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Binding to Cells of Virosomes Containing Herpes Simplex Virus Type 1 Glycoproteins and Evidence for Fusion

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Envelope proteins and lipids were extracted from purified herpes simplex virus type 1 virions with octyl glucoside and mixed with phosphatidylcholine for preparation of virosomes by removal of the detergent. Greater than 85% of the extracted envelope proteins, including all the glycoproteins and the nonglycosylated protein designated VP16, were associated with virosomes, which ranged in density from ca. 1.07 to 1.13 g/cm^3. All the glycoproteins except gC were as susceptible to degradation by added protease in virosomes as in virions, indicating similar orientations in both. Approximately 30 to 40% of radiolabel incorporated into virosomes bound to HEp-2 cells within 1.5 h at either 4 or 37°C. The cell-bound virosomes were enriched for gB and deficient in other glycoproteins, in comparison with unbound or total virosomes. Binding of virosomes to HEp-2 cells could be inhibited by purified virus, heparin, and monospecific antiviral antibodies. Polyclonal and monoclonal anti-gB antibodies were more effective at inhibiting virosome binding than were anti-gD or anti-gC antibodies. Virosomes depleted of gB or gD did not bind to cells as efficiently as did virosomes containing all the extracted enveloped components; this loss of binding activity was especially pronounced on depletion of gB. The binding of herpes simplex virus type 1 virosomes to cells is discussed in relation to possible heterogeneity of the virosomes and comparisons with binding of virions to cells. We also present electron microscopic evidence that bound virosomes can fuse with the cell surface.

Herpes simplex virus type 1 (HSV-1) specifies at least four glycoproteins designated gB, gC, gD, and gE, the genes of which have all been mapped in the viral genome (see for review: P. G. Spear, *in* B. Roizman, ed., *The Herpesviruses*, vol. 3, in press). The nucleotide sequences of genes for gB, gC, and gD have been previously reported (5, 12, 44), as has a partial amino acid sequence for gD (9). Therefore, although a great deal is known about the primary structures of these glycoproteins, we know little about how they function in the virion envelope or in cell membranes. Because the glycoproteins are the only viral products so far detected on the surfaces of virions and infected cells, it seems likely that they play important roles in the adsorption and entry of virions into host cells and in recognition of infected cells by the immune system.

Only limited information is available as to the roles of individual HSV glycoproteins in the adsorption and entry process. Studies of the temperature-sensitive mutants *ts*B5 and *ts*J12 implicate gB in this process, specifically at the level of entry (25, 37). Both mutants, whose mutations have been mapped to the gB gene (5, 8, 18, 24a, 25, 36), produce noninfectious virions at the nonpermissive temperature. These virions, which are deficient in normal gB but probably contain an aberrant form of gB, can bind to cells but do not initiate infection. Treatment of the virion-cell complexes with polyethylene glycol permits a fraction of the adsorbed virions to initiate infection (25, 37). Other results also implicate gB in the process of entry. Specifically, an assay was devised to measure the rate of entry of wild-type HSV after adsorption; by using this assay, differences in the rate of entry were noted among HSV strains, and a genetic difference controlling rate of entry was mapped to the gB gene (8). Still other experiments suggest the possibility that gB may also have a role in adsorption. Specifically, heparin blocks the adsorption of HSV-1 and HSV-2 to cells (15, 31, 41, 43); HSV virions bind to heparin-Sepharose, and after extraction of envelope proteins from virions, only gB binds quantitatively to heparin-Sepharose (P. G. Spear and M. L. Parish, manuscript in preparation).

Several lines of evidence also implicate gD in the adsorption and entry process. First, anti-gD monoclonal antibodies, but not antibodies specific for other HSV glycoproteins, were shown to block HSV-induced cell fusion (32), a process which may be analogous in some respects to the virion-cell fusion required for entry. Second, both polyclonal anti-gD antibodies and certain monoclonal anti-gD antibodies inhibited the binding of virus to cells better than did antibodies directed against other HSV glycoproteins (A. O. Fuller and P. G. Spear, manuscript in preparation). Finally, gD has been shown to elicit the production of neutralizing antibodies (1, 10, 17, 33, 35, 39). The significance of this latter observation for assessing the physiological role of gD remains uncertain until additional information is available. It is necessary to have comparative information on the ability of the several HSV glycoproteins to induce neutralizing antibodies under similar conditions and also to know more about the mechanism of neutralization.

No roles for gC or gE in adsorption and entry have been identified, although gC has been shown to bind the C3b component of complement (10a) and gE binds to the Fc regions of immunoglobulin G (IgG) (2). Because mutants unable to produce detectable forms of gC are viable in cell culture (6, 13, 17, 27, 46), it has been suggested that gC may play no essential role in infectivity for cultured cells.

To approach the question of how HSV-1 glycoproteins function in the adsorption and entry process, we extracted the glycoproteins from purified virions and reconstituted the glycoproteins along with a nonglycosylated virion polypeptide, VP16, into lipid vesicles which have been called virosomes (28). Virosomes, composed of a lipid bilayer in which are inserted viral glycoproteins extracted from virion

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envelopes, have been previously prepared in a number of laboratories (11, 14, 19–22, 28, 30, 34, 42), and in several cases the preparations were found to be active in binding to and fusing with cells (11, 19, 22, 28, 30). The virosomes we prepared from HSV-1 glycoproteins were able to bind to cells, and that binding was inhibited by HSV-1 virions, antibodies directed against viral glycoproteins, and heparin. In other experiments, virosomes were prepared with extracts depleted of gB or gD, and binding of virosomes was found to be reduced. We also present preliminary evidence that virosomes can fuse with cell surface membranes. Therefore, the virosomes possess activities which are thought to be associated with virion envelope glycoproteins, providing a system in which the activities of individual HSV-1 glycoproteins, alone or in combination, may be studied.

MATERIALS AND METHODS

Cells and virus. HEp-2 and African green monkey kidney cells (Vero) were grown as monolayer cultures in Dulbecco modified Eagle minimal essential medium supplemented with 10% fetal calf serum, both from KC Biologicals, Lenexa, Kans. The virus strains used were HSV-1(HFEM), obtained from A. Buchan (University of Birmingham, Birmingham, England), HSV-1(HFEM)syn, a syncytial derivative of the HFEM strain isolated in this laboratory (2), and HSV-1(MP), a syncytial HSV-1 strain defective in the synthesis of gC (13, 16, 27). Virus strains were passaged at low multiplicity in HEp-2 cells.

