the form of sialic acid. Our results showed that a similar amount of radioactive glucosamine (36%) was converted to sialic acid in infected cells.

Does the conversion of pD to D involve a change in electrical charge as well as molecular weight? The results presented so far suggest that the processed form of D is sialyated and the unprocessed form is not. Thus, the precursor and product should differ in charge as well as molecular weight. To test this prediction, we subjected the [^{35}S]methionine-labeled pulse and chase extracts to two-dimensional gel electrophoresis. Although several changes were noted in the position of labeled polypeptides of the pulse and chase, the complexity of the patterns made it impossible to pinpoint where pD and D were located. We therefore employed our antisera as immunological probes to select the pD and D components out of this complex mixture of proteins. Anti-CP-1 serum immunoprecipitated pD (Fig. 10A; cf. Fig. 1A, track 5) which had an isoelectric point (pI) of 8.0. In addition, there was a trailing edge of protein extending into the lower pH range. This antiserum immunoprecipitated D as a heterogeneous molecule of 59K to 64K, having an average pI of 6.4 (Fig. 10B; cf. Fig. 1A, track 7). It should be noted that the higher-molecular-weight material had a

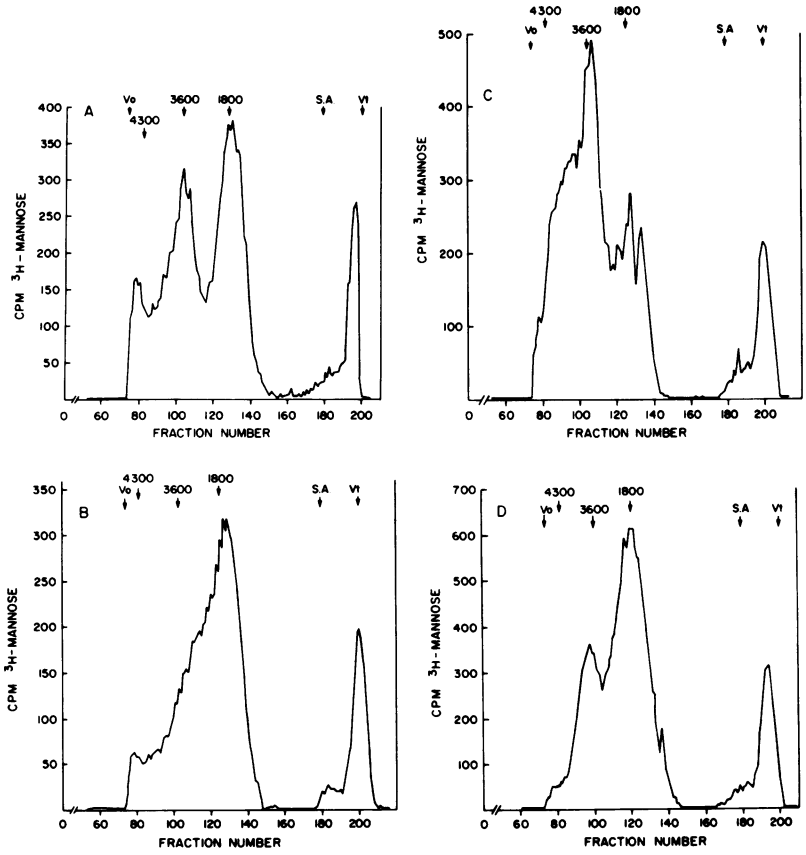

FIG. 7. *Gel filtration chromatography on Bio-Gel P6 of pronase digests obtained from HSV-infected cells pulse-labeled with [2-³H]mannose for 30 min and chased with unlabeled medium for 5 h. Glycopeptides were obtained from: (A) the total cytoplasmic extract of infected cells; (B) the total cytoplasmic extract treated with 0.05 M H_2SO_4 at 90°C for 1 h; (C) glycoprotein D excised from a polyacrylamide gel; and (D) glycoprotein D from the gel treated with 0.05 M H_2SO_4 at 90°C for 1 h.*

greater negative charge, possibly due to the fact that it was more heavily sialyated. Figure 10C shows the results of two-dimensional electrophoresis of [^{35}S]methionine-labeled polypeptides extracted with NP40 from purified virions. Several labeled components were seen, including: (i) a 59K to 64K molecule, pI 6.4 (D); (ii) a 130K component with an average pI of 5.0; and (iii) a component(s) with a pI of approximately 6.0 and a molecular weight of approximately 70K to 90K. Thus it appears that the processed form of D found in the virion has the same pI and molecular size as it does in the chase. We conclude that the conversion of pD to D involves a change in electrical charge as well as molecular weight, and that both of these alterations are due to the addition of carbohydrate, especially sialic acid, to the protein.

