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Preparation and Characterization of Specific Antisera to Individual Glycoprotein Antigens Comprising the Major Glycoprotein Region of Herpes Simplex Virus Type 1

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The major glycoprotein complex (VP123) of herpes simplex virus type 1 resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was purified and further fractionated into two major and two minor components by chromatography of the isolated VP123 region on SDS-hydroxylapatite columns. The two major components (gC and gA/gB) were purified free of other polypeptides and used to prepare specific antisera to these glycoproteins. Radioimmune precipitation demonstrated that these antisera were specific for the antigens used in their production. These two antisera as well as an anti-VP123 serum were further characterized by immunoprecipitation, neutralization, and membrane immunofluorescence techniques. Results indicate that both of the major glycoprotein antigens are expressed on the surface of virions as well as on the surface of infected cells.

The existence of two serotypes of herpes simplex viruses (HSV), type 1 (HSV-1) and type 2 (HSV-2), has been firmly established (19, 25). The two virus types differ in certain biological, biochemical, and immunological characteristics, while at the same time retaining many properties in common. Notably, several investigators have demonstrated by cross-adsorption of antiviral sera that the two HSV serotypes have a set of antigens common to both HSV-1 and HSV-2 (type common) and a set unique to each virus type (type specific) (11, 27, 28). The immunological cross-reactivity between both the surface and internal antigens of the two virus types coupled with the extensive immunity of the general population to HSV-1 has been a major problem in seroepidemiological studies attempting to define the relationship of HSV-2 to cervical carcinoma (12, 14, 22, 26). As has been demonstrated by a number of investigators, the viral surface glycoproteins are the major if not exclusive targets of neutralizing antibodies (4, 11, 15, 20, 27). Despite the obvious importance of the viral glycoproteins in host immunity, studies on the immunological aspects of the individual glycoproteins which constitute the major viral glycoprotein region have been limited (15, 32). If it is assumed that individual glycoproteins may bear exclusively or predominantly type-specific or type-common antigenic sites, separation and purification of a relatively type-specific antigen should be possible, thus allowing a more reliable method of serotyping to be developed. A major obstacle to this type of study has been the difficulty in separating these antigens from host cell and other viral proteins as well as from one another. To date no methods which are readily adaptable to purification of the individual glycoproteins of HSV-1 in preparative amounts have been described.

HSV-1-specific glycoproteins have been shown to be present in both the virion envelope and the membranes of infected cells (10, 17). The glycoproteins present in infected cells appear to be indistinguishable from those present in virions based on both immunological and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) studies. When examined by SDS-PAGE, the HSV-1 glycoproteins fall into two major glycoprotein regions having average apparent molecular weights of 123,000 (VP123) and 58,000 (VP58) and a minor 88,000-molecular-weight glycoprotein region (VP88) (4). The VP123 region is the predominant glycoprotein region of both virions and infected cells. Spear (29, 30) has shown that the VP123 region is comprised of three distinct components, which have been designated gA, gB, and gC. Using immune precipitation and SDS-PAGE, Spear also identified two partially glycosylated precursors to the glycoproteins of the VP123 region. The subsequent glycosylation of these precursors to yield the three stable glycoproteins which together constitute the VP123 region has been clearly demonstrated. Similarly, it has been shown that the VP58 region also

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represents more than a single glycoprotein species (2, 29, 30).

In this communication, we use the technique of SDS-hydroxylapatite (HTP) chromatography as described by Moss and Rosenblum (13) to fractionate the purified VP123 complex into individual components which correspond to HSV-1-specific glycoproteins described by other investigators (16, 29, 30). We also describe the purification in preparative amounts of the major glycoproteins of the VP123 complex, the production of specific antisera to each of these antigens, and the characterization of these antisera.

(This research was carried out in partial fulfillment of the requirements for the Ph.D. degree of R.E. from the Baylor College of Medicine.)

MATERIALS AND METHODS

Viruses, cell cultures, and media. The KOS strain of HSV-1 was used throughout these studies. Procedures used in the production and titration of virus stocks were as previously described (1). Serially propagated VERO and HEp-2 cells were cultured in Eagle medium supplemented with 10% donor calf serum (DCS) and 0.075% $NaHCO_3$. In all experiments, cells were grown in 60- or 100-mm plastic petri dishes and maintained in Eagle medium supplemented with 2% DCS and 0.225% $NaHCO_3$ (maintenance medium). In experiments involving labeling with ^{14}C-labeled amino acids, Eagle medium with 0.1× amino acids supplemented with 1× arginine and glutamine, 2% DCS, and 0.225% $NaHCO_3$ was used. Where [^{35}S]methionine was used, Eagle medium supplemented with 1× amino acids without methionine, 0.1× methionine, 1× glutamine, 2% DCS, and 0.225% $NaHCO_3$ was used.

Chemicals and radioisotopes. Chemicals used for analytical and preparative PAGE and HTP chromatography were purchased from Bio-Rad Laboratories, Richmond, Calif. D-[6-^{3}H]glucosamine (specific activity, 10 to 25 Ci/mmol), [*U*-^{14}C]-labeled amino acids mixture (specific activity, 57 Ci/mmol), and [^{35}S]-methionine (specific activity, 1,200 Ci/mmol) were obtained from Amersham Corp., Arlington Heights, Ill.

Infection, labeling, and detergent extraction. Cell cultures were infected with virus stock at an input multiplicity of 10 to 20 PFU/cell. After a 1-h adsorption at 37°C, the inoculum was removed and 5 ml of maintenance medium was added. Unless specifically noted otherwise, all radioisotopes were present from 4 to 24 h postinfection. Cells were harvested by scraping into the medium, washed twice with cold phosphate-buffered saline (PBS), suspended in cold, sterile water at 2×10^7 cells/ml, sonicated, and stored at −20°C until used.

Infected-cell extracts were prepared by extraction of infected cells with 1% sodium deoxycholate and 1% Tween 40 for 1 h with constant shaking at 37°C. Insoluble material was removed by high-speed centrifugation at 100,000 × *g* for 1 h. The resulting detergent extracts (supernatant) were either run on preparative SDS-PAGE or used as antigen for immunoprecipitation studies.

Discontinuous SDS-PAGE. Details of the methods used for SDS-PAGE have been previously described (21). Unless otherwise noted, all slab gels presented are 8.6% acrylamide cross-linked with 0.23% *N,N'*-diallyltartardiamide (DATD) as described by Gibson and Roizman (8). Kodak No-Screen NS-2T X-ray film was used for detection of radioactivity in slab gels. Preparative SDS-PAGE methods were modified from those used formerly (4) in that urea was omitted from both the running buffers and gels. Peaks eluted from preparative SDS-polyacrylamide gels were located; then the appropriate fractions were pooled and concentrated, using an Amicon ultrafiltration cell equipped with a PM10 membrane.

SDS-HTP chromatography. The methods used for SDS-HTP chromatography were essentially those described by Moss and Rosenblum (13). Briefly, HTP was washed to remove fines in 0.1% SDS–0.01 M phosphate buffer, pH 6.4, resuspended in the same buffer, poured into columns, allowed to settle, and washed with 2 column volumes of 0.01 M phosphate buffer (pH 6.4) containing 0.1% SDS and 1 mM dithiothreitol. Samples for SDS-HTP chromatography were dialyzed against 0.01 M phosphate buffer, pH 6.4, containing 0.1% SDS and heated at 100°C for 30 s before loading on SDS-HTP columns. Material was then eluted from the columns using linear gradients of increasing phosphate molarity, in 0.1% SDS–1 mM dithiothreitol–phosphate buffer, pH 6.4. The flow rate obtained by gravity was 10 to 15 ml/h; 120-drop (approximately 1.5-ml) fractions were collected, and 0.1 ml of each was assayed for radioactivity. The refractive index was determined on every fifth fraction. Peak fractions were concentrated and dialyzed against 0.1% SDS–0.01 M phosphate buffer, pH 6.4, at 4°C unless the samples were to be used for radioimmune precipitation, in which case dialysis was against 1% Triton X-100 in PBS, pH 7.2.

