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Identification of Herpes Simplex Virus Type 1 Glycoproteins Interacting with the Cell Surface

JOACHIM E. KÜHN,[1]* MICHAEL D. KRAMER,[2] WOLFGANG WILLENBACHER,[1] ULRIKE WIELAND,[1] EVA U. LORENTZEN,[1] AND RÜDIGER W. BRAUN[1]

Institute of Medical Virology, University of Heidelberg, Im Neuenheimer Feld 324,[1] and Department of Dermatology, University Clinics of Heidelberg,[2] D-6900 Heidelberg, Federal Republic of Germany

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To investigate the interaction of herpes simplex virus type 1 (HSV-1) with the cell surface, we studied the formation of complexes by HSV-1 virion proteins with biotinylated cell membrane components. HSV-1 virion proteins reactive with surface components of HEp-2 and other cells were identified as gC, gB, and gD. Results from competition experiments suggested that binding of gC, gB, and gD occurred in a noncooperative way. The observed complex formation could be specifically blocked by monospecific rabbit antisera against gB and gD. The interaction of gD with the cell surface was also inhibited by monoclonal antibody IV3.4., whereas other gD-specific monoclonal antibodies, despite their high neutralizing activity, were not able to inhibit this interaction. Taken together, these data provide direct evidence that at least three of the seven known HSV-1 glycoproteins are able to form complexes with cellular surface structures.

The initial events in the infectious cycle of herpes simplex virus (HSV) are adsorption to the cell surface and penetration into the cytoplasm by fusion of its envelope with the plasma membrane (10, 25, 27). Both attachment and viral entry are thought to be mediated by glycoproteins (31). At least seven different species of glycoproteins, designated gB, gC, gD, gE, gG, gH, and gI, are located in the envelope of HSV (1, 4, 12, 13, 28, 31). Of these glycoproteins, only gB, gD, and gH are indispensable for viral growth in cell culture. Defective viral mutants lacking gB, gD, or gH adsorb to the cell surface; however, they are unable to penetrate into the cytoplasm (6, 8, 11, 20, 21, 24, 35). HSV glycoproteins indispensable for virus attachment have not yet been identified. Previous studies, however, suggest a role of gC, gB, and gD in viral attachment (9, 15). Binding sites necessary for adsorption of HSV have been found to be rather abundant ($\geq 10^4$ per cell) and are present on a wide variety of mammalian cells (14). Although the exact chemical structure of these cellular receptors is unknown, a heparinlike structure seems to play a critical role in viral attachment (39). Furthermore, the presence of a cellular receptor essential for the initiation of penetration has recently been postulated (14). The consumption of specific receptor sites may also play a role in the prevention of reinfection of cells already infected with HSV (5, 16, 26). Thus, the available data suggest that attachment and entry of HSV probably are mediated by multiple interactions between viral glycoproteins and cellular surface components.

The chemical derivatization of macromolecules with biotin has been shown to be a valuable tool in the analysis of interactions between receptor molecules and their ligands, as recently reviewed by Wilchek and Bayer (38). To mention just a few advantages of this system, biotin residues can be selectively introduced into a number of different chemical groups, such as amines, imidazoles, phenols, sulfhydryls, and aldehydes. In addition, biotinylation is normally achieved under mild chemical conditions and therefore generally will not interfere with the biological functions of a given macromolecule (2, 37). Furthermore, the extremely high affinity of biotin or biotinylated molecules for avidin ($\geq 10^{15}$ M^{-1}) greatly facilitates the isolation of complexes containing biotinylated molecules. We have adopted this experimental approach to the analysis of complex formation between ^{14}C-labeled HSV type 1 (HSV-1) envelope proteins and biotinylated cellular surface components in vitro. The results of these studies identify HSV-1 structural proteins interacting with cell surface components as gC-1, gB-1, and gD-1.

MATERIALS AND METHODS

Cells and viral strains. The cell lines used were as follows: human diploid fibroblasts (Wi-38), human epithelioma (HEp-2) cells, African green monkey kidney (RC-37) cells, rat transformed Schwann (TSC) cells, rat neurinoma (N2A) cells, murine fibroblasts (L929), and murine mastocytoma (P815) cells.

