JOURNAL OF VIROLOGY, Apr. 1996, p. 2138–2145 Vol. 70, No. 4
0022-538X/96/$04.00+0

Identification of Cell Surface Molecules That Interact with Pseudorabies Virus

AXEL KARGER AND THOMAS C. METTENLEITER*

Institute of Molecular and Cellular Virology, Friedrich Loeffler Institutes, Federal Research Centre for Virus Diseases of Animals, D-17498 Insel Riems, Germany

Received 3 October 1995/Accepted 21 December 1995

The alphaherpesvirus pseudorabies virus (PrV) has been shown to attach to cells by interaction between the viral glycoprotein gC and cell membrane proteoglycans carrying heparan sulfate chains (HSPGs). A secondary binding step requires gD and presumably another, hitherto unidentified cellular receptor. By use of a virus overlay protein binding assay (VOPBA), cosedimentation analyses, and affinity chromatography, we identified three species of cell membrane constituents that bind PrV. By treatment with EDTA, peripheral HSPGs of very high apparent molecular mass (>200 kDa) could be extracted from Madin-Darby bovine kidney cells. Binding of PrV to these HSPGs in the VOPBA was sensitive to enzymatic digestion with heparinase or papain. Cosedimentation analyses indicated that binding between PrV and high-molecular-weight HSPG depended on the presence of gC in the virion. In addition, adsorption of radiolabeled PrV virions to cells could be inhibited by the addition of purified high-molecular-weight HSPG. By using urea extraction buffer, a second species of HSPG of approximately 140 kDa could be solubilized. Binding of PrV to this HSPG in the VOPBA was also dependent on the presence of heparan sulfate, since reactivity was abolished after suppression of glycosaminoglycan biosynthesis with $NaClO_3$ and after heparinase treatment. In addition to HSPG, in cellular membrane extracts obtained by treatment with mild detergent, a 85-kDa membrane protein was demonstrated to bind to PrV in the VOPBA and affinity chromatography. In summary, we identified three species of cell membrane constituents that bind PrV: a peripheral HSPG of high molecular weight, an integral HSPG of approximately 140 kDa, and an integral membrane protein of 85 kDa. It is tempting to speculate that interaction between PrV and the two species of HSPG mediates primary attachment of PrV and that the 85-kDa protein is involved in a subsequent attachment step.

Several alphaherpesviruses, such as herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) (52), varicella-zoster virus (54), pseudorabies virus (PrV) (36, 42), and bovine herpesvirus 1 (BHV-1) (39), as well as the betaherpesvirus human cytomegalovirus (7) and the gammaherpesvirus BHV-4 (51) attach to target cells by interaction of the virion with heparan sulfate-carrying proteoglycans (HSPGs) of the cell surface. In HSV-1, PrV, and BHV-1, the viral glycoprotein gC is primarily responsible for this interaction (10, 15, 28, 36, 39). Attachment of wild-type virus is significantly reduced by addition of competing heparin during the attachment process, by digestion of the target cell membrane with heparinase, or by the absence of gC in the virion. In PrV, gC^- mutants have been shown to be resistant to heparin inhibition (36). In addition, mutated murine L cells with defects in heparan sulfate biosynthesis can be infected with gC^- PrV with the same efficiency as wild-type cells, indicating that in PrV gC represents the only viral glycoprotein productively interacting with cell surface heparan sulfate during the attachment process (23). Interaction with other glycosaminoglycans (GAGs), e.g., chondroitin sulfate, appears to play a minor role, if any. Therefore, gC^- PrV initiates infection by a pathway which is independent of cell surface proteoglycans. In contrast, gC^- HSV-1 mutants remain sensitive to heparin competition and lack of cell surface GAGs because of an interaction between virion gB and cellular proteoglycans (1, 14).

Attachment of PrV to Madin-Darby bovine kidney (MDBK) cells is a biphasic process (55) requiring gC for an initial binding step which is sensitive to competition by exogenous heparin. For a second, heparin-resistant attachment, gD is necessary (22), presumably by interaction with a hitherto unidentified cellular receptor. A similar finding has been reported for BHV-1 (22). In HSV-1, cumulative evidence also indicates that gD is a receptor-binding protein (30). HSV-1 gD is required for stable virus attachment to the cell surface (11). A truncated water-soluble form of gD inhibits penetration but not adsorption of HSV-1 and binds to a limited number of sites on Vero cell surfaces (18). HSV-1 gD has been shown to bind to the mannose 6-phosphate receptor (4, 5), although the significance of this interaction for viral entry is unclear. The basic fibroblast growth factor (bFGF) receptor has also been described to act as an HSV-1 receptor (19). However, the results could not be reproduced, and the role of this protein in HSV-1 infection is questionable (37, 38, 44). Recently, a receptor-binding function has also been proposed for the BHV-1 gB, although a corresponding cellular receptor has not been identified (27).

Therefore, aside from cellular HSPG, no specific receptor for any alphaherpesvirus has been unequivocally identified. This could at least partially be due to the fact that attachment of alphaherpesviruses to cells is a complex process in which several components of the viral membrane interact with different cell membrane constituents. Examples of multiple interactions in ligand-receptor binding are known. For example, two distinct binding sites for the bFGF receptor in addition to a heparin-binding domain are present on bFGF protein. Interaction of all three receptor-binding domains with their cellular counterparts is required for mitogenic activity of bFGF (47). In human immunodeficiency virus type 1, the formation of a ternary complex between the viral glycoprotein gp120 and two proteins of the cell surface, i.e., CD4 and an unidentified HSPG, has been shown to occur (41).

* Corresponding author. Phone: 49-38351-7102. Fax: 49-38351-7151.