Preparation of antisera. Antigen preparations to be used for immunization of rabbits were electrophoresed into cylindrical 7% polyacrylamide gels. The gels were removed from the tubes, and all but one were frozen at −20°C. The remaining gel was stained with Coomassie brilliant blue, destained, and used as a guide to remove 6- to 8-mm sections from the frozen gels which contained the antigen. For immunization, one gel section was brought to 0.5 ml with sterile PBS, emulsified with an equal volume of complete Freund adjuvant (GIBCO Laboratories, Grand Island, N.Y.), and injected into both hind footpads of 2- to 3-kg white rabbits. Booster immunizations using incomplete Freund adjuvant given intramuscularly were administered at 2-week intervals until all gel plugs had been injected (four to six plugs total). Starting at 2 weeks after the fourth injection, rabbits were bled weekly.

Treatment of sera. Rabbits were bled from the marginal ear vein into sterile tubes, and the blood was allowed to clot for 1 h at 37°C and overnight at 4°C. The serum was then centrifuged to remove residual cells, divided into equal portions, and stored at −20°C. For immunofluorescence studies, both rabbit antisera and fluorescein isothiocyanate-conjugated goat anti-rabbit gamma globulin (FITC-GAR) (Hyland Laboratories, Inc., Costa Mesa, Calif.) were adsorbed with rabbit liver powder and a VERO–HEp-2 (1:1) cell

pellet. FITC-GAR was filtered through a 0.45-μm filter immediately before use.

RIP. Diluent used for radioimmune precipitation (RIP) was PBS, pH 7.2, containing 1% Triton X-100 and 0.5% gelatin (Difco Laboratories, Detroit, Mich.). Samples (10 to 30 μl) of the antigen preparation were reacted with 25 μl of rabbit antisera in a final volume of 200 μl for 1 h at 37°C and an additional 18 to 24 h at 4°C. Goat anti-rabbit serum was then added in amounts predetermined to give maximal precipitation of the rabbit immunoglobulin and incubated for an additional 18 to 24 h at 4°C. Immune complexes were pelleted by centrifugation at 1,600 × *g* for 20 min and washed twice with cold PBS. The final pellet was solubilized in 100 μl of 2% SDS–2% β-mercaptoethanol–1 M urea at 100°C for 2 min and counted in 5 ml of toluene-based scintillation cocktail. The percentage of precipitation was determined by the following formula:

$$\frac{\text{cpm in immunoprecipitate}}{\text{cpm in input antigen}} \times 100 = \%\ \text{precipitation}$$

When immunoprecipitation from infected cell extracts for analysis on slab SDS-PAGE was performed, the final precipitate was solubilized in 35 μl of 2% SDS–2% β-mercaptoethanol–1 M urea.

Membrane immunofluorescence. Cover slip cultures of VERO cells in 60-mm plates were seeded 36 h before infection so as to be approaching confluency at the time of staining. Monolayers were infected with an input multiplicity of 0.1 to 0.01 PFU/cell, and the virus was allowed to adsorb for 1 h at 37°C, after which the cells were washed with 1% DCS-PBS, pH 7.2, and 5 ml of maintenance medium was added. At 18 h postinfection, cover slips were washed three times with PBS at room temperature, excess PBS was removed, and 15 μl of adsorbed rabbit antiserum was added. After 30 min at room temperature in a humidified chamber, cover slips were washed three times in PBS, and the procedure was repeated with FITC-GAR. After the final washings, cover slips were mounted on slides with PBS-glycerol (4:1). Fluorescence was observed within 12 h on a Ziess microscope. Photographs were taken with Kodak Ektachrome daylight ASA 400 film.

Neutralization. All sera were heat inactivated at 56°C for 30 min before use in neutralization tests. Fresh guinea pig serum served as the complement source. Virus stocks of HSV-1 were diluted to 10^6 PFU/ml for use. Eight units of complement (0.2 ml) and 0.2 ml virus stock were added to 0.4 ml of the antiserum dilutions, mixed, and incubated at 37°C for 1 h. At the end of the incubation period, samples were immediately diluted 1:100 in ice-cold diluent, and 0.1 ml was plated in duplicate on subconfluent VERO cell monolayers. After a 1-h adsorption period at 37°C, plates were overlaid with media containing 1% methylcellulose and stained with neutral red as previously described (1). Results are expressed as plaque reduction, which was calculated as follows:

$$100 - \frac{n}{n_0} \times 100 = \%\ \text{plaque reduction}$$

where n_0 is the number of PFU remaining in absence of serum and n is the number of PFU remaining in the presence of serum.

Molecular weight determinations. Standard protein markers for molecular weight determinations were obtained from Bio-Rad Laboratories and contained β-galactosidase (130,000), phosphorylase A (94,000), bovine serum albumin (68,000), and ovalbumin (43,000). The major capsid protein of HSV-1 (VP154, 154,000) was also included as a protein standard. Approximately 1 μg of each of these markers was coelectrophoresed with purified glycoproteins on 7 and 5% acrylamide gels cross-linked with methylenebisacrylamide and on 6.5 and 8.6% acrylamide gels cross-linked with DATD. Gels were stained for 3 h in 0.05% Coomassie brilliant blue and destained overnight. Gels were transferred to 3M Whatman filter paper, dried for 30 min under vacuum, and exposed to Kodak NS2T film for autoradiography. The apparent molecular weights of the purified glycoproteins were calculated from their electrophoretic mobility relative to those of the standard proteins used as markers of known molecular weight.

RESULTS

Fractionation and analysis of the purified HSV-1 VP123 glycoprotein region. HEp-2 cells were infected with HSV-1 and double labeled with ^{14}C-labeled amino acids and [^{3}H]glucosamine from 4 to 24 h postinfection. At 24 h postinfection, the whole-cell fraction was harvested and electrophoresed in preparative SDS-PAGE as described above. The VP123 region was pooled, concentrated, dialyzed, and further fractionated by SDS-HTP chromatography (Fig. 1). In this profile, the gC and gA/gB peaks were readily resolved; however, only a very small amount of the gC(105) component was observed. It should be noted that the gC peak has a higher glucosamine-to-amino acid ratio relative to that of the gA/gB peak. A third peak of primarily amino acid-labeled material, designated NG (nonglycosylated), was eluted from the column at a higher molarity of sodium phosphate than was the gA/gB peak. This material could not be further resolved into distinct components by additional SDS-HTP chromatography. To further purify the glycoproteins resolved on the initial SDS-HTP column, each of these three peaks was pooled and subsequently run on additional SDS-HTP columns. Fractions containing the three major peaks were pooled, concentrated, dialyzed, and then electrophoresed on SDS-slab gels to determine differences in the migration of each of the peaks resolved by SDS-HTP chromatography. Each of the components migrated within the VP123 region and exhibited a distinct pattern of migration within this region (Fig. 2). The gC component migrated as a diffuse band, whereas the gA/gB component migrated as two distinct bands of similar molecular weight. The amino acid-labeled component also