HEp-2, Wi-38, TSC, N2A, and L929 cells were held in Dulbecco modified Eagle medium (Biochrom, Berlin, Federal Republic of Germany) supplemented with 10% fetal calf serum (FCS) (Biochrom), 100 U of penicillin per ml, and 100 μg of streptomycin (Biochrom) per ml at 37°C in a humidified atmosphere containing 5% CO_2. For growth of RC-37 cells, basal medium Eagle (Biochrom) with 10% FCS was used. P815 cells were cultured in RPMI 1640 (Biochrom) supplemented with 10% FCS.

The HSV strains HSV-1 ANG (23), a gC-gE double-negative variant of HSV-1 ANG (kindly provided by H.-C. Kaerner; 7), HSV-1 MP (17), HSV-1 Kos 321 (30), and HSV-1 tsJ12 (21) were used.

Virions from the supernatants of infected cell cultures were obtained as described by Spear and Roizman (32). Virions metabolically labeled with ^{14}C-amino acids and [^{14}C]glucosamine were prepared as previously described (18). Labeling of HSV-1 ANG virions with [^3H]thymidine and adsorption experiments were carried out essentially as described earlier (19).

Biotinylation of the cellular surface. To introduce biotin residues onto the surface of intact monolayer cells, we used biotinyl-ε-aminocaproic acid *N*-hydroxysuccinimide ester (biotin-X-NHS; Calbiochem, Frankfurt am Main, Federal

* Corresponding author.

Republic of Germany) (2, 3). After extensive washing of the cell monolayers with phosphate-buffered saline (PBS; pH 7.2), cells were incubated for 20 min at 4°C with soft agitation in PBS (pH 7.2) containing 0.5 mg of biotin-X-NHS per ml. The remaining free reagent was then blocked and removed by four washes with cold PBS (pH 7.0) containing 10 mM glycine. To inactivate the last traces of unreacted biotin-X-NHS, cells were incubated for another 4 h at 37°C in the appropriate culture medium supplemented with 10% FCS. Finally, the cell monolayers were washed twice with PBS (7.0).

Lysis of cells and virions. To obtain cellular membrane lysates, cells were harvested by agitation with glass beads, washed twice with ice-cold PBS (pH 7.0) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), suspended in 40 mM Tris hydrochloride (pH 7.0) containing 1 mM EDTA and 0.2 mM PMSF, and incubated for 30 min on ice. Insoluble material was washed thrice in the same buffer. Subsequently, lysis buffer consisting of 40 mM Tris hydrochloride (pH 7.0), 1 mM EDTA, 0.2 mM PMSF, and 10 mM 3[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS; Boehringer GmbH, Mannheim, Federal Republic of Germany) was added, cellular material was incubated with continuous agitation at 4°C for 30 min, and detergent-insoluble material was removed by centrifugation at 200,000 × *g* for 60 min in an SW60 rotor (Beckman, Heidelberg, Federal Republic of Germany). Detergent-soluble material was stored in small samples at −80°C until use.

Lysates of viral proteins were prepared by incubating purified virions in lysis buffer for 30 min on ice. Insoluble material was removed at 200,000 × *g* as described above, and the supernatants were stored at −80°C until use.

Adsorption of viral proteins to cellular surface components. Lysates of viral proteins and biotinylated cellular surface components prepared as described above were incubated under continuous agitation at 4°C in siliconized glass tubes. The final incubation volume was adjusted to 500 μl with lysis buffer. In a typical experiment, ^{14}C-labeled viral proteins corresponding to approximately 10^7 PFU were allowed to adsorb to biotinylated surface structures of approximately 10^6 lysed cells. To identify viral proteins interacting with the cellular surface in immunoblots, virus lysates corresponding to approximately 10^9 PFU were incubated with lysates of approximately 2×10^7 biotinylated HEp-2 cells.