Purification of virus. Monolayers of HEp-2 cells growing in roller bottles were infected with virus at 10 PFU per cell in phosphate-buffered saline (10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 140 mM NaCl, 3 mM KCl, 0.5 mM $MgCl_2 \cdot 5H_2O$, 1 mM $CaCl_2$ [pH 7.4]) containing 1% inactivated calf serum and 0.1% (wt/vol) glucose for 2 h at 37°C. The virus was removed, and medium 199 supplemented with 1% calf serum (199V) was added to monolayers. Typically, 4 roller bottles of a total of 20 were labeled with [^{35}S]methionine by removing the 199V 4 h after infection and adding 199V containing 1/100 of the usual concentration of methionine and [^{35}S]methionine at 50 μCi/ml. Cells were incubated at 37°C for 20 to 24 h, harvested by scraping, and pelleted at low speed. Virus was purified from cells as described by Cassai et al. (7), except that the hypotonic buffer was 1 mM Tris-hydrochloride (pH 7.4) instead of 1 mM phosphate buffer (pH 7.4). The virus was concentrated by centrifugation at 23,000 rpm in an SW27 rotor for 2 h.

Extraction of virus. Pelleted virions were extracted with Tris-saline (50 mM Tris-hydrochloride [pH 7.5], 100 mM NaCl)–50 mM octyl-β-D-glucopyranoside (octyl glucoside; Calbiochem-Behring, La Jolla, Calif.)–1 mM EDTA–1 mM phenylmethylsulfonyl fluoride (PMSF)–200 U of aprotinin per ml (Trasylol; Mobay Chemical Corp., New York, N.Y.), sonicated three or four times for 30 s, and then incubated for 8 to 14 h on ice and sonicated briefly. The lysate was centrifuged at 30,000 rpm in a Beckman 50 Ti rotor for 1 h at 4°C, and the supernatant was used in reconstitution experiments.

Reconstitution of viral polypeptides into virosomes. Samples of extracted viral polypeptides were assayed for protein concentration, using the Bio-Rad protein assay kit and bovine serum as standard (Bio-Rad Laboratories, Richmond, Calif.). Phosphatidylcholine (150 μg/100 μg of viral protein) (type V-3; Sigma Chemical Co., St. Louis, Mo.) and octyl glucoside (500 μg/100 μg of viral protein), both dissolved in chloroform, were dried onto the surface of a glass test tube and then dissolved by the addition of the viral polypeptide extract, usually 1 to 2 ml containing 100 to 300 μg of protein. The solution of lipid, detergent, and protein was sonicated several times and dialyzed for 36 h against two 2-liter changes of 10 mM Tris-hydrochloride (pH 7.5)–1 mM $MgCl_2$ and for 12 h against 2 liters of Tris-saline–1 mM $MgCl_2$. The virosomes were sonicated briefly and stored at 4°C. Virosomes and liposomes labeled with ^{3}H-lipids were prepared in the same manner, except that the viral polypeptides were not labeled or were absent (liposomes), and ^{3}H-labeled lipids, extracted with chloroform-methanol (2:1) from HEp-2 cells labeled with [9,10-^{3}H(N)]palmitic acid (100 μCi/ml; New England Nuclear Corp., Boston, Mass.) in growth medium, were added to the phosphatidylcholine (ca. 1 to 3 μg [based on dry weight determination of a larger sample] of ^{3}H-labeled lipids per 100 μg of phosphatidylcholine).

Equilibrium density centrifugation. Virosomes labeled with [^{35}S]methionine or ^{3}H-lipids were layered onto 20 to 50% (wt/wt) linear sucrose gradients prepared in Tris-saline–1 mM $MgCl_2$ and centrifuged at 25,000 rpm in a Beckman SW50L rotor for 18 h at 4°C. Fractions were collected (38) and counted on glass filters.

Protease treatment of virosomes and virions. [^{35}S]methionine-labeled virosomes (3 μg of viral protein) or virions (25 μg of viral protein) were treated with a mixture of trypsin (type III; Sigma) and chymotrypsin (type VII; Sigma), each present at 166, 50, 10, 5, or 1 μg/ml in 30 μl of Tris-saline–1 mM $MgCl_2$. After 15 min at 37°C, bovine serum albumin (1 mg/ml), soybean trypsin inhibitor (250 μg/ml; type I-S; Sigma), chymostatin (200 μg/ml; Sigma), aprotinin (500 U/ml), and 1 mM PMSF were added for 3 min. In some samples, protease inhibitors were added before the proteases to test for efficacy of inhibition. An equal volume of a solution containing 4% sodium dodecyl sulfate (SDS), 4% β-mercaptoethanol, 20% glycerol, and a trace of bromophenol blue dye (2× SDS extraction buffer) was added, and the samples were immediately boiled for 5 min. The samples were then frozen at −20°C until shortly before electrophoresis in SDS-polyacrylamide gels.

Binding of virosomes or liposomes to cells. HEp-2 or Vero cells growing in 24-well dishes (ca. 5×10^5 cells per well) were incubated with ^{35}S-labeled virosomes (2 to 4 μg of viral protein) or ^{3}H-labeled liposomes (3 to 6 μg of lipid calculated on the basis of the amount of phosphatidylcholine added) in Tris-saline–1 mM $MgCl_2$–1 mM $CaCl_2$ containing 100 μg of bovine serum albumin (binding buffer) per ml for 1.5 h at 4 or 37°C. Unbound virosomes or liposomes were then removed, and the cells were washed with Tris-saline–1 mM $MgCl_2$–1 mM $CaCl_2$. The cells were disrupted with Tris-saline containing 2% SDS and 2% β-mercaptoethanol. The unbound and cell-associated radioactivity was quantitated by drying samples of the unbound material plus washings or cell lysate on glass filters for liquid scintillation counting. Material not bound to cells was prepared for electrophoresis on SDS-polyacrylamide gels by diluting the sample with an equal volume of 2× SDS extraction buffer. Cell-associated material was diluted with an equal volume of 1× SDS extraction buffer.

Inhibition of binding of virosomes by virions. HEp-2 cells growing in 24-well dishes were incubated with purified HSV-1(HFEM)syn virions in 200 μl of binding buffer for 1.5 h at 4°C. The virions were removed, except in one sample in which the virions were left on the cells and virosomes were added, and the cells were washed with Tris-saline–1 mM $MgCl_2$–1 mM $CaCl_2$. [^{35}S]methionine-labeled virosomes (2 to 4 μg of viral protein) in 220 μl of binding buffer were added

to cells for 1.5 h at 4°C. In one case, the virions and virosomes were preincubated for 1.5 h at 4°C and then added to cells for 1.5 h at 4°C. The binding of virosomes was quantitated as described above.