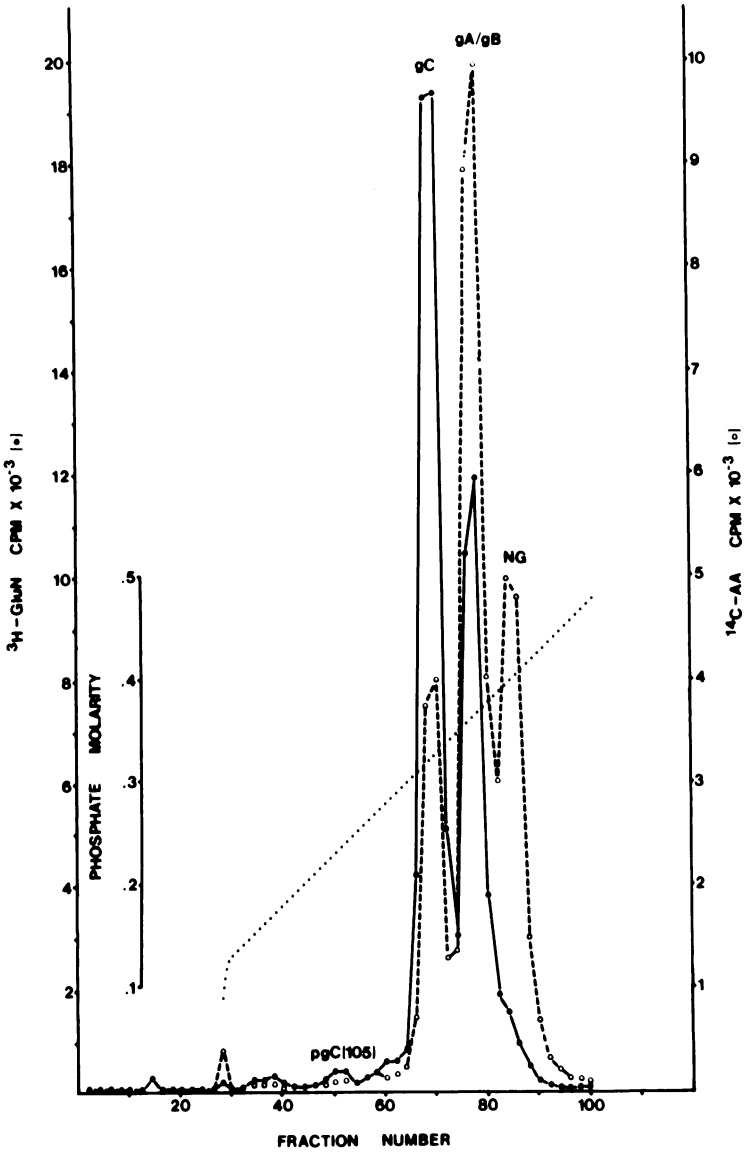

FIG. 1. *SDS-HTP fractionation of the major glycoprotein region of HSV-1. HSV-1-infected HEp-2 cells were labeled from 4 to 24 h postinfection and harvested at 24 h. The detergent extract was then electrophoresed on preparative SDS-PAGE. The VP123 region was pooled, concentrated, and reelectrophoresed on a second preparative gel. The purified VP123 region was pooled, concentrated, and dialyzed against 0.01 M phosphate buffer, pH 6.4, containing 0.1% SDS. The sample was then chromatographed on an SDS-HTP column (0.9 by 15 cm), using a 0.1 to 0.5 M phosphate gradient.*

migrated as distinct components which were resolved from the VP123 region.

Molecular weight determinations of the major virus-specific glycoproteins. The glycoprotein components from the major HSV-1 glycoprotein region were purified by repetitive chromatography on SDS-HTP columns. The apparent molecular weight of each of the glycoprotein components was estimated from their electrophoretic mobilities relative to standard proteins of known molecular weight. Since acrylamide gels cross-linked with DATD were primarily used in subsequent studies and by other authors, molecular weights were determined in both the DATD and the conventional methylenebisacrylamide-cross-linked SDS-gel systems. The average apparent molecular weights presented in Table 1 were derived from multiple

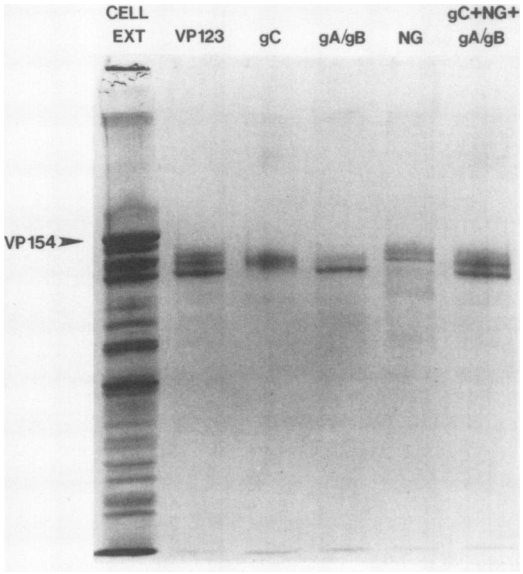

FIG. 2. *SDS-PAGE analysis of the HSV-1 VP123 components resolved by SDS-HTP chromatography. Glycoprotein components purified by repetitive SDS-HTP chromatography as described in Fig. 3 were electrophoresed on a 7% polyacrylamide slab gel cross-linked with methylenebisacrylamide. An infected-cell lysate which served as starting material and the VP123 region isolated by preparative SDS-PAGE are included for reference. Each of the three major SDS-HTP peaks (gC, gA/gB, and NG) was electrophoresed individually as well as recombined to demonstrate that these components account for all of the components present in VP123 region. VP154 (the major capsid protein of HSV-1) is indicated for reference.*

determinations made in gels of two different acrylamide concentrations, using each of the cross-linking agents. The apparent molecular weight of glycoproteins which appeared on SDS-PAGE as highly diffuse bands (gC) listed in Table 1 represent the average molecular weight of the entire band. Included in Table 1 are the apparent molecular weights previously assigned to the various HSV-1 glycoproteins by Spear (30).

Antiserum production. After the initial identification of the individual components which together comprise the major VP123 glycoprotein complex of HSV-1, it was of interest to examine the immunological characteristics of both the VP123 region as a whole and of the individual SDS-HTP-derived components. To address this problem, we have produced specific antisera to the VP123 region and to the two major components derived from this region by SDS-HTP chromatography. These components have been designated gC and gA/gB, since they contain the gC glycoprotein and the gA and gB glycoproteins, respectively.

TABLE 1. *Molecular weight estimates of the major virus-specific glycoproteins*

HSV-1 component[a]	Apparent mol wt ($\times 10^{-3}$)[b]		
	Methylenebisacrylamide	DATD	DATD[c]
gA	118	115	119
gB	127	122	126
gC	128	127	Approx 135
gC(105)	103	104	102–110

[a] Glycoprotein antigens were purified by SDS-HTP chromatography and coelectrophoresed on analytical slab gels with proteins of known molecular weight.

[b] Apparent molecular weights of each of the glycoproteins were determined on 6.5 and 8.6% gels cross-linked with DATD and on 5.0 and 7.0% gels cross-linked with methylenebisacrylamide.

[c] These apparent molecular weight estimates are from Spear (30).

The antigens used to produce antisera were prepared as described in Materials and Methods. Briefly, approximately 10^9 HEp-2 cells were infected with HSV-1 (KOS) at a multiplicity of 10 to 20 PFU/cell. One-fourth of the cells were labeled with [^{3}H]glucosamine (GluN) (5 μCi/ml) and ^{14}C-labeled amino acids (3 μCi/ml) from 4 to 24 h postinfection. At 24 h postinfection, cells were harvested and frozen in portions of 10^8 cells. The glycoproteins were solubilized with 1% sodium deoxycholate and 1% Tween-40 at 37°C for 1 h, followed by high-speed centrifugation at 100,000 $\times$ *g* for 1 h. Each supernatant was electrophoresed on a 7% preparative SDS-polyacrylamide gel (Fig. 3A). The VP123 region was identified by the [^{3}H]GluN label, pooled, and concentrated. All of the resulting VP123 preparations were combined and reelectrophoresed on the preparative SDS-PAGE system (Fig. 3B). VP123 antigen at this stage of purification was used to produce antisera to the VP123 region. For preparation of antisera to the major VP123 components, purified VP123 was dialyzed against 0.01 M phosphate buffer containing 0.1% SDS and chromatographed on an SDS-HTP column (2.4 by 15 cm) (Fig. 3C). The peaks designated gC and gA/gB were pooled, concentrated, dialyzed against 0.1% SDS in 0.01 M phosphate buffer, and sequentially rechromatographed on a series of two to four smaller (1.0 by 15 cm) SDS-HTP columns, using shallower gradients of phosphate molarity to increase resolution, until a single, homogeneous peak was obtained as judged by both SDS-HTP chromatography and SDS-PAGE (Fig. 3D,E). These purified HTP components were then used to