In competition experiments with nonlabeled virions, lysates of biotinylated cells were preincubated with the appropriate viral lysates for 1 h at 4°C in lysis buffer under continuous agitation.

Effects of monospecific antisera and monoclonal antibodies against HSV glycoproteins were studied by preincubation of radioactively labeled viral lysates with antibodies for 1 h at 4°C under continuous agitation in lysis buffer. Concentrations of monoclonal antibodies and monospecific rabbit antisera used in competition experiments are given in Results.

Isolation of complexes on streptavidin agarose. After incubation of biotinylated cell lysates with radioactively labeled viral lysates, approximately 80 μl of streptavidin agarose (Sigma Chemical Co., St. Louis, Mo.) suspended in 200 μl of lysis buffer was added for another incubation period of 60 min at 4°C with continuous agitation. Finally, unbound material was removed by washing the streptavidin agarose four times vigorously with cold lysis buffer.

Gel electrophoresis, electroblotting, and autoradiographs. Complexes among viral and cellular proteins bound to streptavidin agarose were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was carried out essentially as described by Thomas and Kornberg (33). Briefly, streptavidin beads were resuspended in 80 μl of twofold SDS-PAGE sample buffer, boiled for 3 minutes, and pelleted for 5 min at 10,000 × *g*. The supernatants were loaded on discontinuous 10% polyacrylamide gels, and electrophoresis was performed at 4°C and a total of 1,200 V h^{-1}. After electrophoresis had been completed, proteins were transferred by electroblotting to polyvinyldifluoride membranes (Millipore, Eschborn, Federal Republic of Germany) as described by Towbin et al. (34). To obtain autoradiographs, membranes were subsequently dried and exposed to Kodak XAR-5 film.

Immunoblotting procedures. Immunoblotting with rabbit antisera and monoclonal antibodies was performed as previously described (19). The HSV-1 type-specific rabbit serum was purchased from Dako Diagnostica, Hamburg, Federal Republic of Germany. To detect viral proteins in immunoblots, rabbit antisera were used in a dilution of 1:100 and monoclonal antibodies were used at a concentration of approximately 1 μg/ml (unless otherwise indicated). Biotinylated cellular proteins were directly detected by blocking the membranes after electrotransfer for 2 h at 4°C in Tris-buffered saline (TBS) (pH 7.4) containing 5% nonfat dry milk powder and 2% bovine serum albumin. Subsequently, membranes were washed three times with TBS and incubated for another 2 h in TBS (pH 7.4) containing 1% bovine serum albumin and streptavidin-horseradish complexes (Amersham Buchler AG) diluted 1:1,000. After three washes with TBS, membrane-bound peroxidase activity as visualized with 4-chloronaphthol (Sigma)-H_2O_2.

Purification of ^{14}C-amino acid-labeled gD by affinity chromatography. Approximately 2 mg of monoclonal antibody IV 3.4. was coupled to 2 ml of AminoLink gel (Pierce, Bender & Hobein, Heidelberg) according to the instructions of the manufacturer. Subsequently, the gel was packed onto 5-ml columns (Pierce) and equilibrated with degassed lysis buffer. Lysates of purified HSV-1 virions labeled by ^{14}C-amino acids and obtained as described above were allowed to adsorb to the column for 2 h at 4°C, unbound material was removed by washing with cold lysis buffer, and proteins specifically bound were eluted with 0.1 M glycine hydrochloride HCl (pH 2.8)–0.5 M NaCl–10 mM CHAPS. Fractions of 500 μl were collected; protein-containing fractions (as determined by A_{280}) were pooled, neutralized by the addition of a small volume of 1 M Tris hydrochloride (pH 9.0), and dialyzed overnight against lysis buffer. The purity of each preparation was controlled by SDS-PAGE and autoradiography.

RESULTS

Effect of cell surface biotinylation on viral infectivity. To exclude a possible impairment of cellular functions in virus replication resulting from the biotin-X-NHS treatment, we analyzed the infectivity and adsorption kinetics of HSV-1 ANG on biotinylated HEp-2 cells in comparison with mock-biotinylated cells. The results of these preliminary experiments showed that the labeling protocol used in this study did not lead to a measureable reduction of adsorption (Fig. 1), infectivity, and viral progeny formation (data not shown).