To identify possible cellular PrV receptors, we developed a virus overlay protein binding assay (VOPBA) for use with fractionated cell membrane extracts. These studies, as well as affinity chromatography on immobilized virion glycoproteins and cosedimentation analyses, identified three species of PrV-binding cell surface components which we propose play a role in the initiation of PrV infection of target cells.

MATERIALS AND METHODS

Cells and viruses. Wild-type PrV strain Ka (21) and an isogenic gC deletion mutant, PrV-gC$^-$ (43), were used. Wild-type PrV and PrV-gC$^-$ were propagated and radiolabeled on pig kidney (PSEK) cells. For titration, adsorption assays and preparation of cell extracts, MDBK cells were used.

Adsorption assay. Adsorption assays were performed as described earlier (22). PrV virions were labeled with [^{3}H]thymidine by the procedure of Kaplan and Ben-Porat (20) and purified in a sucrose step gradient as described previously (22). To test inhibition of attachment by anion-exchange chromatography fractions I, IIA, IIB, and IIB-H, [^{3}H]thymidine-labeled PrV (70,000 cpm) was mixed with the indicated amount of protein and diluted to a final volume of 60 μl in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) (PBS-A). After 1 h of incubation on ice, 50 μl of the mixture was added to 200 μl of PBS-A and used for the adsorption assay. [^{3}H]thymidine-labeled PrV preincubated in PBS-A served as a control. Only total binding was determined (22).

Anion-exchange chromatography. For anion-exchange chromatography (modified as described in references 13 and 53), a column of 5 ml of DEAE-Sephacel (Pharmacia, Freiburg, Germany) was washed with urea extraction buffer {7.2 M urea, 0.1% 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate (CHAPS), 50 mM sodium acetate-acetic acid (pH 5.8), 5 mM benzamidine, 0.1 mM *N*-ethylmaleimide, 0.2 mM phenylmethylsulfonyl fluoride} and subsequently with urea extraction buffer supplemented by 1 M NaCl and equilibrated in urea extraction buffer. Urea extracts from labeled (10^6 cpm) or unlabeled (5 to 10 mg of protein) MDBK cells were applied to the column at a flow rate of 0.5 ml/min. Unbound material was removed with a minimum of 10 column volumes of urea extraction buffer. Bound protein was eluted with a linear gradient from 0 to 1,000 mM NaCl in urea extraction buffer at a flow rate of 0.5 ml/min. Elution of proteins was monitored at 280 nm, and fractions of 0.5 ml were collected. When radiolabeled urea extracts were fractionated, aliquots of 50 μl were taken from each fraction and radioactivity was determined by liquid scintillation counting. Single fractions of the unlabeled material were dialyzed against deionized water, freeze-dried and analyzed by VOPBA.

Anion-exchange high-pressure liquid chromatography (HPLC) of EDTA-soluble membrane proteins of MDBK cells. The anion-exchange column (LKB 2133-500 Glas Pac column TSK DEAE 5 PW 8x75) was washed with 5 column volumes each of 50 mM Tris-HCl (pH 7.4), TN1000 (50 mM Tris-HCl [pH 7.4], 1 M NaCl), AC50 (50 mM sodium acetate-acetic acid [pH 4.5]), and again 50 mM Tris-HCl (pH 7.4) at a flow rate of 1.0 ml/min. For sample application and separation, a flow rate of 0.5 ml/min was used. EDTA extracts were applied to the column, and the column was washed with TN150 (50 mM Tris-HCl [pH 7.4], 150 mM NaCl) until baseline levels were monitored at 280 nm and then washed with AC50 until baseline was again reached. After equilibration with 5 column volumes of TN150, proteins were eluted by a linear NaCl gradient. Fractions of 0.5 ml were collected, concentrated, and desalted in Centricon-10 microconcentrators (Amicon, Beverly, Mass.). For analysis in the VOPBA and in cosedimentation and adsorption inhibition assays, buffer was changed to PBS and pools were concentrated to a protein content of approximately 1 mg/ml in Centricon-10 microconcentrators. For chemical degradation of GAGs with trifluoromethanesulfonic acid (9), aliquots of fraction IIB were diafiltrated with deionized water in Centricon-10 microconcentrators and freeze-dried.

Biotinylation of PrV virions. Purified PrV (22) was pelleted and resuspended in biotinylation buffer (50 mM Na_2CO_3-$NaHCO_3$ [pH 8.5], 150 mM NaCl; modified as described in reference 17) at a protein concentration of 1 mg/ml. After brief sonication, 20 μg of NHS-LC-Biotin (Pierce, Rockford, Ill.) was added per mg of PrV protein from a 10-μg/μl stock solution in dimethyl sulfoxide. The mixture was shaken for 1 h on ice, and the reaction was terminated by addition of Tris-HCl (pH 8.5) to a final concentration of 50 mM. Biotinylated virus was collected by ultracentrifugation (TLA45 rotor, 45,000 rpm, 4°C), washed once in PBS, and resuspended in PBS at a protein concentration of 2 mg/ml. After biotinylation, the titer of PrV was reduced by 50% at maximum as measured by plaque assay. Biotinylated virions were stored at −70°C.

Cosedimentation analysis. $Na_2{}^{35}SO_4$-labeled fraction IIB from the anion-exchange HPLC was diluted to 100 μl in PBS and clarified (TLA45 rotor, 45,000 rpm, 45 min, 4°C) to remove any precipitates. Thereafter, 100 μg of purified wild-type PrV or PrV-gC$^-$ virions was resuspended in the clarified solution. After 1 h of incubation on ice, the mixture was layered onto a 1-ml cushion of 40% sucrose in 10 mM Tris-HCl (pH 7.4)–1 mM EDTA in a test tube for a TLS55 rotor. After centrifugation (40,000 rpm, 1 h, 4°C), the supernatant was carefully removed, the pellet was resuspended in 50 μl of sample buffer, and proteins were separated by gel electrophoresis and then subjected to fluorography.