DISCUSSION

The results presented in this study firmly establish a structural relationship between the two forms of the CP-1 antigen, pD and D. Tryptic fingerprint profiles of methionine- and arginine-labeled proteins show that both precursor and product forms share these peptides. The processed form of CP-1 (D) found in the cytoplasm has the same methionine peptides as that found in purified virions. Moreover, the tryptic fingerprint profile of D differs from that of the gp130 complex. Thus, it would appear that D is not a breakdown product of a larger-molecular-weight glycoprotein. The present data confirm and extend previous observations (2, 19) that pD and D are antigenically related to each other and are unrelated to the 130K glycoproteins of HSV.

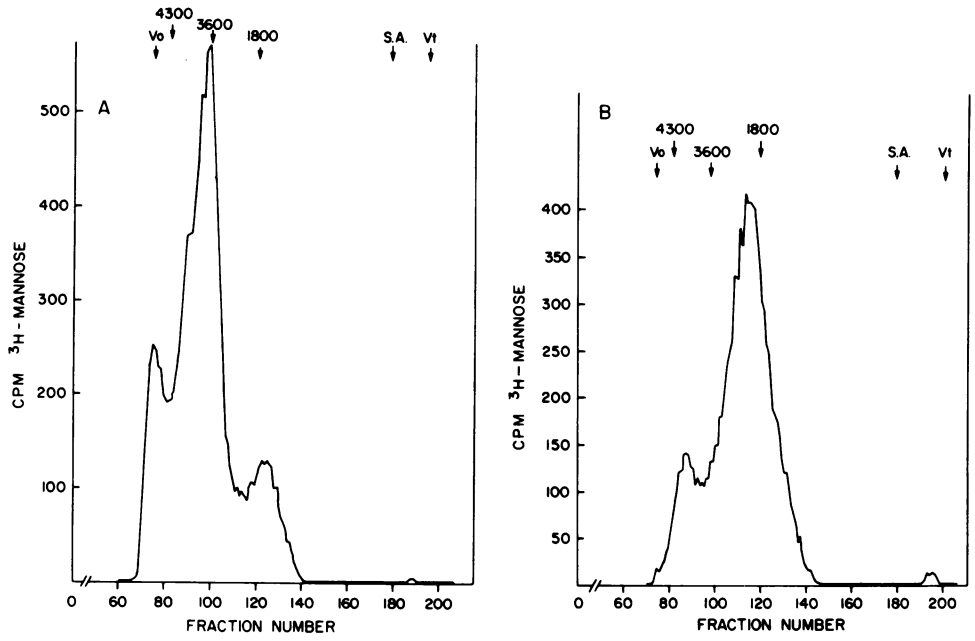

FIG. 8. *Gel filtration chromatography on Bio-Gel P6 of pronase digests obtained from [2-³H]mannose-labeled purified HSV virions. Glycopeptides were obtained from: (A) the NP40 extract of purified virions; and (B) the NP40 extract of purified virions treated with 0.05 M H_2SO_4 at 90°C for 1 h.*

Mannose labeled in the 2-tritium position appears to be an excellent choice as a carbohydrate precursor since: (i) it is rapidly incorporated into oligosaccharides in the form of mannose or other sugars (17); (ii) it is rapidly incorporated into viral glycoproteins; (iii) it is incorporated into the initial core oligosaccharide allowing the initial steps of processing to be studied; and (iv) it is not converted into sialic acid (Fig. 7B) (26). Using [2-³H]mannose as the carbohydrate probe, we have shown that processing of pD to D as well as the processing of the other glycoproteins of HSV involves alterations of the carbohydrate portion of the molecule. By analyzing pronase digests of pD and D, we were able to follow the flow of mannose label from an 1,800-molecular-weight moiety added to the protein within 5 min of synthesis (unpublished data) to larger, more heterogeneous carbohydrate components. A similar flow was found for the unfractionated mixture of glycoproteins in the pulse and chase, suggesting that the other HSV glycoproteins may be processed in a fashion similar to D. These results agree with those of Honess and Roizman (7), who studied the processing of oligosaccharides by using glucosamine as the label in a mixture of HSV glycoproteins. However, before we can conclude that all HSV glycoproteins are processed similar to D, it will be necessary to examine the processing of each of the other glycoproteins individually. Spear (19), for example, reported that the processing of A differs from that of the other glycoproteins and in fact does not change in molecular weight. That processing of the oligosaccharide portion of D involves extensive sialyation was shown in several ways. First, mild acid hydrolysis of the larger mannose-labeled glycopeptides converted them to lower-molecular-weight forms. Second, when glucosamine-labeled glycopeptides were subjected to this treatment, 36% of the label eluted from Bio-Gel P6 at the same position as sialic acid.