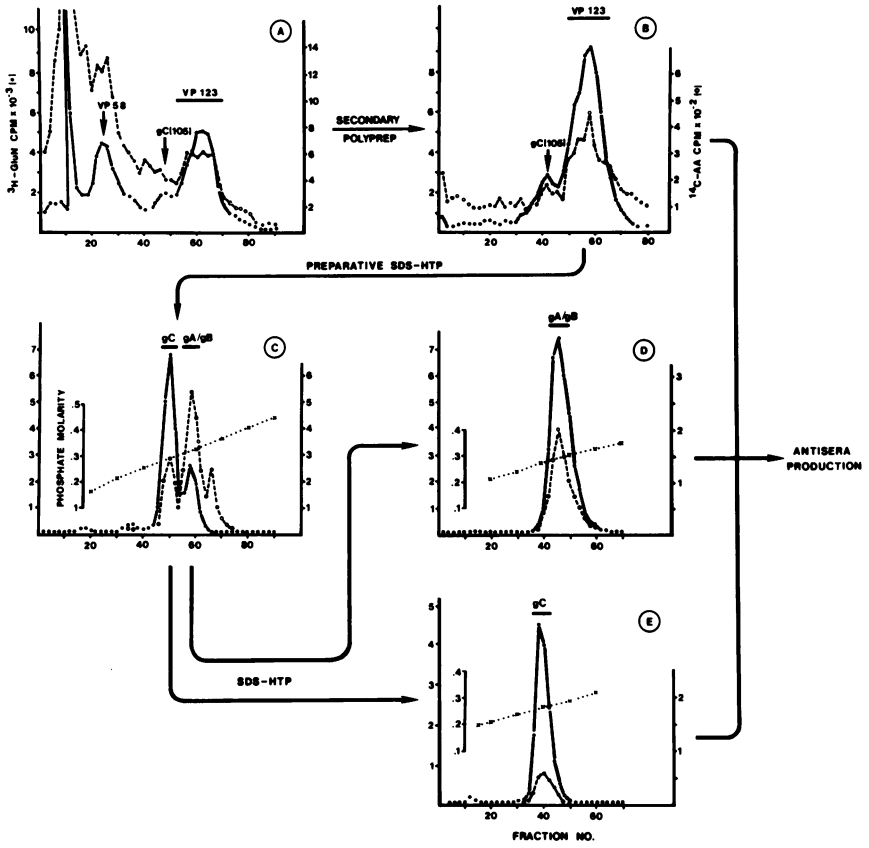

FIG. 3. *Purification of glycoprotein antigens for antisera production. Approximately one-fourth of the infected cells used were labeled with [^{3}H]GluN (10 μCi/ml) and ^{14}C-labeled amino acids (3 μCi/ml) from 4 to 24 h postinfection. Labeled and unlabeled cells were mixed and electrophoresed on preparative SDS-PAGE in portions of 10^8 cells/gel (A). All VP123 regions were combined and reelectrophoresed on the preparative SDS-PAGE system (B). The resulting VP123 preparations were used either for the preparation of anti-VP123 sera or as the starting material in the purification of the individual glycoprotein antigens. To purify the gC and gA/gB antigens, purified VP123 was chromatographed on preparative SDS-HTP columns (C); then the gC and gA/gB peaks were pooled separately and rechromatographed on a series of smaller SDS-HTP columns until single homogeneous peaks were obtained (D, E). This material was analyzed on SDS-PAGE for purity and used to produce antisera to each of the glycoproteins. Symbols: ●, [^{3}H]GluN; ○, ^{14}C-labeled amino acids; ····, phosphate molarity.*

prepare specific antisera. Each antigen preparation was electrophoresed into multiple cylindrical 7% acrylamide gels, and 5- to 8-mm segments containing the antigen were removed and used as immunogens as described in Materials and Methods.

RIP. All antisera were initially tested by indirect RIP to determine the specificity of these antisera for the antigens used in their production (Table 2). As a positive control, an antiserum made against an HSV-1-infected cell lysate was included. This anti-HSV-1 serum efficiently precipitated all of the purified virus-specific glycoprotein antigens tested. The antiserum raised to the unfractionated VP123 region precipitated only the VP123 antigen, the gC antigen, and the gA/gB antigen; the purified VP58 antigen was not significantly precipitated by this antiserum. The two HTP component antisera (anti-gC and anti-gA/gB) each exhibited definite, although not complete, precipitation of the VP123 antigen. Furthermore, both of these antisera efficiently precipitated the purified homologous SDS-HTP antigen while precipitating only background amounts of the purified heterologous SDS-HTP antigen. Again, as expected, neither of these antisera reacted significantly with the VP58 antigen.

Since the purification procedure used to isolate the glycoprotein immunogens relied on met-

TABLE 2. *Specificity of HSV-1 glycoprotein antisera*

Antiserum	Antigen				
	VP 123	gC	gA/gB	VP 58	Uninfected-cell VP 123
Preimmune	2.7[a]	2.0	10.1	2.9	4.0
Anti-HSV-1[b]	69.0	79.5	66.7	39.2	ND[c]
Anti-VP123	71.4	81.6	56.1	4.6	5.0
Anti-gC	27.3	48.5	11.0	5.4	4.0
Anti-gA/gB	25.4	3.4	87.9	4.0	3.0

[a] Percentage of precipitation of [^{3}H]GluN cpm based on cpm added to the reaction.

[b] Antisera prepared to cells infected with HSV-1.

[c] Not done.

abolic labeling of infected cells after host cell protein synthesis had been shut down by the virus, any host cell glycoproteins contaminating the final antigen preparations would not have been detected except at the final stage, when each preparation was analyzed on SDS-polyacrylamide gels by staining for protein. Although stained bands corresponding only to the radiolabeled virus-specific glycoproteins were observed in each antigen preparation, the presence of any host proteins which coelectrophoresed with the viral antigens still would not have been detected. To address this problem, uninfected HEp-2 cells were used to prepare an antigen analogous to the VP123 region. Using this uninfected cell antigen, none of the antisera could be shown to specifically precipitate significant amounts of this host cell material, thus demonstrating a lack of any detectable activity in these sera to host cell antigens. Further evidence for a lack of specific reactivity with host cell antigens is presented below.

Immunoprecipitation. Although RIP tests using the purified glycoprotein antigens yielded results which suggested strict specificity of the antiglycoprotein sera tested, immunoprecipitation of purified polypeptides was, in itself, insufficient to demonstrate the exact specificity of these antisera. Therefore, the three HSV-1 antiglycoprotein sera were used to immunoprecipitate polypeptides from detergent extracts of infected cells labeled with [^{35}S]methionine (1 μCi/ml) from 4 to 24 h postinfection. The resulting immune precipitates were then analyzed by analytical SDS-PAGE (Fig. 4).

The immunoprecipitate of the preimmune rabbit serum defined the background levels of labeled proteins found in the immunoprecipitates with this method. All three of the immune sera precipitated the same background proteins nonspecifically in amounts comparable to those observed in the preimmune precipitate. However, the anti-VP123 serum also specifically precipitated from the infected-cell extract three polypeptides which comigrated with the purified VP123 marker. Both the marker and immune precipitate consisted of a single rather diffuse band (gC) and two well-defined bands of slightly greater electrophoretic mobility (gA and gB). Precipitates obtained with the anti-gC serum contained only the diffuse, higher-molecular-weight gC glycoprotein, whereas the anti-gA/gB serum serum precipitated only the two lower-molecular-weight bands of the VP123 region (gA and gB).

Analysis of similar immune precipitates on 5% gels revealed the presence of a fourth polypeptide within the VP123 region (Fig. 5). This protein, which probably corresponds to the nonglycosylated VP7.5 polypeptide previously described by Sarmiento et al. (24), migrated between the gC and gB glycoproteins. This polypeptide was present in the anti-VP123 immune precipitate but was absent from immune precipitates of both the anti-gC and anti-gA/gB sera.

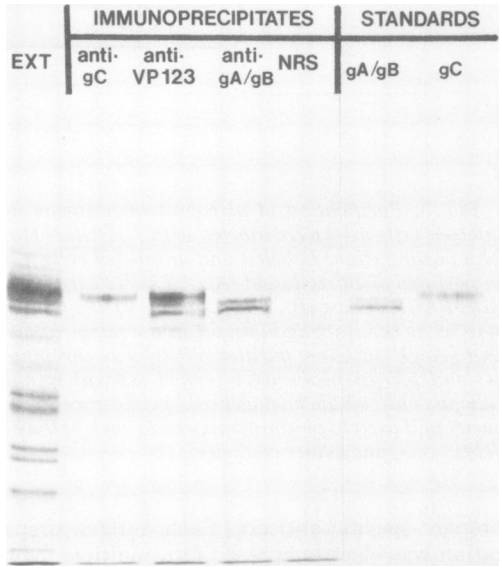

FIG. 4. *Immunoprecipitation of HSV-1 glycoproteins, using antisera prepared to the purified glycoprotein antigens. Infected cells labeled with [^{35}S]methionine (1 μCi/ml) from 4 to 24 h postinfection were extracted with 1% sodium deoxycholate and 1% Tween-40 as described in the text. Antisera prepared to the three HSV-1 glycoprotein antigens (VP123, gC, and gA/gB) and a nonimmune serum were used to immunoprecipitate polypeptides from the detergent extracts. The immune precipitates were analyzed on 8.6% DATD-cross-linked polyacrylamide gels. The detergent extract used as the antigen preparation and SDS-HTP-purified gC and gA/gB antigens are included for reference.*