Electrophoresis and protein blotting. Standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (24) was used at an acrylamide concentration of 8%. All samples were incubated at 95°C for 5 min in a reducing sample buffer immediately before the electrophoretic run. After electrophoresis, proteins were blotted onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) by submarine blotting (49) for 16 h at 0.1 A in transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol).

Extraction of MDBK cells. To obtain peripheral membrane proteins, monolayers of 10^7 MDBK cells were washed with PBS lacking divalent cations (PBS-S) and incubated with 5 ml of PBS-EDTA (PBS-S, 1 mM EDTA) for 30 min at room temperature. Detached cells were sedimented by low-speed centrifugation (1,000 × *g*, 10 min, 4°C) and used for the preparation of biotinylated integral membrane proteins (see below). The supernatant was clarified by ultracentrifugation (50Ti rotor, 1 h, 40,000 rpm, 4°C) and concentrated, and the buffer was changed to TN50/150 in Centricon-30 microconcentrators, yielding the EDTA extract.

After treatment with EDTA (see above), remaining cell surface proteins were biotinylated as described previously (34), with the following modifications. Cells were resuspended in biotinylation buffer (50 mM Na_2CO_3-$NaHCO_3$ [pH 8.5], 150 mM NaCl) at 2×10^7 cells per ml and kept on ice for 15 min; 25 μg of NHS-LC-Biotin (stock solution of 10 mg/ml in DMSO; Pierce) was added per 10^7 cells. After 30 min of incubation on ice with occasional shaking, biotinylation was terminated by addition of NH_4Cl from a 1 M stock solution to a final concentration of 10 mM. After 10 min, cells were pelleted by low-speed centrifugation and washed three times with Tris-buffered saline (50 mM Tris-HCl [pH 7.4], 150 mM NaCl). Biotinylated surface proteins were extracted by resuspending 10^7 biotinylated cells in 1 ml of lysis buffer (1% CHAPS–1% Nonidet P-40–0.1% sodium deoxycholate–0.2 mM phenylmethylsulfonyl fluoride–10 mM *N*-ethylmaleimide in PBS) and incubating the mixture for 1 h on ice. Cells and cellular debris were removed by ultracentrifugation (TLA45 rotor, 45 min, 4°C, 45,000 rpm). The extract was concentrated to approximately 5 mg of protein per ml in Centricon microconcentrators.

For extraction of total membrane proteins, 10^7 MDBK cells were washed with PBS and then incubated at room temperature for 1 h with 10 ml of urea extraction buffer. Cells and cellular debris were removed by low-speed centrifugation, and the supernatant was cleared by ultracentrifugation (50Ti rotor, 45,000 rpm, 1 h, 4°C), resulting in the urea extract. Extraction with urea extraction buffer yielded approximately 5 mg of protein per 10^7 cells.

Enzyme reactions. For heparinase treatment, monolayers of MDBK cells grown in 24-well tissue culture dishes were washed with PBS-A, overlayed with 250 μl of PBS-A containing 2.5 U of heparinase, and incubated at 30°C for 2 h. Monolayers were then washed with PBS-A and used for an adsorption assay as described above. Aliquots of fractions from anion-exchange chromatography and anion-exchange HPLC containing 10 μg of protein were digested with 10 U of heparinase and 1 U of heparitinase per ml in a volume of 50 μl of PBS for 2 h at 30°C. For chondroitinase treatment, 1 U/ml was used. For papain digestion, 10 μg of protein of fraction IIB was diluted to 50 μl in papain buffer (100 mM sodium acetate-acetic acid [pH 6.2], 50 mM EDTA), a grain of cysteine-HCl was added, and the reaction was performed with 1 U papain at 56°C for 24 h. After 8 h, an additional unit of papain was added. All enzymes were purchased from Sigma, Deisenhofen, Germany.

Inhibition of GAG biosynthesis with $NaClO_3$. MDBK cells were passaged twice in SO_4-deficient minimum essential medium (MEM) supplemented with 10% dialyzed fetal calf serum and 30 mM $NaClO_3$. Cells were then incubated in SO_4-deficient MEM supplemented with 100 μCi of $Na_2{}^{35}SO_4$ (Amersham-Buchler, Braunschweig, Germany) per ml for 72 h. For chromatography or anion-exchange HPLC, labeled cells were extracted with urea extraction buffer or with PBS-EDTA as described for unlabeled cells. Control cells were passaged and grown in MEM containing 0.811 mM $NaSO_4$ and additional 30 mM NaCl.

VOPBA. Cell extracts were separated by SDS-PAGE and blotted to nitrocellulose. Unspecific binding was blocked by incubation with PBS-A for 1 h at room temperature followed by incubation in 5% dry milk powder in PBS for 1 h. Nitrocellulose sheets were washed in binding buffer (125 mM Tris-HCl [pH 7.0], 50 mM NaCl, 10 mM $MgCl_2$, 1% BSA) and overlaid with biotinylated PrV at a concentration of 10 μg/ml. After 16 h incubation at 4°C, unbound virus was removed by three brief washes with precooled binding buffer. Nitrocellulose sheets were then treated with streptavidin-biotinylated horseradish peroxidase complex (diluted 1:2,500 in binding buffer; Amersham) for 20 min at 4°C. After three washes with binding buffer, bound PrV was detected by using an Amersham ECL kit. Usually, exposure times of 1 min were sufficient.