Inhibition of binding of virosomes by antibodies and heparin. The rabbit antisera used were R no. 67, specific for gB (23), and an antiserum prepared by Eisenberg et al. (10) against affinity-purified gD. The mouse monoclonal antibodies used included I-206, II-436, and II-886 specific for gD (32), II-512 specific for gC, and I-59 specific for gB (M. F. Para, A. G. Noble, K. Snitzer, M. L. Parish, and P. G. Spear, manuscript in preparation). Polyclonal and monoclonal antibodies were purified from rabbit serum or mouse ascites fluids by chromatography on DEAE-Affigel blue (Bio-Rad) as described by Bruck et al. (4), followed by precipitation with 50% saturated $(NH_4)_2SO_4$. Some of the monoclonal antibodies (I-206, II-886, and II-512) were also purified by affinity chromatography on protein A-Sepharose (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) as described by Noble et al. (32). The protein concentrations in purified antibody preparations were determined by using the Bio-Rad protein assay kit and mouse or rabbit IgG (Sigma) as standards. [^{35}S]methionine-labeled virosomes (2 to 4 μg of viral protein) were incubated with various quantities of purified IgG or heparin for 1 h at 4°C in binding buffer, and then binding was assayed at 37°C as described above.

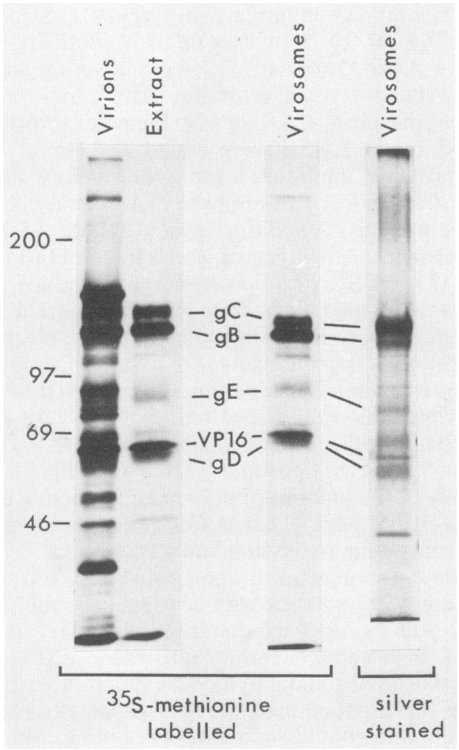

FIG. 1. Polypeptide composition of virions, octyl glucoside extracts, and virosomes. HSV-1(HFEM)syn virions, labeled with [^{35}S]methionine and purified as described in the text, were extracted with Tris-saline containing 50 mM octyl glucoside, 1 mM EDTA, 1 mM PMSF, and 200 U of aprotinin per ml. Virosomes were prepared from the extract as described in the text. Virions, extract, and virosomes were all prepared for electrophoresis by the addition of 2× SDS extraction buffer and electrophoresed on SDS-polyacrylamide gels. Gels to be subjected to fluorography ([^{35}S]methionine-labeled samples) were impregnated with PPO, dried, and placed in contact with X-ray film. Silver staining of gels was performed with a kit from Bio-Rad. Molecular mass markers were myosin (200 kd), phosphorylase B (97 kd), bovine serum albumin (69 kd), and ovalbumin (46 kd).

Depletion of gB or gD from octyl glucoside extracts and binding of virosomes prepared from these extracts. Immunoaffinity columns were prepared with purified mouse monoclonal IgGs, I-59 specific for gB, and II-436 specific for gD. Immunoglobulins were coupled to CNBr-activated Sepharose 4B (Pharmacia) by procedures prescribed by the manufacturer except that the coupling buffer was 0.1 M sodium citrate (pH 6.5)–0.3 M NaCl. The gel was washed with coupling buffer, incubated with 1 M ethanolamine (pH 8.0) for 2 h at 23°C, and then washed sequentially with (i) 0.1 M sodium acetate buffer (pH 4.0)–1 M NaCl, (ii) 0.1 M sodium borate buffer (pH 8.2)–1 M NaCl, (iii) 0.1 M sodium acetate buffer (pH 4.0)–1 M NaCl, and (iv) Tris-saline containing 30 mM octyl glucoside. Octyl glucoside extracts of virions were passed over immunoaffinity columns five times at 4°C, and the unbound material was used in the preparation of virosomes. The column was washed for 5 h with 20 mM Tris-hydrochloride (pH 7.5)–0.5 M NaCl–0.1% Nonidet P-40 (NP-40; BDH Chemicals, Ltd., Poole, England)–0.1 mM PMSF, and the bound material was eluted with 3 M KSCN (10). The eluted material was mixed with 0.1% NP-40 and dialyzed against 2 liters of Tris-saline containing 0.1% NP-40 for 18 h at 4°C. Samples of the extracts were passed over the column, and the eluates were prepared for SDS-polyacrylamide gel electrophoresis by the addition of 2× SDS extraction buffer.

Electron microscopy. Virosomes were adsorbed to HEp-2 cells grown in six-well dishes (10^6 cells per well) in binding buffer at 4°C for 1.5 h. The cells were washed with Tris-saline–1 mM $MgCl_2$–1 mM $CaCl_2$ and fixed immediately or incubated at 37°C for 10 or 30 min before washing and fixation with 2% glutaraldehyde–0.1 M *S*-collidine (pH 7.4) for 1 h at 4°C. The cells were scraped from the plastic dishes, pelleted, fixed in 1% osmium tetroxide buffered with *S*-collidine at pH 7.4, dehydrated with a gradient of ethanol and propylene oxide, and embedded in Epon (26). Sections were cut on a Porter-Blum microtome and poststained with uranyl acetate and lead citrate before examination in a Siemans Elmiskop 102 microscope.

SDS-polyacrylamide gel electrophoresis and silver staining. Samples were prepared for electrophoresis in SDS extraction buffer, and the proteins were denatured by boiling for 5 min shortly before being loaded onto 8.5% polyacrylamide gels cross-linked with *N,N'*-diallyltartardiamide as described previously (13). Gels were infused with PPO (2,5-diphenyloxazole) by the procedures of Bonner and Laskey (3) and placed in contact with Cronex medical X-ray film. Gels were silver stained with a kit from Bio-Rad.