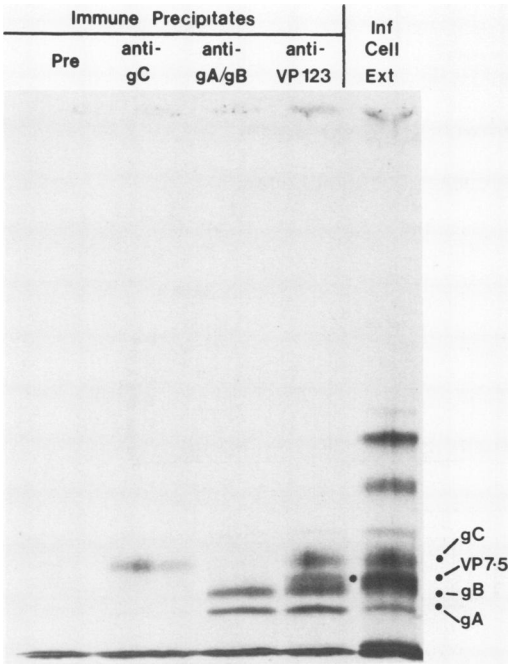

FIG. 5. *High-resolution analysis of the immune precipitates obtained by using the antiglycoprotein sera. Immunoprecipitations were carried out as described in the text. Immune precipitates were analyzed on a 5.0% acrylamide gel cross-linked with DATD. A nonglycosylated protein (VP7.5) which was not detected on 8.6% gels was resolved on 5% gels in both the detergent extract and the anti-VP123 immune precipitate.*

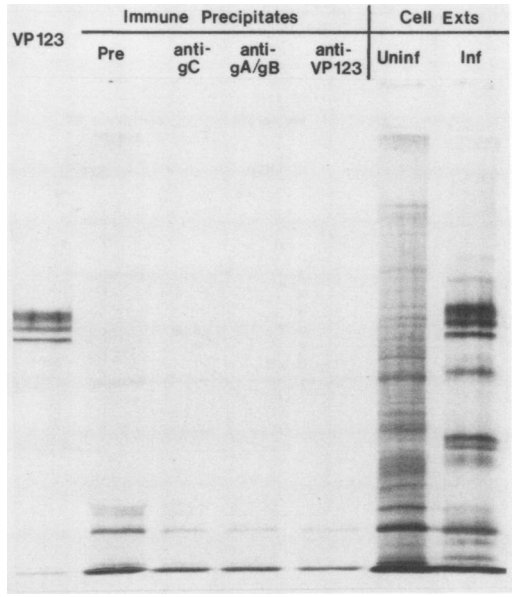

FIG. 6. *Immunoprecipitation from uninfected-cell extracts, using antisera prepared to the HSV-1 glycoproteins. Mock-infected cells were labeled with [^{35}S]methionine (1 μCi/ml) from 4 to 24 h postinfection, and a detergent extract was prepared. Immune precipitations were carried out and analyzed on 8.6% gels cross-linked with DATD. An infected-cell extract and purified VP123 are included for reference.*

These data further demonstrate the restricted specificity of the anti-gC and the anti-gA/gB sera for the gC and the gA and gB glycoproteins, respectively.

To further examine the reactivity of the glycoprotein antisera with uninfected HEp-2 cell antigens, immunoprecipitation from extracts of uninfected cells labeled with [^{35}S]methionine (3 μCi/ml) for 20 h was performed (Fig. 6). None of the antiglycoprotein sera could be shown to specifically precipitate any polypeptides from uninfected HEp-2 cell extracts. These results again showed a lack of detectable activity directed against host cell antigens in any of the antiglycoprotein sera.

Although the glycoprotein antisera all specifically precipitated discrete protein bands from infected-cell extracts which, when analyzed by slab SDS-PAGE, coelectrophoresed with the glycoprotein peaks isolated by SDS-HTP chromatography, it was desirable to demonstrate that the proteins immunoprecipitated by the antisera were actually identical to those obtained from SDS-HTP columns. Figure 7 presents the results of an experiment designed to demonstrate such identity. Immunoprecipitates obtained from [^{35}S]methione-labeled infected cells by using each of the three glycoprotein antisera and a preimmune serum were solubilized and divided into two portions. One portion of each of the immune precipitates was mixed with a purified [^{3}H]GluN-labeled VP123 preparation (which served as an internal standard) and was chromatographed on a small (0.5 by 8 cm) SDS-HTP column. The peaks from each of these columns which contained [^{35}S]methionine label were pooled, concentrated, dialyzed, and analyzed on SDS-PAGE in parallel with the remaining half of the original immune precipitate.

The results of this experiment clearly demonstrated that the material precipitated by the anti-gC serum both coeluted from SDS-HTP columns and coelectrophoresed on SDS-PAGE with the material comprising the gC peak of the VP123 region. Similarly, the material precipitated by the anti-gA/gB serum coeluted from the SDS-HTP columns with the gA/gB peak of the ^{3}H-labeled VP123 marker. Both the original anti-gA/gB precipitate and the material eluted

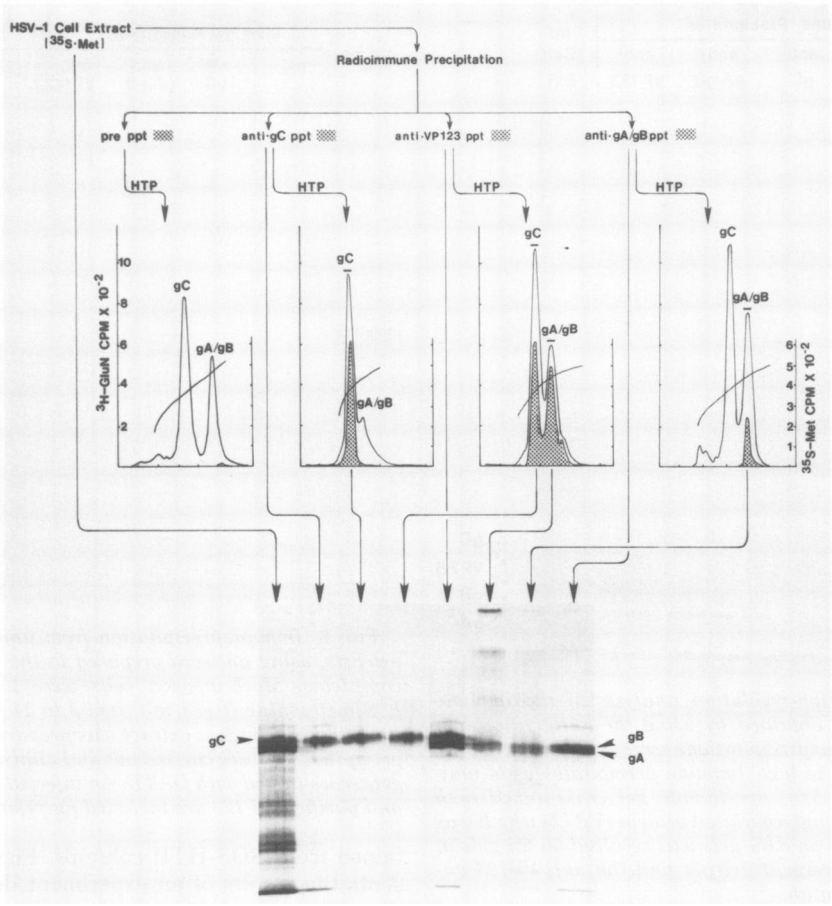

FIG. 7. *Identity of SDS-HTP components and the glycopolypeptides observed on SDS-PAGE. Cells infected with HSV-1 were labeled with [^{35}S]methionine (1 μCi/ml) from 4 to 24 h postinfection. Antiglycoprotein sera were used to immunoprecipitate specific antigens from detergent extracts of the infected cells. One half of each of the resulting immune precipitates was cochromatographed with purified [^{3}H]GluN-labeled VP123 (solid lines) on SDS-HTP columns (0.5 by 8 cm). The [^{35}S]methionine-containing peaks (hatched areas) were pooled and electrophoresed on 8.6% gels cross-linked with DATD along with the remaining half of the original immune precipitates. The gC, gB, and gA glycoproteins in the infected-cell extract are identified for reference.*