Affinity chromatography of biotinylated integral membrane proteins on a PrV affinity matrix. For the preparation of a PrV affinity matrix, 10 mg of gradient-purified wild-type PrV virions were extracted with 10 ml of lysis buffer for 1 h on ice. Insoluble material was removed by ultracentrifugation (50Ti rotor, 49,000 rpm, 1 h, 4°C). The supernatant buffer was changed to coupling buffer (100 mM $NaHCO_3$, 1% Nonidet P-40, 500 mM NaCl; adjusted to pH 8.3 with HCl) by diafiltration in Centricon-30 microconcentrators. If precipitates were observed, they were removed by ultracentrifugation (see above). The clarified supernatant usually contained approximately 3 mg of protein. Coupling of the extracted PrV envelope proteins to CNBr-activated Sepharose 4B (Pharmacia) was performed

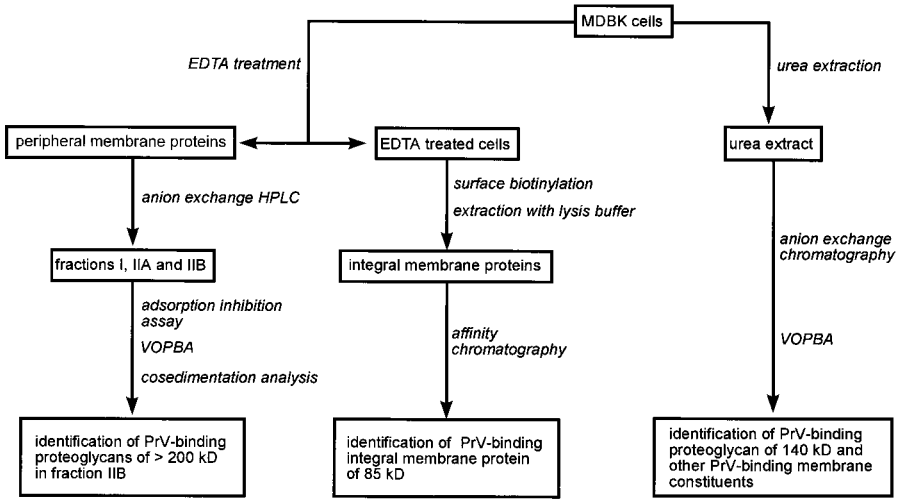

FIG. 1. Experimental design. Total membrane proteins of MDBK cells were extracted with urea extraction buffer, separated by anion-exchange chromatography, and analyzed by VOPBA, leading to identification of an approximately 140-kDa PrV-binding proteoglycan and additional PrV-binding membrane constituents. Peripheral membrane proteins were released by treatment of cells with EDTA and separated by anion-exchange HPLC. Analysis by adsorption inhibition assay, VOPBA, and cocentrifugation with purified virions identified >200-kDa PrV-binding HSPG(s). Integral membrane proteins were obtained from EDTA-extracted cells after surface biotinylation and extraction with detergent-containing lysis buffer. They were further analyzed by affinity chromatography on immobilized PrV envelope proteins, resulting in the demonstration of an 85-kDa PrV-binding protein.

as recommended by the manufacturer, with the modification that all buffers were supplemented with 1% Nonidet P-40 to solubilize integral membrane proteins. For one column, 3 mg of protein was coupled to 300 mg of CNBr-activated Sepharose 4B (1-ml gel bed volume). PrV envelope protein extracts were tested by immunoblotting in comparison with purified whole virions to ensure correct presence of viral glycoproteins gB, gC, gD, gE, and gH, for which specific immunological reagents were available (data not shown).

Biotinylated integral membrane proteins were fractionated by affinity chromatography immediately after extraction without prior freezing, which was found to lead to precipitation and loss of material. The buffer for samples containing 1 mg of biotinylated integral membrane proteins was changed to TC (50 mM Tris-HCl [pH 7.4], 0.1% CHAPS). Precipitates were removed by ultracentrifugation (TLA45 rotor, 45,000 rpm, 45 min, 4°C), and the volume was adjusted to 1 ml. After equilibration of the affinity matrix with TC, 1 mg of biotinylated integral membrane proteins in 1 ml of TC was applied, and the mixture was shaken in an overhead tumbler for 2 h at room temperature. Unbound material was removed with TC (five times with 5 ml each time) and TC150 (150 mM NaCl in TC; five times with 5 ml each time), and bound proteins were eluted by three sequential washes with 1 ml of TC150-Hep (50 μg of heparin per ml in TC150), TC500 (500 mM NaCl in TC), TC1000 (1,000 mM NaCl in TC), and 4 M urea. Eluates were concentrated, and buffer was changed to TC in Centricon-30 microconcentrators. Concentrated samples were subjected to SDS-PAGE and blotted to nitrocellulose, and unspecific binding was blocked by 0.1% Tween-20 in PBS. After addition of streptavidin-peroxidase, biotinylated proteins were detected by chemiluminescence.

RESULTS

Experimental design. The experimental approach is depicted in Fig. 1. For the isolation and analysis of all cell membrane constituents, urea extraction followed by anion-exchange chromatography was performed. To separate integral and peripheral membrane proteins, cells were treated with EDTA, which released cell surface structures bound by divalent cations. These molecules were further separated by anion-exchange HPLC and characterized by adsorption inhibition assay, cosedimentation analysis, and VOPBA. EDTA-treated cells were surface biotinylated and extracted in detergent-containing lysis buffer to solubilize integral membrane proteins, which were further analyzed by affinity chromatography on immobilized PrV envelope proteins.