Detection and identification of viral proteins forming complexes with cell surface components. Lysates of purified HSV virions were allowed to react with lysates of biotinylated HEp-2 cells; protein complexes were purified on streptavidin agarose columns and finally analyzed by SDS-PAGE and

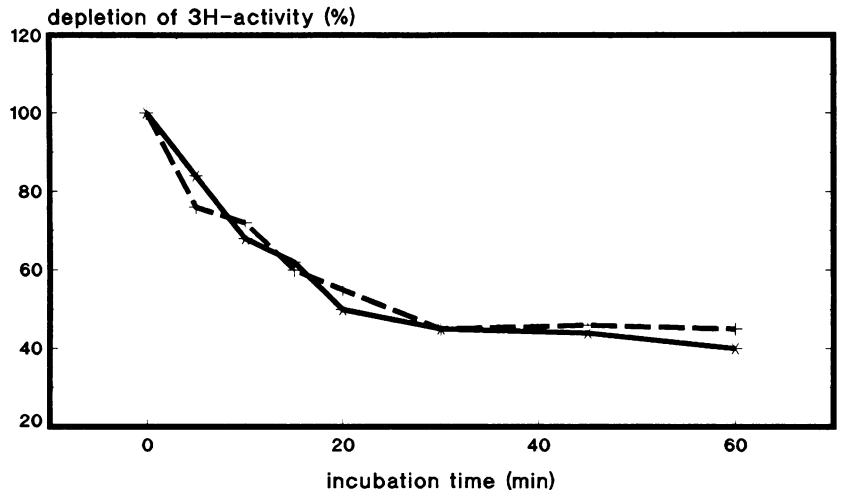

FIG. 1. Adsorption of purified [^{3}H]thymidine-labeled HSV-1 ANG virions to mock-biotinylated HEp-2 cells (——) and HEp-2 cells biotinylated with biotin-X-NHS (– – –). Experimental details are given in Materials and Methods.

immunoblotting as described in Materials and Methods. Using an HSV-1 type-specific rabbit serum, viral proteins with apparent molecular masses of 130, 120, and 60 kilodaltons were found to bind to cell surface components (Fig. 2a). By the use of monospecific and monoclonal antibodies, these viral proteins were identified as gC-1, gB-1, and gD-1, respectively (Fig. 2b to f). Control experiments with mock-biotinylated cells and with free biotin confirmed the specificity of the results obtained (Fig. 2a to e, lanes 3; Fig. 2a, lane 5).

Incubation of biotinylated cell lysates with lysates of ^{14}C-amino acid-labeled purified virions and subsequent analysis of complexes between viral and cellular components by SDS-PAGE and autoradiography gave results identical to those obtained by immunoblotting: the HSV-1 proteins gC, gB, and gD were found to interact with cell surface components (Fig. 3a).

In a modification of the experimental procedure usually performed in this study, cells and virions were lysed after adsorption of intact radioactively labeled virions to the surface of biotinylated cells; again, binding of gC, gB, and gD was observed (Fig. 3b and c). Specific binding of viral proteins to the cell surface was further shown by competition experiments in which the binding of radioactive viral proteins was blocked by the addition of an excess of nonlabeled viral proteins (data not shown).