from the column in the gA/gB peak appeared as a doublet on slab gels, thus demonstrating the specificity of this antiserum for both the gA and gB polypeptides. Material precipitated by the anti-VP123 serum contained protein species which eluted from the SDS-HTP columns and coelectrophoresed on SDS-PAGE with both the gC and gA/gB components. In addition, much more nonglycosylated material (which eluted from the SDS-HTP column at a higher phosphate molarity than the gA/gB component) was present in the anti-VP123 immune precipitate than was present in the immune precipitates of the anti-gC, anti-gA/gB, or preimmune sera. This suggests a specific recognition by the anti-VP123 serum of some nonglycosylated proteins which electrophoreses within the VP123 region on SDS-PAGE. Subsequent SDS-HTP chromatography of the VP123 region would have eliminated this material from both the gC and gA/gB antigen preparations by virtue of its elution at a higher phosphate molarity than the glycosylated gA, gB, and gC polypeptides. SDS-HTP chromatography of the preimmune immunoprecipitate revealed only background amounts of [^{35}S]methionine-labeled material which eluted predominantly with the nonglycosylated proteins. Thus, antisera prepared to the gC and gA/gB glycoprotein components lack reactivity to (i) the heterologous SDS-HTP-derived glycoprotein antigen and (ii) nonglycosylated proteins which are also found in the VP123

region (see also Fig. 5). These results further define the restricted specificity of the antisera for the individual glycoprotein component used in their production.

Since the glycoprotein antisera were produced by using SDS-denatured glycoprotein antigens, it was important to determine whether these antisera would show any reactivity with their respective antigens in their nondenatured or native state. To address this question, a detergent extract of HSV-1-infected cells labeled with [^{35}S]methionine was divided into two portions. One half of this preparation was made 1% SDS (denatured antigen), and an equal volume of water was added to the remaining half (native antigen). Immunoprecipitations were then carried out in parallel, using the two antigen preparations, and the resultant immunoprecipitates were analyzed on slab SDS-PAGE (Fig. 8). Although all three of the anti-HSV-1 glycoprotein sera precipitated their respective antigens from the two extracts, it should be noted that the glycoproteins were precipitated with three- to fourfold greater efficiency from nondenatured extracts than from SDS-treated extracts. Immunoprecipitated polypeptides from native and denatured extracts were indistinguishable from one another by SDS-PAGE analysis. Identical results were obtained with extracts prepared with 1% Triton X-100 or 1% Nonidet P-40 (data not presented). Thus, it is evident that these antisera are capable of recognizing the native glycoprotein antigens even though SDS-denatured glycoproteins were used for production of the antisera.

Membrane immunofluorescence. The three HSV-1 glycoprotein antisera were examined by indirect membrane immunofluorescence to determine their ability to recognize and react with their respective antigens when in the in vivo conformation, i.e., situated in the plasma membrane of the infected cell. VERO cell cover slip cultures were infected at a multiplicity of 0.1 PFU/cell, harvested at 18 h postinfection, and stained immediately. As a positive control, an antiserum prepared to cells infected with HSV-1 (anti-HSV-1) was included (Fig. 9B). All three of the HSV-1 glycoprotein antisera gave bright

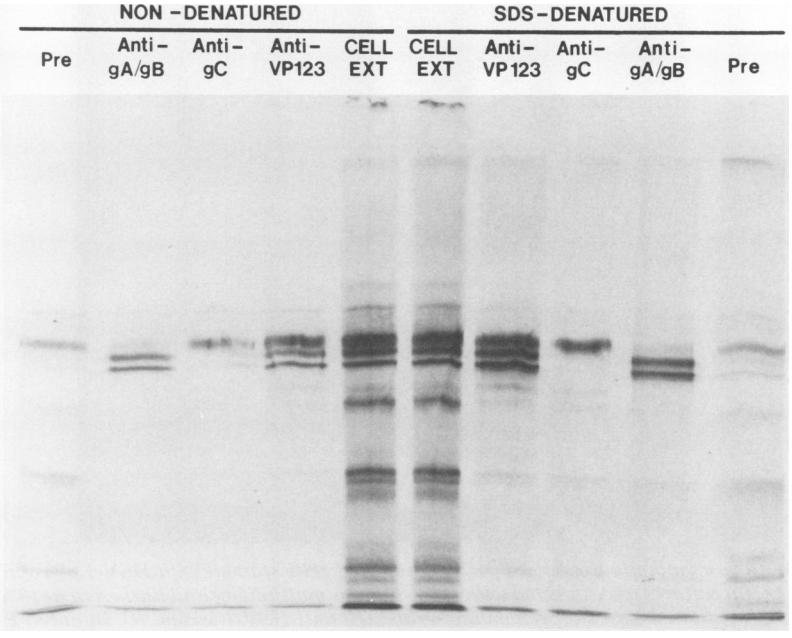

FIG. 8. *Immunoprecipitation of detergent-solubilized nondenatured glycoprotein antigens by antiglycoprotein sera. Infected cells were labeled with [^{35}S]methionine (1 μCi/ml) from 4 to 24 h postinfection, and a detergent extract was prepared. One half of the extract was made 1% SDS (denatured), and an equal amount of water was added to the remaining half (nondenatured). Immunoprecipitations from the two extracts were carried out in parallel, and the precipitates analyzed on 8.6% gels cross-linked with DATD. To prevent overloading of the gel with the isotopically labeled protein, approximately half of the native immune precipitates were run on this gel as compared with all of the precipitates from SDS-denatured extracts. In addition, twice the amount of the preimmune serum precipitates were loaded in the end lanes on this particular gel to emphasize the specificity of the precipitation of HSV glycoproteins by the immune sera.*

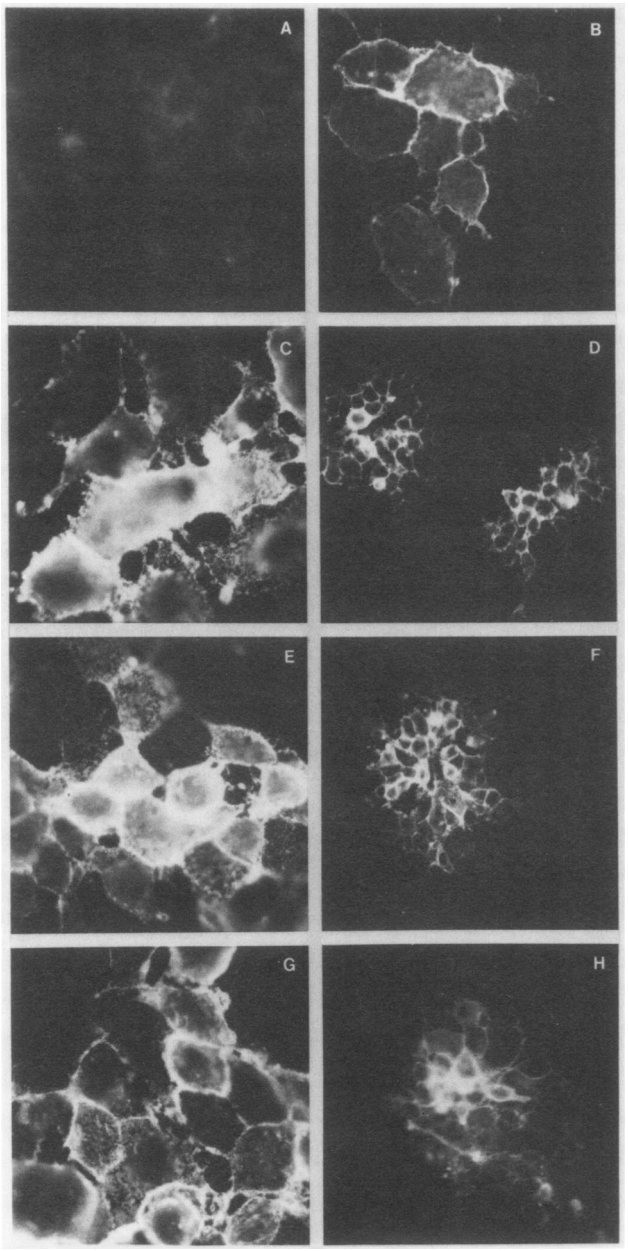

FIG. 9. *Membrane immunofluorescence of HSV-1-infected cells stained with HSV-1 glycoprotein antisera. VERO cell cover slip cultures were infected with HSV-1 at low multiplicity and harvested at 18 h postinfection. The cover slips were stained with (A) preimmune serum; (B) anti-HSV-1 serum; (C, D) anti-VP123 serum; (E, F) anti-gC serum; (G, H) anti-gA/gB serum. (A, B, C, E, G) ×400 magnification; (D, F, and H) ×100 magnification.*

positive reactions (Fig. 9C–H). Of the three antiglycoprotein sera, the anti-VP123 serum exhibited the most intensive staining of the infected cells (Fig. 9C). Even when lower dilutions of the anti-gC (Fig. 9E) and anti-gA/gB (Fig. 9G) sera were used, the fluorescence obtained was not as intense as that observed with the anti-VP123 serum, possibly reflecting the recognition by the anti-VP123 serum of multiple antigens (the gA, gB, and gC glycoproteins). Preimmune rabbit sera did not stain infected cells (Fig. 9A). None of the sera, preimmune or

immune, gave a positive reaction with uninfected cells. This is readily apparent in the photomicrographs where a lower magnification was used (Fig. 9D,F,H). Only the foci of infected cells were stained by the immune sera; surrounding uninfected cells did not react with the antisera.