This finding predicted that processing of pD to D involved not only a change in molecular weight but also a significant alteration in molecular charge. Two-dimensional gel electrophoresis studies indicate that pD is a basic molecule with a pI of 8.0, whereas the processed form, D, is more acidic (average pI = 6.4). In this regard, it is worth noting that Thompson et al. (22) reported that the surface charge of HSV-infected cells differs from that of uninfected cells as well as the envelope of purified virions. They found that, whereas uninfected control cells focused at a pI of 4.48, infected cells became less acidic, focusing at a pI of 4.58. The pI of the purified virion was still higher (5.0 to 5.8). From

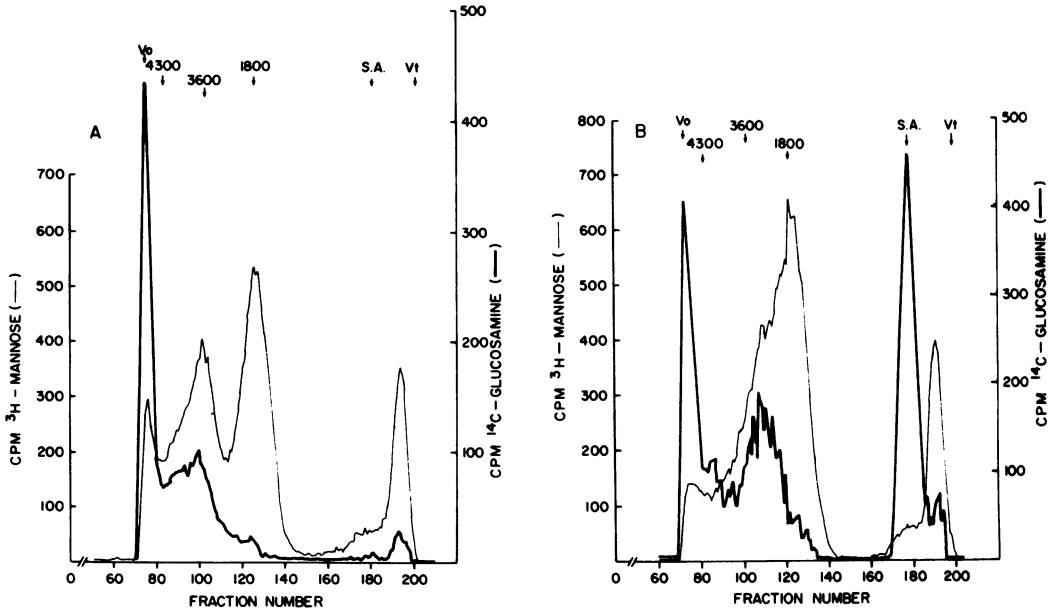

FIG. 9. *Gel filtration chromatography on Bio-Gel P6 of pronase digests obtained from HSV-infected cells. The cells were pulse-labeled for 30 min with [2-³H]mannose (narrow line) or [¹⁴C]glucosamine (heavy line), then chased for 5 h. Glycopeptides were obtained from: (A) cytoplasmic extract; and (B) total cytoplasmic extract treated with 0.05 M H_2SO_4 for 1 h at 90°C.*

these data as well as direct analysis of the number of amino groups, they concluded that the plasma membrane of HSV-infected cells, as well as the virion envelope, were modified by addition of basic amino groups, possibly present in virion glycoproteins. Our isoelectric focusing data suggest that glycoprotein D has a significant number of basic amino groups, and therefore this glycoprotein may contribute substantially to the electrokinetic properties of the plasma membrane and virion envelope. It is evident that a detailed analysis of the amino acid composition of D is required. However, based on the data in Fig. 3, we can at least speculate about the minimum number of arginine residues in D. Assuming that the peak with the smallest number of counts (Fig. 3, fractions 102 through 107) represents one arginine peptide (12), we estimated that there are approximately 30 arginine residues in the molecule. Assuming that the core protein (minus carbohydrate) has a molecular weight of 50,000 (L. Pizer, G. H. Cohen, and R. J. Eisenberg, unpublished data) and contains 416 amino acids each with an average molecular weight of 120, then approximately 7% of the D protein is arginine. The major drawback of this estimation is that peak heights are not always reliable.