Identification of PrV-binding proteoglycans in urea extracts of MDBK cells. MDBK cells were extracted in urea extraction buffer, a buffer especially suitable for the solubilization of proteoglycans (53), and extracts were fractionated by anion-exchange chromatography on DEAE-Sepharose in a linear NaCl gradient. The elution profile is shown in Fig. 2A. When cellular GAGs had been labeled with $Na_2{}^{35}SO_4$ prior to extraction, radioactively labeled material appeared in fractions 30 to 40, indicating that these fractions contained proteoglycans (Fig. 2A). Every second fraction was tested for PrV-binding proteins in the VOPBA (Fig. 2B). Biotinylated Pr virions bound to several protein species in fractions eluting at different salt concentrations. In particular, in fractions 22 to 28 eluting at medium salt concentrations, several PrV-binding proteins, including proteins of 85 and 90 kDa, were present. In fractions 30 to 40, which also contained the material which could be metabolically labeled with $Na_2{}^{35}SO_4$ (Fig. 2A), a protein of approximately 105 kDa was detected, as well as a smear of material ranging from 116 to 170 kDa with a maximum intensity at approximately 140 kDa. In addition, material with a molecular mass of >200 kDa was also detected in fractions 28 to 34. A similar profile was obtained when African green monkey (Vero) cells were analyzed under identical conditions (data not shown).

To identify proteoglycans among the PrV-binding cell membrane constituents, cells were incubated in medium containing $NaClO_3$, which inhibits biosynthesis of cellular GAGs, and extracted in parallel with the untreated cells analyzed as described above. As shown in Fig. 2C, inhibition of GAG biosynthesis did not affect or only marginally affected detection of proteins in fractions 22 to 28 as well as reactivity of the 105-kDa protein in fractions 30 to 32. In contrast, reactivity with the material resulting in the broad bands in Fig. 2B, as well as reactivity with the >200-kDa species, was completely lost. This result shows that in the VOPBA, biotinylated PrV is able to interact with cell surface GAGs and that the reactivity is lost when biosynthesis of GAGs is inhibited. This correlates with a decrease in attachment of radiolabeled Pr virions on chlorate-treated cells by 90% compared with untreated controls (data not shown). Reactivity in the VOPBA could also be abolished when corresponding fractions were digested with heparinase-heparitinase (data not shown).

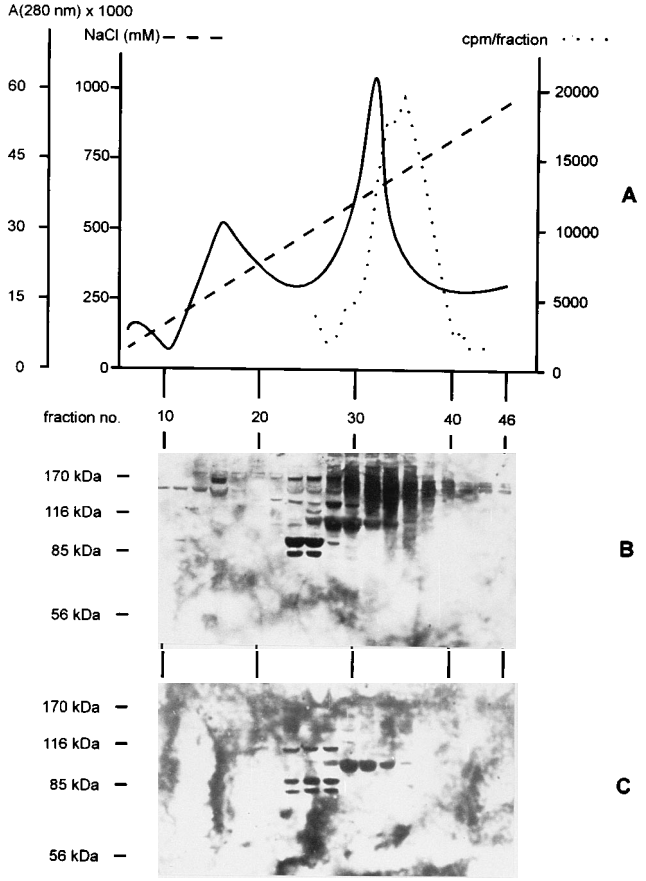

FIG. 2. Anion-exchange chromatography of urea extracts on DEAE-Sepharose. (A) Elution profile of proteins monitored at 280 nm (——) in a linear NaCl gradient (– – –). ·····, material which could be metabolically labeled by $Na_2{}^{35}SO_4$. (B) VOPBA of single fractions (every second fraction) from the chromatography with biotinylated PrV virions after separation in SDS-PAGE. Fraction numbers are given. Positions of molecular weight marker proteins in SDS-PAGE are indicated. (C) VOPBA as in panel B except that cells were grown in medium supplemented by $NaClO_3$ to inhibit GAG biosynthesis.

Identification of water-soluble peripheral PrV-binding proteins of MDBK cells. Treatment of MDBK cells with EDTA released radioactively labeled material when cells had been incubated in medium supplemented with $Na_2{}^{35}SO_4$, indicating the presence of proteoglycans in the EDTA extract. Fractionation by anion-exchange HPLC yielded three major fractions designated I, IIA, and IIB (Fig. 3A), of which fraction IIB contained all material that could be labeled by $Na_2{}^{35}SO_4$. To assay for biological activity, abilities of the different fractions to inhibit attachment of PrV to target cells were assayed. As shown in Fig. 3B, when different amounts of proteins of fractions I, IIA, and IIB were mixed with radiolabeled Pr virions and virus attachment was compared with that of an untreated control, only fraction IIB was able to inhibit attachment of PrV to MDBK cells in a dose-dependent manner, whereas fractions I and IIA showed no effect (Fig. 3B). After digestion of fraction IIB with heparinase and an additional chromatography (IIB-H), the inhibitory activity of fraction IIB was eliminated, indicating that the proteoglycans present in fraction IIB which are responsible for the inhibition of the attachment of PrV to MDBK cells carried heparan sulfate GAGs. Again, this result correlates with attachment studies on intact cells in which treatment of cell surfaces with heparinase reduced attachment by approximately two-thirds (36).