Neutralization. Since, as evidenced by membrane immunofluorescence and immunoprecipitation, the three HSV-1 glycoprotein antisera were capable of reacting with their respective antigens when in their native conformation, the ability of these antisera to neutralize infectious virus was also of interest. To investigate this problem, each of the antisera as well as a preimmune serum were tested in both the presence and absence of complement for their ability to neutralize HSV-1 (KOS). The percentage of reduction of infectious virus at various dilutions of each antiserum both with and without complement are presented in Fig. 10. In the absence of complement, both the anti-VP123 and anti-gA/gB sera exhibited a strong capacity to neutralize infectious virus. However, only at low serum dilutions was any neutralizing activity detected in the anti-gC serum. In the presence of complement, the neutralizing titer of the anti-gC serum was markedly enhanced. In contrast, the neutralizing titer of both the anti-gA/gB and anti-VP123 sera in the presence of active complement was only slightly greater than that observed in the absence of complement. Results identical to these were obtained with a second set of antisera prepared separately to the VP123 region, the gC glycoprotein and the gA/gB glycoproteins. From these results, it may be concluded that both the gC and gA/gB antigens are expressed on the surface of infectious virions. As indicated by the high neutralizing titer of the anti-gC serum in the presence of complement, the gC antigen is present on the surface of the virion and accessible to antibody. However, the lack of neutralization by anti-gC sera in the absence of complement implies that the gC glycoprotein may not be directly involved in determining the infectivity of the virion. The complexing of the gA/gB antigen (almost exclusively gB in mature virions) with antibody is, in itself, sufficient to effectively neutralize virion infectivity, thereby suggesting an important role for this antigen in the adsorption or penetration process as has been previously proposed (24).

DISCUSSION

Various approaches have been used to purify certain of the HSV-specific glycoproteins from virions or from infected cells. These include: the use of PAGE to purify the VP123 region of HSV-1 (3, 5, 20); the use of density gradient centrifugation to purify gB of HSV-1 (22); the use of crossed immunoelectrophoresis to purify the glycoproteins of the VP123 and VP58 regions

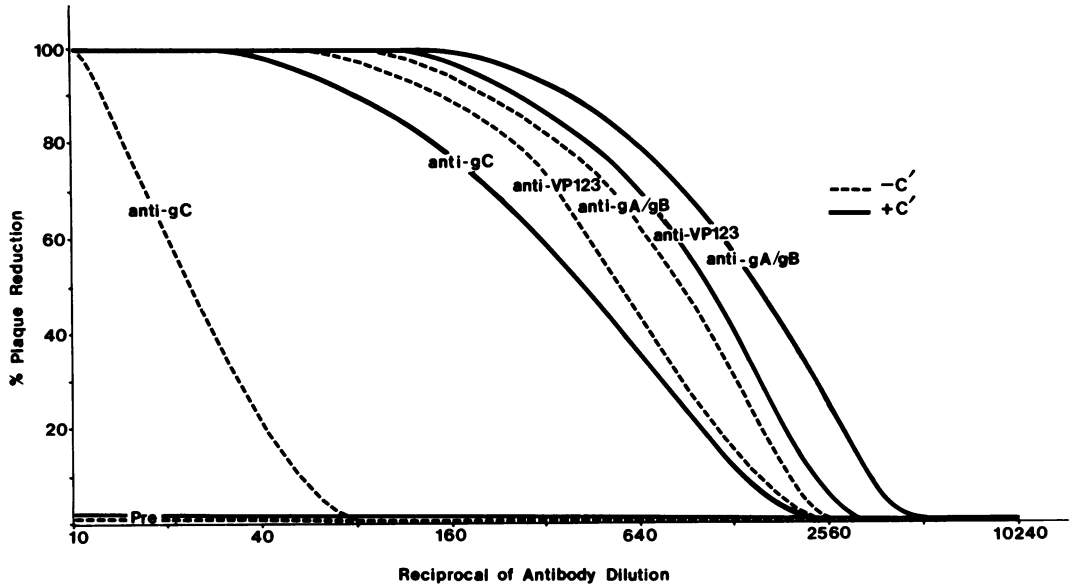

FIG. 10. *Neutralization of HSV-1 by antisera to HSV-1 glycoproteins. Serial dilutions of each antiserum were combined with 8 U of fresh or heat-inactivated complement and 2 × 10^5 PFU of HSV-1. The neutralization reaction was carried out at 37°C for 1 h. Results are expressed as the percentage of plaque reduction compared with a control sample containing no antiserum. The results presented here represent average values obtained from multiple determinations.*

(16, 32); and the use of HTP chromatography to purify the glycoproteins gD (2) found within the VP58 region. In this report, we describe a different approach to the purification of HSV-specific glycoproteins which is suitable for the isolation of the individual HSV-specific glycoproteins found within the infected cell. It appears that the combined use of preparative SDS-PAGE and SDS-HTP chromatography offers several advantages for isolating and purifying the HSV-specific glycoproteins in preparative amounts. First, the combined use of SDS-PAGE and SDS-HTP chromatography resolves the glycosylated proteins away from all nonglycosylated proteins. In these studies, the glycosylated proteins have always eluted at lower molarities of sodium phosphate than have the nonglycosylated proteins. Second, the separation obtained by SDS-HTP chromatography appears to resolve various species of glycoproteins based on their degree of glycosylation. Thus, the use of SDS-HTP chromatography has essentially provided a second dimension of resolution additional to the first separation based strictly on differences in molecular weight (SDS-PAGE).

As discussed by Moss and Rosenblum (13), the factors responsible for the separation of SDS-containing proteins by HTP are not well understood. The necessary increase in ionic strength for elution of SDS-treated polypeptides and the dependence on ionic strength of the interaction of the SDS-treated monomer and the protein (23) are probably significant factors. Our observation of the elution at lower molarity from SDS-HTP columns of the glycoproteins suggests to us that glycosylated polypeptides, especially those which are highly glycosylated, may bind less SDS per unit length of protein than do polypeptides which are not glycosylated. The elution from SDS-HTP columns of all nonglycosylated polypeptides in a single peak at a higher phosphate molarity than any of the glycosylated species would appear to support this hypothesis.

The capacity to resolve and isolate the HSV-specific glycoproteins in preparative amounts has made it possible to further characterize the immunological and biochemical properties of each of the individual glycoprotein components. RIP tests using purified, radiolabeled glycoproteins as antigens failed to detect specific precipitation of any antigen other than the VP123 region or its subcomponents when examining the reactivity of the antiserum made to the SDS-PAGE-purified VP123 complex. Similarly, antisera prepared to the gC and gA/gB glycoprotein components did not cross-react, nor did they precipitate significant amounts of the VP58 region or uninfected-cell glycoproteins. Should any cross-reactivity exist between the gC and gA/gB antigens, precipitation of the purified glycoprotein antigens should be the most sensitive means for its detection. The failure to detect any specific precipitation of the heterologous SDS-HTP antigen with either the anti-gC or anti-gA/gB sera clearly indicates that the gC and gA/gB glycoproteins are antigenically distinct from one another.