Reactivity of viral proteins with freely soluble and detergent-soluble cellular components. To provide further evidence that the observed interaction takes place with cellular surface components, cellular lysates were fractionated in

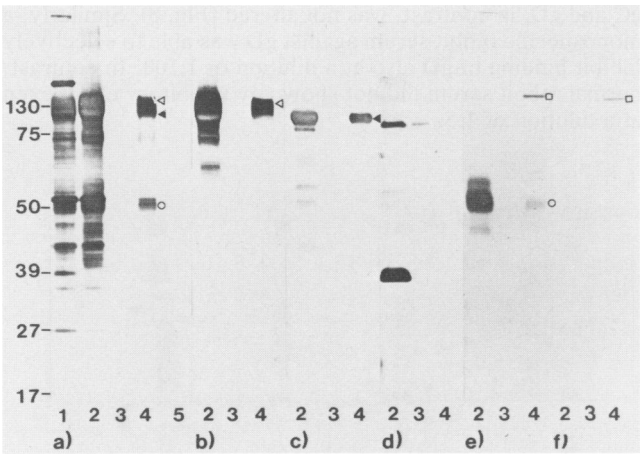

FIG. 2. Identification of viral proteins forming complexes with biotinylated cell surface components. Shown is detection of viral proteins by an HSV-1 type-specific rabbit serum (a), monoclonal antibody against gC (b), monoclonal antibody against gB (c), monospecific rabbit serum against gH (d), monoclonal antibody against gD (e), and rabbit normal serum (f). Lanes: 1, purified HSV-1 ANG virions; 2, lysates of HSV-1 ANG virions; 3, incubation of viral proteins with mock-biotinylated HEp-2 cells; 4, incubation of viral proteins with biotinylated HEp-2 cell lysates; 5, competition with free biotin. Experimental details are given in Materials and Methods. Specific binding of viral proteins is indicated (◁, gC; ◀, gB; ○, gD). □, Nonspecific recognition of viral proteins.

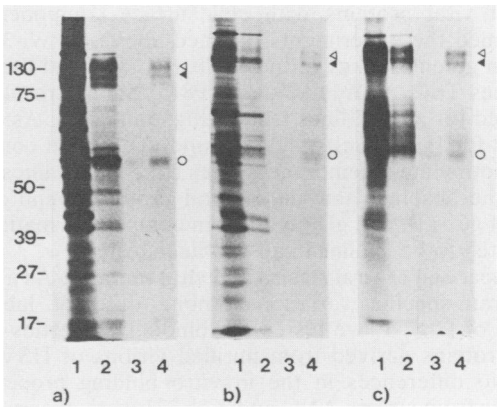

FIG. 3. Interactions of ^{14}C-labeled virions with cell surface components. (a) Complex formation of ^{14}C-amino acid-labeled virus lysates with biotinylated cell lysates; (b) adsorption of intact ^{14}C-amino acid-labeled virions to the cell surface before lysis; (c) adsorption of intact[^{14}C]glucosamine-labeled virions to the cell surface before lysis. Lanes: 1, purified HSV-1 ANG virions; 2, virus lysates; 3, adsorption to mock-biotinylated HEp-2 cells; 4, adsorption to biotinylated HEp-2 cells. Complex formation of gC, gB, and gD is indicated as in Fig. 2.

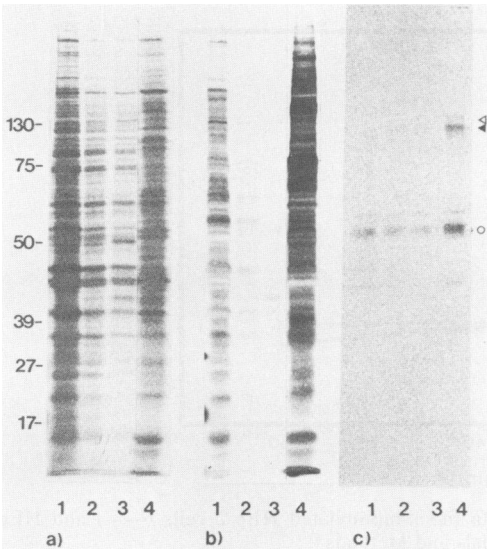

FIG. 4. Reactivity of viral proteins with freely soluble and detergent-soluble HEp-2 cell components. (a) Total protein stain with Coomassie blue G250; (b) detection of biotinylated cellular proteins; (c) complex formation among viral proteins and cellular components. Lanes: 1, freely soluble cellular proteins; 2, supernatant from first wash; 3, supernatant from second wash; 4, detergent-soluble cellular material. Experimental details are given in Materials and Methods; binding of viral proteins is indicated as in Fig. 2.

freely soluble and detergent-soluble material. As expected, the majority of biotinylated proteins was found in the strictly detergent-soluble fraction of cellular components, indicating that predominantly cell surface molecules were labeled by the biotinylation procedure (Fig. 4). Also, the reactivity of cellular components with viral proteins occurred predominantly in this detergent-soluble fraction (Fig. 4c). This result provides further evidence that this assay allows the analysis of complex formation between viral proteins and the cellular surface.