To further analyze fractions I, IIA, and IIB, 10 μg of protein from each fraction was separated by SDS-PAGE and analyzed by VOPBA. As shown in Fig. 4A, only fraction IIB contained material which was able to bind biotinylated PrV virions (lane 3), whereas no PrV-binding components were identified in fraction I (lane 1) or IIA (lane 2). Reactivity of fraction IIB was significantly reduced after treatment with heparinase-heparitinase (Fig. 4B, lane 2) compared with the untreated control (Fig. 4B, lane 1). In contrast, digestion with chondroitinase (Fig. 4B, lane 3) did not substantially impair binding of Pr virions. Combined heparinase-heparitinase-chondroitinase digestion (Fig. 5B, lane 4) resulted in loss of all reactivity, as was found after degradation with papain (Fig. 4B, lane 5), or trifluoromethanesulfonic acid (Fig. 4B, lane 6). In summary, these data show that proteoglycans with apparent molecular masses of >200 kDa, predominantly those carrying heparan sulfate, are able to specifically bind Pr virions in the VOPBA.

Since EDTA-detachable high-molecular-weight HSPGs are solubilized in the absence of detergent, it was possible to analyze their binding to intact PrV virions in suspension. After incubation with radiolabeled fraction IIB, wild-type PrV and PrV-gC$^-$ were sedimented through a 40% sucrose cushion, and radiolabeled PrV-binding proteins were identified by flu-

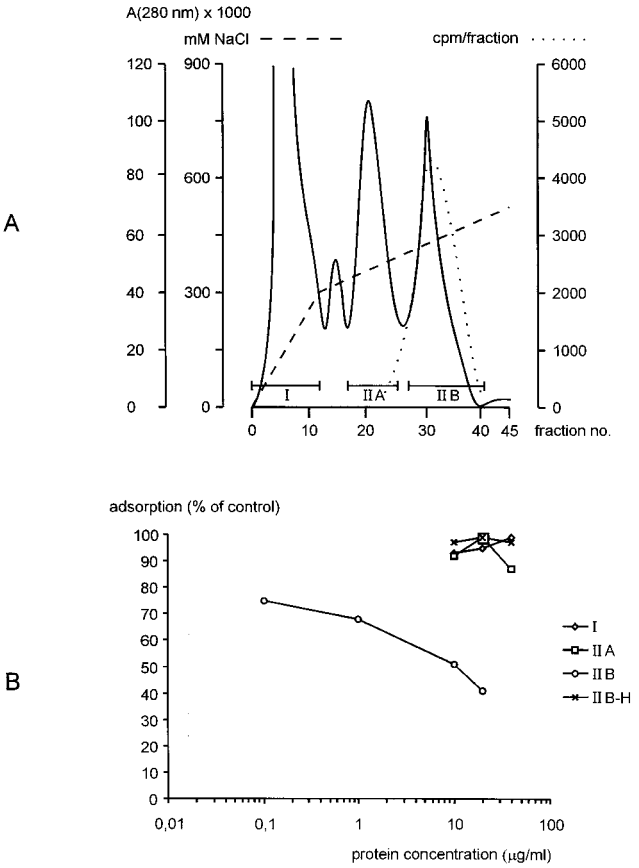

FIG. 3. Anion-exchange HPLC of EDTA extracts from MDBK cells. MDBK cells were extracted with PBS-EDTA, and the extracts were fractionated by anion-exchange HPLC. (A) Elution profile of proteins by the indicated NaCl gradient (– – –) was monitored at 280 nm (——). Three major fractions were obtained and designated I, IIA, and IIB. ·····, material which could be metabolically labeled with $Na_2{}^{35}SO_4$. The fractions were desalted and further characterized in an adsorption inhibition assay (B) using different amounts of protein. Virus adsorption is indicated in relation to the attachment of untreated control samples. IIB-H indicates fraction IIB after heparinase treatment.

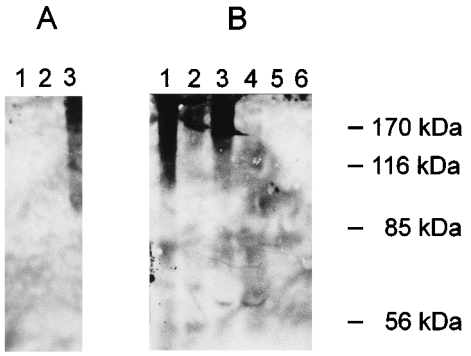

FIG. 4. Characterization of PrV-binding proteoglycans in fraction IIB by VOPBA. (A) Ten-microgram aliquots of protein of fractions I (lane 1), IIA (lane 2), and IIB (lane 3) were separated by SDS-PAGE and tested in the VOPBA. (B) Ten micrograms of fraction IIB (lane 1) was digested with heparinase-heparitinase (lane 2), chondroitinase (lane 3), heparinase-heparitinase and chondroitinase (lane 4), papain (lane 5), and trifluoromethanesulfonic acid (lane 6), separated by SDS-PAGE, and tested in the VOPBA.