RESULTS

Extraction of virion glycoproteins and reconstitution with lipids. HSV-1(HFEM)syn virions were purified by the method of Spear and Roizman (40) as modified by Cassai et al. (7) and extracted with octyl glucoside. The virion glycoproteins, gC, gB, gE, and gD, and a nonglycosylated virion polypeptide, VP16, were selectively solubilized (Fig. 1). In other experiments, it was found that over 90% of gC, gB, and gD were solubilized by octyl glucoside, based on electrophoretic analyses of SDS-solubilized proteins present in samples of the octyl glucoside-solubilized material and octyl glucoside-insoluble residue (data not shown). The detergent-solubilized polypeptides (100 to 300 μg) and any nondialyzed viral

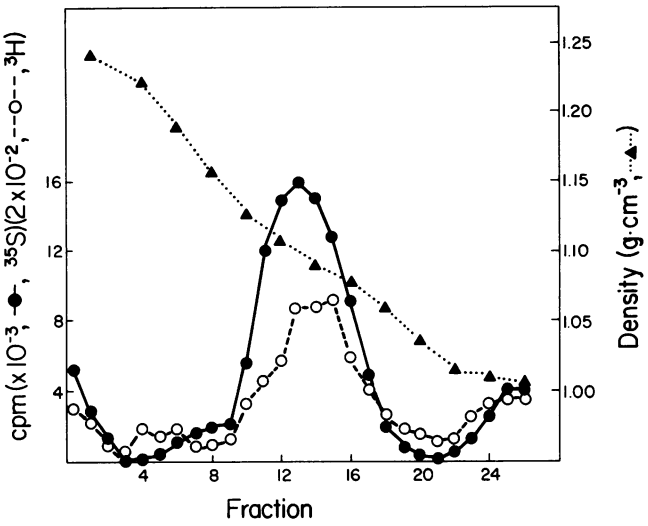

FIG. 2. Equilibrium sedimentation profiles of [^{35}S]methionine- and ^{3}H-labeled lipid virosome preparations. Virosomes labeled with [^{35}S]methionine or ^{3}H-lipids and prepared as described in the text were layered on top of 20 to 50% sucrose gradients containing Tris-saline–1 mM $MgCl_2$. The gradients were centrifuged at 25,000 rpm in a Beckman SW50L rotor for 18 h at 4°C. Fractions were collected from the bottom (fraction 1) and counted. The profiles shown are from two gradients centrifuged in the same rotor and processed under the same conditions. Density measurements were performed with a Bausch & Lomb densitometer.

lipids were reconstituted along with phosphatidylcholine (150 μg/100 μg of viral protein) into lipid vesicles or virosomes upon removal of the detergent by dialysis (14, 34). Very little or no degradation of viral polypeptides occurred during this procedure (Fig. 1). In addition, it was found that greater than 85% of the [^{35}S]methionine-labeled envelope proteins were incorporated into material which could be pelleted at 80,000 × *g* for 1 h. Unlabeled polypeptides from virosomes concentrated by pelleting were analyzed by SDS-polyacrylamide gel electrophoresis, followed by silver staining (Fig. 1). The pattern of stained polypeptides was very similar to that detected in fluorograms of [^{35}S]methionine-labeled polypeptides, although some additional bands were detected.

Virosome preparations labeled with [^{35}S]methionine in the viral polypeptides or with ^{3}H in the lipids were centrifuged to equilibrium on 20 to 50% sucrose gradients to determine the extent of incorporation of labeled polypeptides into membrane structures and the density of these structures (Fig. 2). Over 80% of the ^{35}S and ^{3}H labels were incorporated into a broad peak of material, with an average density of ca. 1.1 g/cm^3. Phosphatidylcholine vesicles containing no viral glycoproteins were found to have a much lower density remaining near the top of the gradient (data not shown).

To investigate whether the glycoproteins and VP16 were oriented in virosomes as in the membrane of intact HSV-1 virion, virosomes and virions were treated with a mixture of the proteases, trypsin, and chymotrypsin, and the proteolytic patterns were compared (Fig. 3). The conditions used here have been shown in other experiments to degrade cell surface HSV-1 glycoproteins to small fragments (data not shown). In contrast, chymotrypsin and trypsin treatment of microsomal membranes containing HSV-1 glycoproteins results only in the removal of small peptides (ca. 3 to 8 kilodaltons [kd]) from gB and gD (29; Johnson and Spear, unpublished data). These small peptides are oriented to the cytoplasmic face of microsomal membranes and are presumably found on the inside surface of the virion envelope. Treatment of virosomes with chymotrypsin and trypsin at 50 μg/ml resulted in degradation of most of gB, VP16, and gD, suggesting that they are all exposed on the surface of the virosome.

gC was somewhat more resistant to proteolysis, suggesting the possibility that a portion of gC is oriented toward the interior of the virosome vesicles. However, if a portion of gC were oriented in this fashion, one might expect to remove the small, cytoplasmic peptide from gC and increase its mobility on SDS-polyacrylamide gels. No shift in the mobility of gC on protease treatment was observed. Inspection of the nucleotide sequence of the gC gene (12) suggests that from 7 to 10 amino acids could be removed from the carboxyl terminus of gC by chymotrypsin and trypsin. We cannot be sure that we would detect a shift in the mobility of gC if these few amino acids were removed. It should be noted that gC appears to be relatively resistant to proteolysis in virions as well as in virosomes (Fig. 3), perhaps because gC may be more highly glycosylated than the other glycoproteins (24, 46).

When virions were treated with trypsin and chymotrypsin at 50 μg/ml, gC, gB, and gD were proteolyzed without evidence of degradation of internal polypeptides such as VP5. Interestingly, VP16, which was sensitive to proteolysis in the virosomes, was not proteolyzed in virions. Therefore, gB, gD, and a fraction of the gC extracted from virions were reconstituted into virosomes in an orientation indistinguishable from that in virions by this test. However, VP16 is