Binding of viral proteins to cells of different origins. To detect differences between various cell lines in the interaction of viral proteins with cell surface components, we performed the experiments outlined above on Wi-38 cells, African green monkey kidney cells (RC-37), and the rodent cell lines TSC, N2A, L929, and P815. Similar results were obtained for all cell lines tested (Fig. 5a and b). As demonstrated for HEp-2 cells, gC, gB, and gD formed complexes with biotinylated membrane lysates from all cell lines tested. Both the absolute amount of viral protein bound and the ratio of gC, gB, and gD involved in complex formation were estimated to be similar in all cell lines tested.

Comparison of viral strains and viral mutants. As a control for strain-specific variations among different laboratory strains of HSV-1, we tested the binding properties of viral glycoproteins derived from purified virions of HSV-1 Kos 321. No differences in the in vitro binding properties of proteins from strains ANG and Kos 321 were observed (Fig. 6a and d).

Viral mutants were used to determine whether cooperation between gC, gB, and gD was necessary for binding to the cell surface. Interestingly, the binding of gB and gD in our assay was not altered when a gC-gE double-negative mutant of HSV ANG was used. Similarly, lysates of tsJ12 virions grown at the nonpermissive temperature showed a nonreduced binding efficiency of gC and gD as compared

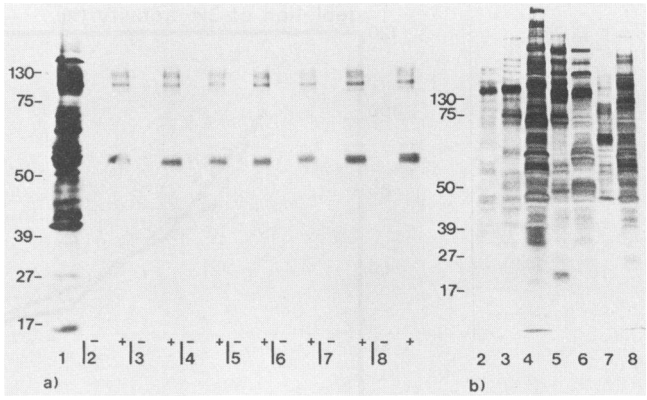

FIG. 5. Comparison of different cell strains. (a) Binding of viral proteins to mock-biotinylated cell lysates (−) and biotinylated cell-lysates (+); (b) detection of biotinylated cellular proteins. Lanes: 1, purified HSV-1 ANG virions; 2, African green monkey (RC-37) kidney cells; 3, human diploid fibroblasts (Wi-38); 4, human epithelioma (HEp-2) cells; 5, rat transformed Schwann (TSC) cells; 6, rat neurinoma (N2A) cells; 7, murine fibroblast (L929) cells; 8, murine mastocytoma (P815) cells.

with the wild-type strains (Fig. 6b and c). We conclude from these data that cooperation between gB and gC is not necessary for their in vitro binding activity. Furthermore, affinity-purified gD was capable of forming in vitro complexes with the cell membrane (Fig. 7). Thus, the interactions of gD, gB, and gC with the cell membrane appear to be independent of each other.

Competition with monospecific and monoclonal antibodies. We finally investigated whether the interaction of viral glycoproteins with the cell surface could be blocked by specific antibodies. Preincubation of viral lysates with a monospecific rabbit antiserum against gB (dilution 1:100) completely inhibited binding of gB. Complex formation of gC and gD, in contrast, was not altered (Fig. 8). Similarly, a monospecific rabbit serum against gD was able to selectively inhibit binding of gD also at a dilution of 1:100. In contrast, normal rabbit serum did not show any inhibitory effect even at a dilution of 1:5.