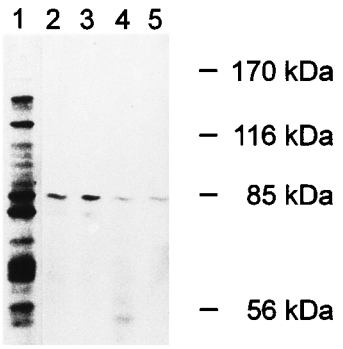

FIG. 6. Affinity chromatography of biotinylated integral membrane proteins from MDBK cells on a PrV affinity matrix. Membrane extracts from gradient-purified wild-type PrV virions were immobilized on Sepharose, and biotinylated integral membrane proteins (lane 1) from MDBK cells were applied. Unbound material was removed by repeated washes with TC and TC150. Bound proteins were sequentially eluted with TC150 supplemented with 50 μg of heparin per ml (lane 2), TC500 (lane 3), TC1000 (lane 4), and 4 M urea (lane 5). Eluates were collected, concentrated by ultracentrifugation, and separated by SDS-PAGE. After blotting to nitrocellulose, biotinylated proteins were detected after incubation with streptavidin-peroxidase by chemiluminescence.

orography after SDS-PAGE of the obtained pellet. As shown in Fig. 5, lane 2, the high-molecular-weight HSPG did indeed bind to wild-type Pr virions in suspension. In the presence of 50 μg of heparin per ml, reactivity of HSPG with Pr virions was abolished (Fig. 5, lane 4), which again correlates with data from attachment assays (22). In the absence of gC (Fig. 5, lane 3), HSPGs from fraction IIB were unable to bind to virions, which is in accordance with data from infectivity experiments using gC-negative PrV mutants and cell lines deficient in GAG biosynthesis (23). Labeled material present on top of the gel in lanes 3 and 4 most likely constitutes insoluble aggregates which pass through the sucrose cushion. Together, these data identify HSPG with a high apparent molecular mass of >200 kDa exhibiting characteristics predicted from biological assays.

Identification of an 85-kDa PrV-binding integral membrane protein. When urea extracts of MDBK cells were fractionated by anion-exchange chromatography and single fractions were analyzed by VOPBA, several proteins were found to be able to bind biotinylated PrV virions (Fig. 2B and C). For further characterization of integral PrV-binding membrane proteins, EDTA-extracted cells (see above) were surface biotinylated and extracted with detergent-containing lysis buffer. Solubilized integral membrane proteins (Fig. 6, lane 1) were then reacted by affinity chromatography with immobilized PrV envelope (glyco)proteins and sequentially eluted with buffer containing 50 μg of heparin per ml and 150 mM NaCl (Fig. 6, lane 2), 500 mM NaCl (Fig. 6, lane 3), 1 M NaCl (Fig. 6, lane 4), or 4 M urea (Fig. 6, lane 5). Eluates were analyzed for the presence of biotinylated proteins after SDS-PAGE and protein blotting. Under the conditions applied, a single protein species of 85 kDa was partially eluted by 50 μg of heparin per ml and 150 mM NaCl (Fig. 6, lane 2). Most of the remaining 85-kDa protein could be recovered by elution with 500 mM NaCl (Fig. 6, lane 3). Harsher elution conditions did not result in an increase in the 85-kDa protein, nor did they lead to recovery of significant amounts of other proteins. Only a weak protein band of 56 kDa was additionally observed in the 1 M NaCl eluate (Fig. 6, lane 4). Therefore, from the biotinylated cell surface proteins remaining after extraction of peripheral membrane constituents with EDTA, an 85-kDa protein which bound to immobilized PrV envelope proteins was identified.

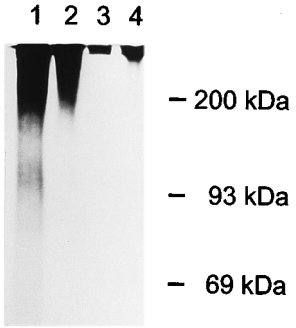

FIG. 5. Cosedimentation of PrV with radiolabeled peripheral proteoglycans extracted from MDBK cells. Fraction IIB of the EDTA extract was metabolically labeled with $Na_2{}^{35}SO_4$ (lane 1). After incubation with wild-type PrV (lane 2) or PrV-gC$^-$ (lane 3) virions for 1 h on ice, virions were sedimented through a 40% sucrose cushion. In lane 4, incubation of wild-type PrV with radiolabeled fraction IIB was performed in the presence of 50 μg of heparin per ml. Proteins that had bound to the virions were analyzed by SDS-PAGE and autoradiography.

DISCUSSION

Initiation of infection by alphaherpesviruses is thought to require a cascade of interactions between different viral and cellular membrane components (11, 35, 46). In HSV-1, PrV, and BHV-1, gC mediates primary attachment by interacting with cell surface components which consist of or contain GAG (15, 36, 39), in particular heparan sulfate (23, 45, 52). Whereas gC appears to be the only PrV virion envelope protein capable of interacting productively with heparan sulfate and thus leading to infection, in HSV-1 and BHV-1, gB is also able to bind cell surface GAG (6, 14). Since gC proteins are nonessential for replication of HSV-1, PrV, and BHV-1, gC$^-$ PrV has to initiate infection by a gC- and GAG-independent mechanism, whereas gC$^-$ HSV-1 and BHV-1 may still use a GAG-dependent attachment mechanism.

Although the analysis of cell lines of either hamster (CHO) or murine (L) origin deficient in GAG biosynthesis demonstrated the importance of these carbohydrates in alphaherpesvirus adsorption (12, 45), biochemical analyses have so far been limited to characterization of the requirements of the GAG for inhibition of virus attachment (31). In this respect, HSV-1 and PrV have been shown to differ (50), presumably as

a result of the additional heparin-binding function of HSV-1 gB. However, respective authentic cellular membrane constituents, i.e., proteoglycans, have not been identified so far.