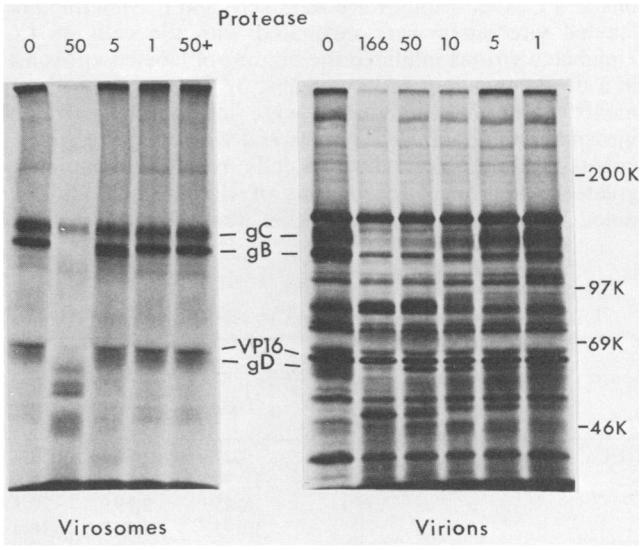

FIG. 3. Protease treatment of virosomes and virions. [^{35}S]methionine-labeled virosomes or virions were treated with a mixture of chymotrypsin and trypsin, each at 166, 50, 10, 5, 1, or 0 μg/ml for 15 min at 37°C. Proteolysis was stopped by the addition of bovine serum albumin (1 mg/ml), soybean trypsin inhibitor (250 μg/ml), chymostatin (200 μg/ml), aprotinin (500 U/ml), and 1 mM PMSF for 3 min at 22°C. Protease inhibitors were added to one sample of virosomes before chymotryspin and trypsin at 50 μg/ml were added for 15 min at 37°C (50+). An equal volume of 2× SDS extraction buffer was added, and the samples were immediately boiled for 5 min and frozen until shortly before electrophoresis in SDS-polyacrylamide gels.

clearly exposed in virosomes, in contrast to its protected location in the virion, and a portion of the gC may be oriented to the interior of the virosomes.

Binding of virosomes to cells. We tested the ability of virosomes containing the viral glycoproteins and VP16 and of liposomes devoid of viral polypeptides to bind to HEp-2 and Vero cells (Table 1). The viral polypeptides were extracted from purified virions of three different strains: HSV-1(HFEM)syn, a fusion-inducing mutant of HFEM, HSV-1(HFEM), and HSV-1(MP), a fusion-inducing mutant virus unable to produce gC. The virosomes prepared from each of these extracts bound equally well to HEp-2 and Vero cells, with ca. 30% of the [^{35}S]methionine label becoming cell associated in 1.5 h at 37°C. Interestingly, virosomes prepared with HSV-1(MP) extracts were able to bind to cells, as do MP virions, although both are missing gC. Phosphatidylcholine liposomes containing no viral polypeptides and labeled with ^{3}H-lipids bound poorly to both cell types. The kinetics of binding of HSV-1(HFEM)syn virosomes at both 4 and 37°C was similar to the binding kinetics of virions at 37°C (Fig. 4).

When [^{35}S]methionine-labeled material bound to cell monolayers was extracted with 2% SDS and electrophoresed on SDS-polyacrylamide gels, the cell-bound material was enriched for gB relative to the other glycoproteins (Fig. 5). The amounts of gC, gB, VP16, and gD bound to cells were quantitated (Table 2), and it was found that 45.9 and 36.1% of gB and VP16, respectively, were cell associated, whereas only 8.4 and 16.5% of gC and gD, respectively, were cell associated.

Inhibition of binding of virosomes by pretreating cells with virions. To determine whether pretreating cells with purified virions could block the binding of virosomes, HEp-2 cells were incubated with various concentrations of purified virions at 4°C, the virions were removed, and [^{35}S]methionine-labeled virosomes were incubated with the cells at 4°C. Unlabeled virions inhibited the binding of labeled virosomes in a dose-dependent fashion (Table 3). Competition experiments, in which the virions were left on the cells and virosomes were added or virions and virosomes were preincubated before being added to cells, resulted in somewhat greater inhibition of the binding of virosomes. It must be noted that although this inhibition was observed at 400 to 1,600 PFU per cell, the numbers of virus particles present were very likely much higher. These results suggest that virosomes may bind at the same sites as do HSV-1 virions.

TABLE 1. Binding of virosomes to HEp-2 and Vero cells

Source of viral envelope proteins	Cells to which virosomes bound[a]	cpm		% Bound
		Input	Bound	
HFEMsyn	HEp-2	9,478	2,908	30.7
		12,316	3,879	31.5
	Vero	8,859	2,595	29.3
		12,922	4,445	34.4
HFEM	HEp-2	1,225	441	36.0
MP	HEp-2	8,061	2,156	26.7
	Vero	9,123	2,627	28.8
No viral glycoprotein (^{3}H-labeled lipid)	HEp-2	2,586	137	5.3
	Vero	1,761	41	2.3

[a] Monolayers of HEp-2 or Vero cells grown in 24-well dishes were washed once with Tris-saline–1 mM $MgCl_2$–1 mM $CaCl_2$ containing bovine serum albumin at 100 μg/ml (binding buffer). Virosomes were adsorbed in 200 μl of binding buffer at 37°C for 1.5 h. The virosomes were removed, and the cells were washed with Tris-saline–1 mM $MgCl_2$–1 mM $CaCl_2$ and then lysed with 200 μl of Tris-saline containing 2% SDS and 2% β-mercaptoethanol. The extent of binding was determined by counting unbound material and cell lysate dried on glass filters.

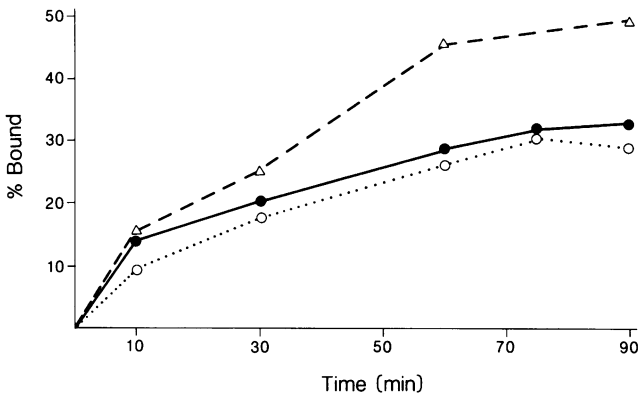

FIG. 4. Kinetics of binding of virosomes and virions at 4 and 37°C. [^{35}S]methionine-labeled virions or virosomes were adsorbed to HEp-2 cells in Tris-saline containing 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 100 μg of bovine serum albumin (binding buffer) per ml at 4 or 37°C for various times. Unbound virions or virosomes were then removed, and the cells were washed once in Tris-saline–1 mM $MgCl_2$–1 mM $CaCl_2$ and lysed in SDS extraction buffer. The extent of binding was calculated by counting the material not bound and the cell lysate. Symbols: △, binding of virions at 37°C; ●, binding of virosomes at 37°C; ○, binding of virosomes at 4°C.