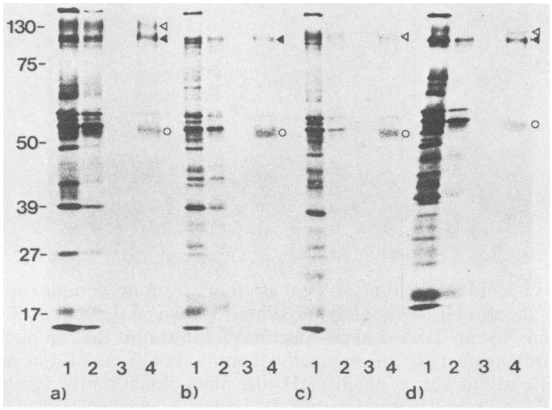

FIG. 6. Comparison of different HSV-1 strains. (a) HSV-1 ANG; (b) gC-gE double-negative derivative of HSV-1 ANG; (c) HSV-1 tsJ12 grown at the nonpermissive temperature; (d) HSV-1 Kos 321. Lanes: 1, purified virions; 2, viral lysates; 3, binding to mock-biotinylated HEp-2 cells; 4, binding to biotinylated HEp-2 cells. Complex formation of viral proteins is indicated as in Fig. 2.

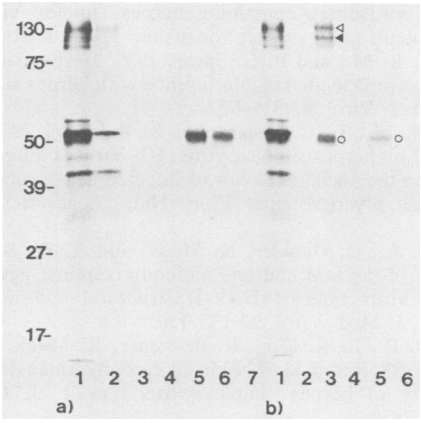

FIG. 7. Binding of purified ^{14}C-amino acid-labeled gD. (a) Purification of ^{14}C-amino acid-labeled gD from HSV-1 ANG virions by affinity chromatography. Lanes: 1, lysates of HSV-1 ANG virions; 2, material not specifically bound to the column; 3 to 8, elution of material specifically bound to the column. Experimental details are given in Materials and Methods. (b) Binding of purified gD to biotinylated cellular components. Lanes: 1, viral lysates; 2, reactivity of viral lysates with mock-biotinylated HEp-2 cells; 3, reactivity of viral lysates with biotinylated HEp-2 cells; 4, reactivity of purified gD with mock-biotinylated HEp-2 cells; 5, reactivity of purified gD with biotinylated HEp-2 cells; 6, addition of 1 mM free biotin during incubation with streptavidin beads. Binding of viral proteins is indicated as in Fig. 2.

We also tested a collection of monoclonal antibodies raised against gC, gB, and gD for their ability to block the interaction of glycoproteins with cell surface components as observed in our assay system. We were not able to identify a monoclonal antibody against gC or gB with the ability to block binding of gC or gB to the cell surface (Fig. 8). In contrast, the gD-specific monoclonal antibody IV 3.4. was able to inhibit binding of gD in concentrations of approximately 10 to 50 μg/ml. Such an effect was not found when other monoclonal antibodies against gD were assessed (Fig. 8).

Interestingly, antibody IV 3.4. had no measurable complement-independent neutralizing activity (data not shown). In contrast, other monoclonal antibodies directed against gD with a strong complement-independent neutralizing activity even at high concentrations had no effect on binding of gD in vitro. This result indicates that the virus-cell surface interaction monitored in our assay system is different from penetration events that were studied in different systems.