Immunoprecipitation from an [^{35}S]methionine-labeled infected-cell detergent extract with the three antiglycoprotein sera has demonstrated the specificity of these antisera by their ability to precipitate only their homologous antigen(s) from all of the proteins, nonglycosylated as well as glycosylated, which are present in infected-cell extracts. The anti-gC serum demonstrates a specificity for the more highly glycosylated antigen of the VP123 complex (glycoprotein gC), a reactivity which is shared, as expected, by the anti-VP123 serum. Occasionally, a glycoprotein of approximately 105,000 daltons was also immunoprecipitated from infected cell extracts by the anti-gC and anti-VP123 sera. This glycoprotein has a high mannose content relative to the gC glycoprotein, suggesting that it may represent a partially glycosylated precursor to the gC glycoprotein as has been suggested by Spear (29, 30). Pulse-chase studies carried out at 6 h postinfection using the anti-gC serum have confirmed that this glycoprotein [designated gC(105)] is indeed a direct precursor to the gC glycoprotein (data not presented).

The precipitation of a doublet (gA and gB) from the infected-cell extract by the anti-gA/gB serum is consistent with data presented that these two polypeptides cochromatograph on SDS-HTP columns as the gA/gB peak of VP123. From this it is apparent that both the anti-gA/gB serum and the anti-VP123 serum specifically react with both the gA and gB glycoproteins. At the onset of the experiments described here, analysis of the VP123 region was routinely carried out by using 7% acrylamide gels cross-linked with methylenebisacrylamide in which these two glycoproteins were not clearly resolved. After the antisera had been produced, 8.6% acrylamide gels cross-linked with DATD as described by Gibson and Roizman (8) were used, thus revealing the multiple polypeptides in the SDS-HTP gA/gB peak material. However, no amount of rechromatography on SDS-HTP columns using shallower gradients or preferential pooling of either the leading or trailing edge of the gA/gB peak could select for one component over the other. In addition, data to be published

elsewhere indicate that the gA and gB glycoproteins probably represent two antigenically related forms of the same polypeptide which differ in the degree of their glycosylation (R. Eberle and R. J. Courtney, manuscript in preparation). This alleviates problems inherent in using antisera which are reactive with more than one polypeptide, since the gA and gB glycoproteins (and the SDS-HTP gA/gB peak) may be dealt with as a single antigen.

The glycoprotein species immunoprecipitated from native detergent extracts of infected cells were indistinguishable on slab SDS-PAGE analysis from parallel immunoprecipitates obtained from an SDS-denatured detergent extract. All three of the antiglycoprotein sera can therefore recognize the nondenatured native antigens even though SDS-treated glycoproteins served as antigens for the production of the antisera. The ability of the sera to bind antigens expressed on the surface of both viable infected cells and infectious virions as measured by membrane immunofluorescence and neutralization demonstrates the ability of these antisera not only to recognize solubilized native antigens but also to specifically bind their respective antigens in the in vivo state, i.e., while situated in a lipid bilayer structure. The antigenic sites responsible for the induction of the antisera could be carbohydrate as reported for certain oncornavirus glycoproteins (31), protein alone, or a combination of the two. Preliminary studies in which the nonglycosylated precursors of HSV-1 gC and gA/gB glycoproteins have been immunoprecipitated suggest that the carbohydrate moiety plays no major role in determining the antigenicity of these polypeptides (E. Wenske and R. Courtney, unpublished data). In addition, recognition of exposed antigenic sites on the glycoproteins by the antiglycoprotein sera indicates either that SDS denaturation does not significantly affect the antigenicity of these sites or that SDS is not bound at all to this particular region of the glycoprotein.

The presence in the infected-cell plasma membrane of the glycoproteins comprising the VP123 region (gA, gB, and gC) has been well documented (10, 29, 30). Similarly, the expression of the VP123 region on the external surface of infected cells has also been shown by using surface-labeling techniques (9, 18). Using our antisera to the VP123 region and the two glycoprotein components in membrane immunofluorescence and antibody-dependent cellular cytotoxicity assays (B. T. Rouse, R. Eberle, and R. J. Courtney, unpublished data), we have confirmed the presence and external orientation of these glycoprotein antigens in the plasma membrane of infected cells. The presence of these same antigens in a similar orientation on infectious virions has been clearly demonstrated by the ability of the antiglycoprotein sera described here to neutralize HSV-1.

Using serial dilutions of these sera in immunofluorescence tests to measure the amount of antibody bound to infected cells, roughly equivalent titers of the gC and gA/gB antisera were indicated. Similarly, the high neutralizing titer of gC antisera in the presence of complement suggests that anti-gC antibody does bind to the virus at the higher dilutions of antiserum used. However, this binding does not appear to be sufficient to neutralize virus. These data suggest that the neutralization observed with anti-gC sera at low dilutions in the absence of complement may in fact be the result of steric hindrance effects caused by the binding of large amounts of antibody rather than the complexing of critical sites by anti-gC antibodies. Another possible explanation for the low neutralization titer of anti-gC sera may be that antibodies specific for critical antigenic sites of the gC glycoprotein are present at low concentrations in the antisera. If so, this would suggest that these sites represent only a small portion of the antigenic determinants of the gC glycoprotein or that this particular determinant is sensitive to denaturation by SDS and was thus not a major determinant in the immunogen.

Despite the apparent unimportance of the gC glycoprotein in determining virion infectivity, this glycoprotein undoubtedly provides some function essential to the virus. SDS-PAGE analysis of immune precipitates from infected cells, using a large number of HSV-1 isolates, has, without exception, demonstrated the ability of all HSV-1 strains to induce the synthesis of a gC glycoprotein which was antigenically and physiochemically similar to the gC glycoprotein of HSV-1 (KOS) (data not presented). The in vitro viability of the laboratory variant HSV-1 (MP) (which lacks a gC glycoprotein) (7) may suggest that the function of the gC glycoprotein is necessary in vivo rather than in vitro.

In contrast to the complement-dependent neutralization observed with the anti-gC serum is the high-titered complement-independent neutralization obtained with the anti-gA/gB serum. The inability of complement to significantly enhance neutralization suggests that virus to which anti-gA/gB antibodies have bound are effectively neutralized. Consequently it would appear that, unlike antibodies specific for the gC glycoprotein, antibodies which recognize the gA and/or gB glycoproteins are binding to antigenic sites which are intimately associated with or

themselves represent the surface sites on HSV-1 virions which determine virus infectivity; i.e., the gA and/or gB glycoproteins are responsible for adsorption to or penetration of host cells by herpes virions. In light of the extremely small amounts of the gA glycoprotein present in purified virions relative to the gB glycoprotein (30; Eberle and Courtney, unpublished data) and studies using a mutant temperature sensitive for production of the gB glycoprotein (24), it appears that the gB glycoprotein is the glycoprotein antigen involved in the adsorption/penetration process. As would be expected of an antigen intimately associated with the infectivity of the virion, antigenically related polypeptides corresponding to the gA and gB glycoproteins of HSV-1 (KOS) are induced on infection of HEp-2 cells by every HSV-1 isolate examined to date regardless of the site of isolation or passage history of the isolate. Preliminary results also indicate that these antigens are cross-reactive with the corresponding gA and gB glycoproteins of HSV-2 which also appear to be closely associated with virion infectivity (Eberle and Courtney, unpublished data). Together, these data suggest that the gA and gB antigens, being essential for virion viability, may represent antigens which have been conserved during the evolutionary divergence of the two HSV serotypes.

The production of antisera specific for individual virus-specific glycoproteins of HSV-1 has permitted the determination of a number of characteristics of these glycoproteins. Similar purification of the glycoproteins which comprise the major glycoprotein region of HSV-2 has also recently been accomplished (Eberle and Courtney, manuscript in preparation). Using these antisera in heterotypic assay systems should permit the immunological characteristics of the individual HSV-1 and HSV-2 glycoproteins to be determined. Since previous studies have all concluded that the VP123 region is relatively type specific in nature (3, 6, 20), the identification of type-specific antigens of HSV-1 and HSV-2 is a real possibility. Being surface antigens of both virions and infected cells, the glycoproteins are undoubtedly exposed to the elements of the host immune system upon infection by these viruses. Consequently, assays using the purified antigens, specific antisera, or both should prove useful in the typing of viral isolates and human immune sera. Immunological characterization of the glycoproteins and the feasibility of incorporating the antigens and antisera into diagnostic tests are currently being investigated.

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