Inhibition of binding of virosomes by antibodies and heparin. To investigate which of the viral glycoproteins were involved in the binding of virosomes to cells, we treated the virosomes with various polyclonal and monoclonal antibodies directed against the HSV-1 glycoproteins and then assayed the binding activities of the virosomes (Fig. 6). The IgG fraction of a rabbit antiserum, R no. 67, and a purified monoclonal antibody, I-59, both directed against gB, were very effective in inhibiting the binding of virosomes. This is, perhaps, not surprising given the observation that gB is the major component of the virosomes which become cell associated (Fig. 5, Table 2). The IgG fraction of a rabbit antiserum prepared against affinity-purified gD (10), three purified monoclonal antibodies directed against gD, and a purified monoclonal antibody directed against gC inhibited the binding of virosomes to a lesser extent. Purified mouse immunoglobulin was without effect. In addition, heparin, which is known to inhibit the binding of HSV-1 and HSV-2 virions (15, 31, 41, 43), had a strongly inhibitory effect on the binding of virosomes.

Binding of virosomes depleted of gB or gD. To examine the effect of removing gB or gD from the virosomes on the ability of the virosomes to bind to cells, we selectively adsorbed gB or gD from octyl glucoside extracts, using immunoaffinity columns before preparation of the virosomes. Most of the gB and gD could be removed by repeatedly passing octyl glucoside extracts over columns containing an anti-gB monoclonal antibody, I-59, or an anti-gD monoclonal antibody, II-436, coupled to Sepharose (Fig. 7). We also observed a partial depletion of VP16 when extracts were passed over the anti-gD affinity column. The adsorbed glycoproteins could be quantitatively eluted with 3 M KSCN (Fig. 7) as described by Eisenberg et al. (10). Virosomes prepared with extracts depleted of either gB or gD were then tested for their ability to bind to HEp-2 cell monolayers (Fig. 7). For both the gB- and gD-depleted

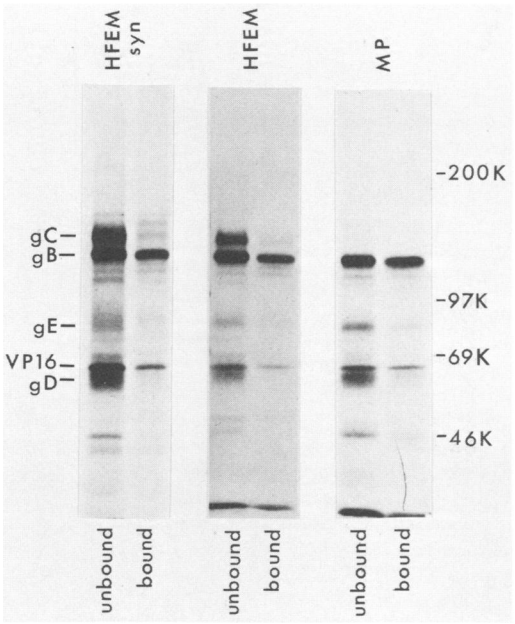

FIG. 5. Analysis of polypeptides in virosomes bound to cells and in unbound virosomes. Virosomes prepared from [^{35}S]methionine-labeled HSV-1(HFEM)syn, HSV-1(HFEM), or HSV-1(MP) extracts were bound to HEp-2 cells as described in Table 1, footnote *a*. Fractions which did not bind to cells (unbound) were diluted with an equal volume of 2× SDS extraction buffer, and cell-associated fractions (bound) were diluted with an equivalent volume of 1× SDS extraction buffer. Both fractions were boiled and electrophoresed on SDS-polyacrylamide gels.

virosomes, the fraction of radiolabel bound to cells was less than that for virosomes containing all the envelope components. The loss in binding activity was particularly pronounced for the gB-depleted virosomes. These results are consistent with those obtained on treatment of virosomes with anti-gB and anti-gD antibodies, suggesting a requirement for gB and, to a lesser degree, gD in the binding of virosomes to cells. We are presently preparing virosomes containing the eluted glycoproteins to assay their ability to bind to cells.

Electron microscopic examination of virosome-cell interactions. HEp-2 cells were incubated with virosomes at 4°C for 1.5 h, and the monolayers were fixed with glutaraldehyde or shifted to 37°C for 10 or 30 min before fixation. In samples in which the cells were fixed directly and prepared for electron microscopy, we observed virosomes, which appeared in a variety of diameters from 0.2 to 1.5 μm, bound to cell surfaces (Fig. 8A). In most cases the virosome binding occurred near regions of the cell membrane in which numerous processes were found. When cells were warmed to 37°C for 10 or 30 min before fixation, virosomes were in many cases still found bound to cell surfaces; however, in these samples virosomes also appeared in coated pits (Fig. 8B) and endosomes. In some cases we observed that the virosome membrane had fused with cell surface membranes, as indicated by continuities between these membranes (Fig. 8C and D).

TABLE 2. Quantitation of gC, gB, VP16, and gD bound to cells treated with virosomes

Polypeptide[a]	Unbound (cpm)	Cell associated (cpm)	% Bound
gC	580	53	8.4
gB	623	529	45.9
VP16	333	188	36.1
gD	353	70	16.5

[a] Samples of unbound and cell-associated labeled polypeptides were obtained after the binding of HSV-1(HFEM)syn virosomes to HEp-2 cells. These samples were electrophoresed on polyacrylamide gels as described in the legend to Fig. 5. [^{35}S]methionine-labeled polypeptides were localized in the dried polyacrylamide gel by a fluorogram, the bands were excised and dissolved in 2% periodic acid, and the radioactivity was counted.

DISCUSSION

We have extracted the glycoproteins from HSV-1 virions and successfully reconstituted these polypeptides along with the nonglycosylated polypeptide, VP16, into lipid vesicles or virosomes. A majority of the extracted viral glycoproteins and VP16 became associated with lipid vesicles upon removal of the detergent, as evidenced by equilibrium sucrose gradient centrifugation. Orientation of the proteins in the virosomes, except for VP16 and a fraction of gC, appeared to be similar to that in virions. The virosomes exhibited activities characteristic of virions: binding to cells and fusion with cell membranes.