DISCUSSION

We have established an in vitro assay system that allows the analysis of formation of complexes between HSV-1 virion proteins and cell surface components. One of the advantages of this approach is the ability to selectively label cell surface components in vivo by biotinylation without detectable loss of cell viability and function. In a second step, this specific label allows the high-resolution purification of biotinylated molecules together with their specific ligands on streptavidin agarose. With this procedure, the HSV-1 proteins gC, gB, and gD were found to bind to cellular surface structures in vitro.

It is likely that our studies reflect the events necessary for virus adsorption rather than for virus penetration. Thus, in vitro analysis of complex formation among viral glycoproteins and cell surface components yielded identical results when lysates of cells and virions were used and when lysis of cells and virions was performed after adsorption of intact virions to the cell surface. Furthermore, gH, which has been reported to play an essential role in viral penetration and cell-to-cell spread (8, 11), showed no in vitro binding activity in our assay (Fig. 2). The specificity of the observed interaction was confirmed in a series of control experiments that included adsorption of virus to mock-biotinylated cells, competition with nonlabeled virions, and addition of free biotin to inhibit the binding of complexes to streptavidin beads. Furthermore, monospecific and monoclonal antibodies could specifically block the binding of gB and gD to cellular surface components. We believe that the interactions of gC, gB, and gD with the cell surface occur independently and do not involve complex formation. Thus, the binding ability of glycoproteins from viral mutants lacking gB or gC and gE was obviously not affected. In addition, radioactively labeled purified gD alone was able to bind in our in vitro assay. Moreover, antibodies against gB and gD blocked binding of the respective proteins in a specific manner.

Attempts to attribute the initial events in virus adsorption to a single HSV-1 glycoprotein have thus far not been successful. Earlier studies, however, in accordance with our results, suggested a role of gC, gB, and gD in viral attachment (9, 15). In contrast, it has been found that mutants of HSV lacking essential viral glycoproteins, i.e., gB, gD, and gH (8, 20, 21), normally adsorb to the cell surface. This may be explained by a possible replacement of functions of a missing glycoprotein by other glycoproteins. In addition, our finding that the nonessential glycoprotein gC may be involved in viral attachment is in accordance with the recent findings in the pseudorabies virus system. In pseudorabies virus, the gC-homologous protein gIII mediates stable attachment of the virion to the cell surface (29, 40). Glycoprotein gIII-negative mutants of PRV show reduced titers,

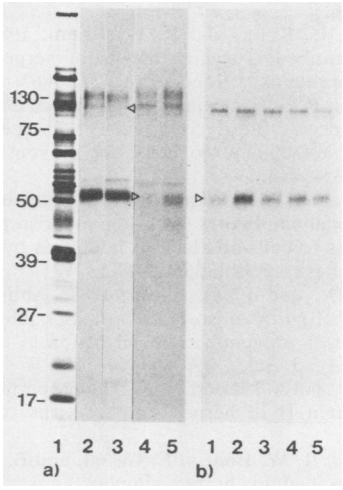

FIG. 8. Effects of monospecific rabbit sera and monoclonal antibodies on the in vitro binding activity of HSV-1 proteins. (a) Monospecific rabbit antisera. Lanes: 1, purified HSV-1 ANG virions; 2, rabbit normal serum (1:10); 3, gB-monospecific rabbit serum (1:100); 4, gD-monospecific rabbit serum (1:100); 5, gD-monospecific rabbit serum (1:1,000). (b) Monoclonal antibodies. Lanes: 1, IV3.4. (gD specific; 50 μg/ml); 2, 2c (gB specific; 50 μg/ml); 3, IE1 (gD specific; 50 μg/ml); 4, IV4.1. (gC specific; 50 μg/ml); 5, IV22.2. (gB specific; 50 μg/ml). Inhibitory effects are marked by Arrowheads.

although they are still infectious in cell culture (36). Therefore, difficulties in delineating single HSV-1 glycoproteins involved in viral attachment might be compounded by the fact that stable attachment to the cell surface is not essential for viral growth in cell culture. Further studies, however, will be needed to locate sites on viral proteins necessary for adsorption and to identify cellular structures involved in viral attachment.

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