Our results suggest that processing of the oligosaccharide(s) of D occurs in a fashion similar to that reported for the G glycoprotein of vesicular stomatitis virus (8, 21) as well as other viral and cell glycoproteins (21, 24). In all cases of eucaryotic glycoprotein synthesis studies to date, asparagine-linked oligosaccharides are formed first by en bloc addition of a "simple" or mannose-rich core containing *N*-acetyl glucosamine and mannose via a dolichol intermediate (24). This is followed by addition of other sugars, including galactose, *N*-acetyl glucosamine, fucose, and sialic acid to form a complex structure. In some cases it has been shown that, before addition of these other sugars, some mannose residues are removed (21). We have no evidence so far to suggest that this step occurs in the conversion of pD to D. Studies of this possible intermediate processing step, involving a more detailed kinetic analysis of the pulse and chase, are underway.

As noted before, D isolated from the cytoplasm is structurally and antigenically similar to D isolated from the virus. However, one difference is evident. D as isolated from the cytoplasm contains mannose label in a low-molecular-weight form (less than 500; see Fig. 7C), whereas none of the mannose label in the virion is in a low-molecular-weight carbohydrate (Fig. 8). The nature of this material is not known presently. The loss of this material might reflect a further processing step in the maturation of the virion

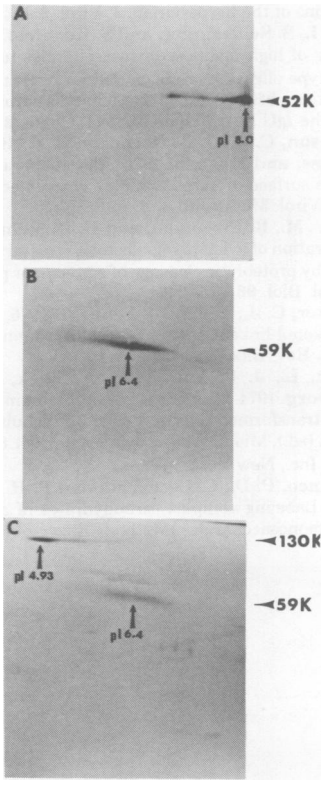

FIG. 10. *Two-dimensional gel electrophoresis of HSV-1 glycoproteins. The immune precipitates in A and B were obtained by using anti-CP-1 serum. (A) Immune precipitates obtained from extracts pulse-labeled with [^{35}S]methionine for 15 min at 5 h after infection (see Fig. 1A, track 5). (B) Immune precipitates obtained from extracts pulse-labeled with [^{35}S]methionine for 15 min at 5 h after infection, then chased with unlabeled medium for 5 h (see Fig. 1A, track 7). (C) NP40 extract of [^{35}S]methionine-labeled purified virions (see Fig. 1B, track 2).*

envelope.

It is noteworthy that the glycopeptides isolated from D contain some of the 1,800-molecular-weight or mannose-rich core oligosaccharide. It is unlikely that band D excised from the gel was contaminated with pD, because the distance between pD and D was sufficiently great to insure that D was isolated free of a contaminating precursor. Thus, it is possible that D may contain more than one asparagine-linked oligosaccharide. Previous experiments with tunicamycin suggested that the unglycosylated form of D has a molecular weight in SDS-PAGE of approximately 50,000 (Pizer et al., unpublished data). In the case of other viruses, the molecular weight of tunicamycin-treated carbohydrate-free polypeptides corresponds closely to the weight predicted by subtraction of the total molecular weight of the oligosaccharides from that of the glycosylated peptide (4, 14). If this additive relationship is valid for protein D of HSV, then there are no more than two to three asparagine-linked oligosaccharide chains present on this molecule.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant DE-02623 from the National Institute of Dental Research. R.J.E. was supported in part by a special grant from the University of Pennsylvania.

We thank William Lawrence and Wesley Wilcox for their help in preparation of this manuscript, Heidi Diggelmann for allowing us to read her manuscript before publication, and Clayton Buck for supplying us with marker glycopeptides. We also wish to thank Walter Taylor for mastering the technique of two-dimensional gel electrophoresis and running the samples, and we thank Madeline Cohen for her excellent technical assistance.

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