Several lines of evidence suggest that the binding of virosomes to cells is similar in some respects to the binding of virions. First, the kinetics and extent of binding of virosomes were very similar to the binding of virions at 37°C. Second, heparin, which is known to inhibit the binding to cells of HSV-1 and HSV-2 virions (15, 31, 41, 43), also inhibited the binding of virosomes. Third, pretreatment of cells with purified virions inhibited virosome binding, suggesting that virosomes and virions bind at the same sites. However, there are also differences between the binding of virosomes and virions. The fraction of virosomes which becomes cell associated at 4 and 37°C differs very little, whereas virions bind and penetrate more efficiently at 37 than at 4°C (M. Wittels, A. O. Fuller, D. C. Johnson, and P. G. Spear, unpublished data). Treatment of virosomes with anti-gB antibodies markedly inhibited the binding of

TABLE 3. Inhibition of binding of virosomes by HSV-1 virions

Pretreatment with virions (PFU)[a]	cpm		% Bound
	Input	Bound	
None	26,048	8,970	34.4
2×10^8	26,294	5,350	20.3
4×10^8	25,980	2,800	10.8
8×10^8	26,092	1,502	5.7
4×10^8; virions not removed[b]	26,864	744	2.8
4×10^8; virions + virosomes preincubated[c]	26,654	798	3.0

[a] HEp-2 cells grown in 24-well dishes (5×10^5 cells per well) were treated with the indicated amounts of purified HSV-1(HFEM)syn virions in 200 μl of binding buffer for 1.5 h at 4°C. The virus titer had been determined after purification but before concentration by centrifugation. The virus was removed, the cells were washed, and the binding of virosomes was assayed as described in Table 1, footnote *a*, except that the binding was carried out in 220 μl for 1.5 h at 4°C.

[b] HEp-2 cells were treated with 10^8 PFU of virus in 200 μl of binding buffer for 1.5 h at 4°C, and virosomes in 20 μl were then added directly to the cells without removing the virus for an additional 1.5 h at 4°C.

[c] Virions (10^8 PFU) and virosomes were preincubated in 220 μl of binding buffer for 1.5 h at 4°C and then added to cells for 1.5 h at 4°C.

virosomes to cells, and anti-gD antibodies inhibited the binding to a lesser extent. In contrast, Fuller and Spear (manuscript in preparation) have recently found that anti-gD antibodies are better able to block the binding of virions to cells than are anti-gB or anti-gC antibodies in experiments carried out at 4°C. It is difficult to gauge the relative importance of either gB or gD in the binding reactions from these data alone, however, because steric hindrances brought about by the binding of antibody molecules to one glycoprotein may interfere with other glycoproteins. In addition, the viral glycoproteins present in the membranes of virosomes may be distributed in a very different fashion than glycoproteins found in the virion envelope.

The finding that virosome material bound to cells at 37°C was enriched for gB, and possibly also for VP16, suggests that either the virosomes as prepared were heterogeneous with respect to protein composition or that interaction of virosomes with cells led to a redistribution of proteins between the cell-bound and unbound fractions. For example, the proteins of virosomes bound to or fused with cells could become nonrandomly distributed within each virosome, and portions of each virosome depleted for gB and

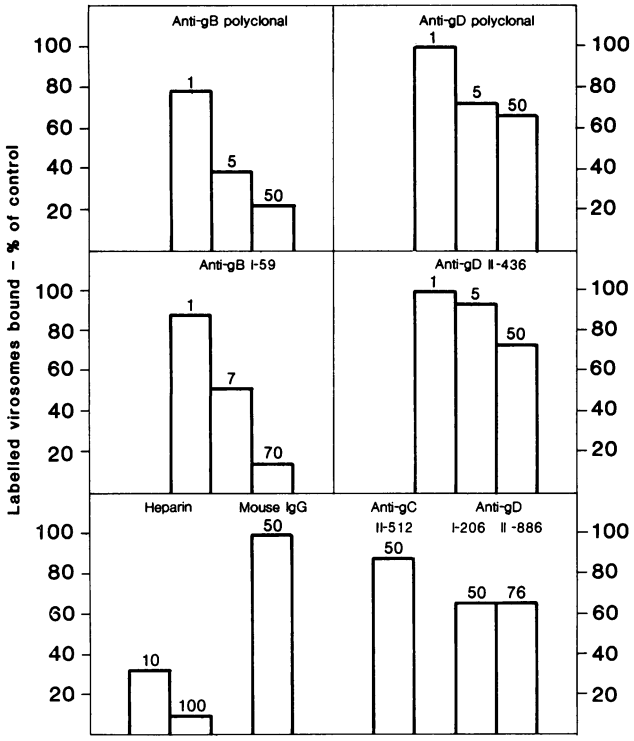

FIG. 6. Inhibition of binding of virosomes by antibodies and heparin. Polyclonal and monoclonal antibodies were purified as described in the text. For each sample, antibody or heparin was preincubated with virosomes (2 μg of viral protein) in 200 μl of binding buffer for 1.5 h at 4°C and then incubated with HEp-2 cells grown in 24-well dishes for 1.5 h at 37°C. The amount of antibody or heparin used (in micrograms per 200 μl) is shown above each bar. The extent of binding of virosomes was determined as described in Table 1, footnote *a*, and compared with binding of virosomes which were not treated with antibody or heparin (control binding, for purposes of comparison, taken to be 100% here was reproducibly ca. 30% of input radioactivity). The anti-gB polyclonal antibody used was R no. 67 (23). The anti-gD polyclonal antibody was described by Eisenberg et al. (10).

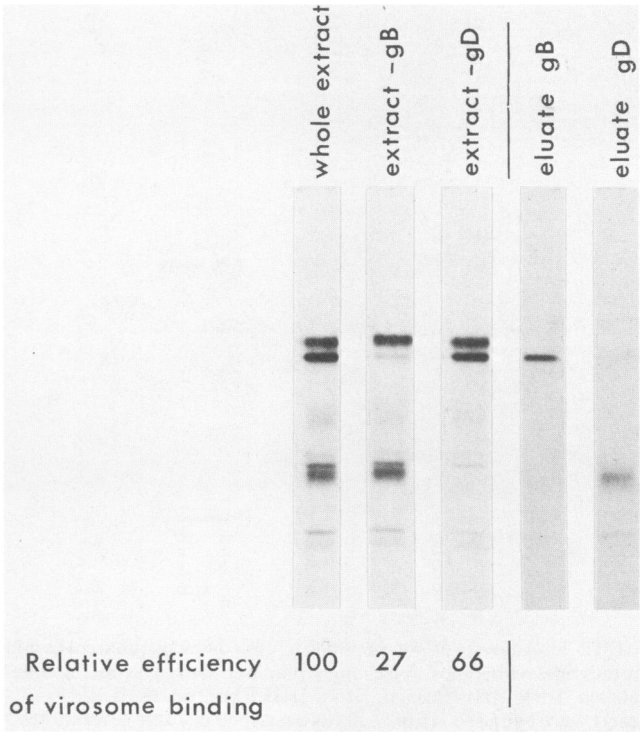

FIG. 7. Binding of virosomes prepared with octyl glucoside extracts depleted of gB or gD. Octyl glucoside extracts of [^{35}S]methionine-labeled HSV-1(HFEM)syn virions were passed over either an anti-gB or an anti-gD affinity column. The affinity columns were washed with 20 mM Tris-hydrochloride (pH 7.5)–0.5 M NaCl–0.1% NP-40–0.1 mM PMSF and eluted with 3 M KSCN, and the eluates were dialyzed against Tris-saline containing 0.1% NP-40. Samples of the unfractionated extract, depleted extracts, and eluates were diluted with an equal volume of 2× SDS extraction buffer and analyzed on SDS-polyacrylamide gels. The remainder of the whole extract, extract-gB, and extract-gD were used to prepare virosomes, and the relative binding activities of these preparations were determined on HEp-2 cells as described in Table 1, footnote *a*.

VP16 could pinch off, to be released from the cell and unable to rebind. We cannot discriminate between the two possibilities mentioned above at present.

Regardless of whether the virosomes were heterogeneous with respect to protein composition, the finding that gB was enriched in the cell-associated fraction of virosomes and the results of the antibody-inhibition and -depletion experiments all implicate gB and, to a lesser extent, gD in mediating the binding of virosomes to cells. Whether VP16, which is probably not normally exposed on the surfaces of virions but may be exposed on the virosomes, contributes at all to the binding activity observed cannot be assessed at present.

The electron micrographs presented here suggest that HSV-1 virosomes can fuse with cell surface membranes. Although fusion was not quantitated in these experiments, we observed a significant number of virosomes apparently fused to cells when virosomes were incubated with cells at 37°C but not at 4°C. We are presently attempting to set up a quantitative assay for virosome-cell membrane fusion.

Demonstration that virosomes possess activities associated with virions is only a first step towards understanding how HSV glycoproteins function in this system. The capacity to alter the polypeptide and lipid composition of the virosomes offers real promise for identifying the glycopro-

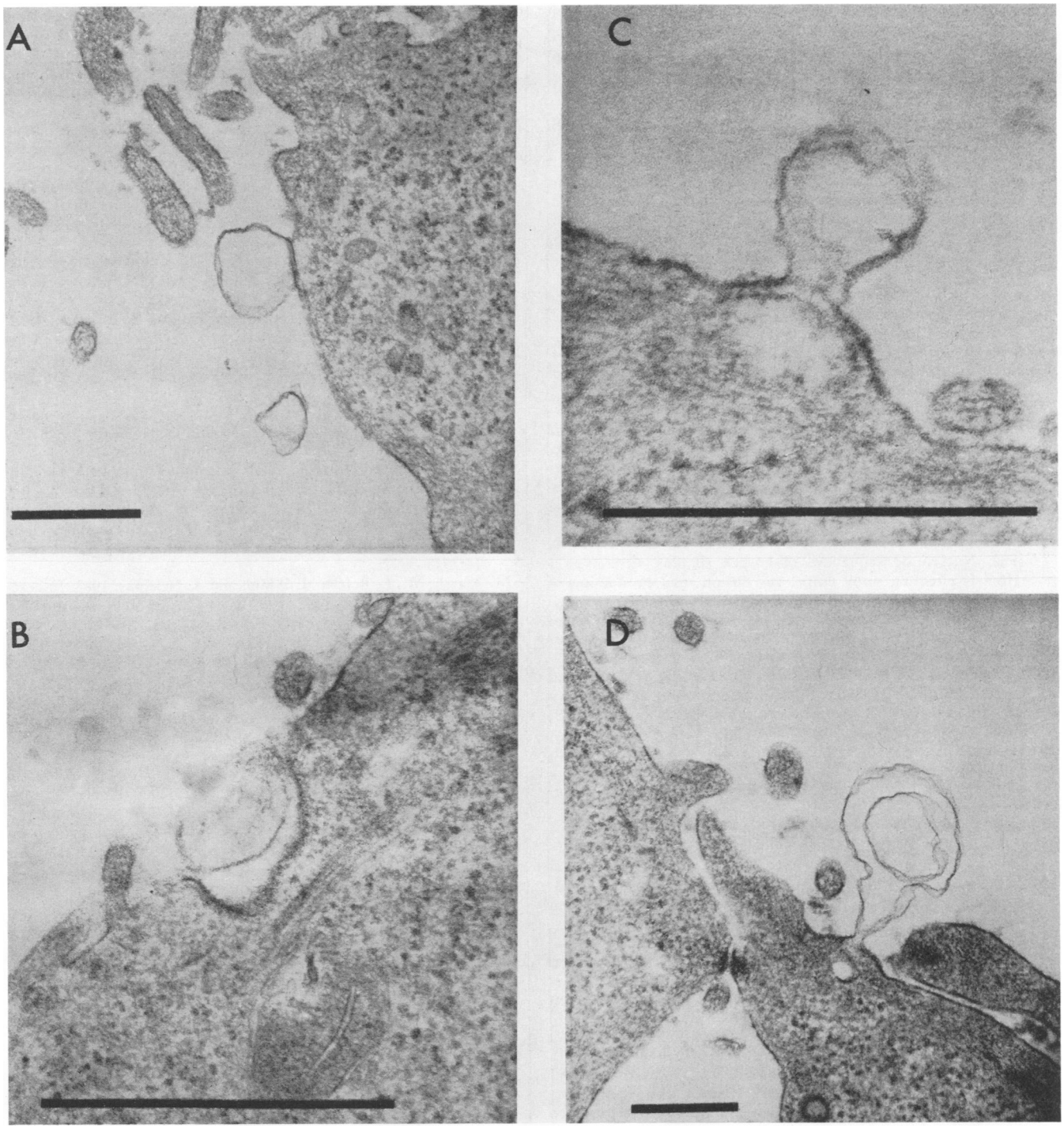

FIG. 8. Electron microscopy of virosomes bound to cells. Unlabeled virosomes were incubated with HEp-2 cells for 1.5 h at 4°C in binding buffer. Some monolayers were immediately washed and fixed with 2% glutaraldehyde (A), and others were incubated at 37°C for 10 min (B and C) or 30 min (D) before washing and fixation. The bars are ca. 1 μm.

teins that mediate various stages of the adsorption and entry process. We have already been successful in depleting gB and gD from virosome preparations, with observed reduction in binding activity of these virosomes. Experiments in progress with purified gB and gD, each alone or in combination in virosomes, should clarify whether binding activity is exhibited by each glycoprotein independently or only in association.

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