Attachment of PrV, HSV-1, and BHV-1 to target cells is an at least biphasic process in which a primary binding step which is sensitive to competition by exogenous heparin converts into a secondary, heparin-resistant binding (22, 33). For PrV, the existence of two binding steps has first been demonstrated for a deletion mutant in which an internal portion of gC encompassing amino acids 157 to 290 had been removed (55). Although overall primary attachment appeared unaltered, binding of this mutant virus to cells was much more sensitive to competing heparin than that of wild-type PrV. A functional attachment domain has been mapped to the amino-terminal one-third of PrV gC (amino acids 25 to 157 [10]), indicating that both domains within gC mediate distinct interactions. In addition, glycoprotein D of PrV, HSV-1, and BHV-1 has subsequently been shown to be required for secondary stable attachment (22, 33). For several years gD has been proposed to interact with a cell surface protein on the basis of the finding that soluble gD can inhibit penetration of HSV-1 by binding to a limited number of sites on the cell surface (18). Recently, soluble truncated HSV-1 gD has been shown to bind to the mannose 6-phosphate receptor in a ligand blotting assay (5). Binding to this protein has also been suggested for varicella-zoster virus (54). However, the significance of this interaction is unclear. In a photoaffinity labeling study, purified BHV-1 and an anti-idiotypic antibody which mimics a BHV-1 gD epitope have been used to screen cell surfaces for BHV-1- or BHV-1 gD-binding proteins. A 60-kDa protein could thus be found only in bovine cell lines which are permissive for BHV-1 infection (48).

Since these studies indicated the interaction of several virion proteins with different cell surface molecules, we used two techniques, i.e., VOPBA (2, 8, 32, 40) and cosedimentation, which allow us to study the attachment of intact PrV virions to isolated cell membrane components, a prerequisite to detect virus-cell interactions involving more than one viral envelope protein. Standard procedures like affinity chromatography or precipitation of receptor-ligand complexes might fail under these circumstances, since experimental conditions (presence of detergents, low concentration of ligands, absence of tertiary structure, absence of lipid envelope) might not support such a complex process. Differential extraction led to the isolation of total, peripheral, and integral cell membrane components. Infectivity assays proved that biotinylation under our conditions did not decrease infectivity of the virus preparation by more than twofold, indicating that the topology of the viral envelope was not overtly disturbed. Our studies identified two species of HSPG which differ in molecular weight and membrane distribution. A >200-kDa HSPG can be released from the cell surface with EDTA, indicating that it represents a peripheral membrane constituent attached to the cell via divalent cations. Several proteoglycans with this property have been described (16). In addition, a presumably integral HSPG of ca. 140 kDa with the capability to bind PrV virions in the VOPBA has been identified.

Binding to HSPG was sensitive to heparinase or proteinase treatment, to competition by exogenous heparin, and to growth of cells in chlorate medium. In cosedimentation, PrV gC^- was deficient in binding to high-molecular-weight HSPG compared with wild-type PrV. Therefore, inhibition of reactivity in VOPBA and lack of cosedimentation with HSPG correlated with inhibition of attachment of PrV to intact cells, indicating that the conditions in the VOPBA reflect the situation with respect to attachment of PrV to cultured cells, at least regarding the parameters tested. Therefore, these data suggest that the two identified species of HSPG participate in the attachment of PrV. It is interesting that the 140-kDa HSPG was detected only after urea extraction of cells. Obviously, it was not solubilized by the mixture of mild detergents contained in the lysis buffer. Glycosylphosphatidylinositol (GPI)-anchored proteins have been reported to be localized in detergent-resistant membrane vesicles and are therefore relatively resistant to extraction with mild detergents (3), and it has been demonstrated that removal of GPI-anchored proteins from the cell surface by phosphoinositol-specific phospholipase C leads to a reduction in binding of HSV-1 to cells (25). Whether GPI-anchored HSPG also play a role in PrV attachment and whether the 140-kDa HSPG is indeed GPI anchored is currently under investigation.

Since interaction of virion gC with cellular HSPG probably represents only the first step in the infection of cells by wild-type PrV, cellular extracts were also analyzed for PrV-binding components other than HSPG. Among several proteins in urea extracts of cell membranes, a 85-kDa polypeptide was found to be capable of binding to biotinylated PrV virions in VOPBA. A protein of identical molecular mass could also be identified by affinity chromatography of cell membrane extracts obtained by mild detergents on immobilized virus envelope proteins. Part of the bound protein eluted in the presence of 50 μg of heparin per ml at physiological conditions (150 mM NaCl); part of the protein required 500 mM NaCl, which indicates an electrostatic interaction between virion protein(s) and the 85-kDa protein, which might thus act as an additional PrV receptor. We are currently analyzing whether this 85-kDa protein correlates with the common saturable receptor for PrV and HSV-1 found on Vero cells which is different from heparan sulfate (26).

The process of initiation of infection by alphaherpesviruses is still poorly understood. Glycoproteins gB, gC, and gD have been implicated in receptor binding (29, 35, 46), and various cellular virus-binding proteins have been described (4, 19, 52). On the basis of the currently available data, we hypothesize that the following events may occur during attachment and infectious entry of PrV. Interaction between virion gC and peripheral high-molecular-weight HSPG (capture receptor) leads to a primary binding of virion to target cells. This binding is stabilized through interaction of gC with the 140-kDa integral membrane HSPG, a process which might be defective in an internal PrV-gC^- mutant lacking amino acids 157 to 290 (55). Interaction of virion gD with another cell membrane component, perhaps related to the 85-kDa protein described here, then further strengthens binding of virus to target cells and initiates fusion between viral and cellular membranes. It needs to be emphasized that except for the initial conversion of heparin-sensitive to heparin-resistant attachment (22), it is unclear whether these postulated interactions occur sequentially or simultaneously.

It appears conceivable that even more viral protein-cellular receptor interactions might take place, and in the VOPBA several other protein species besides the >200- and 140-kDa HSPGs and the 85-kDa protein bound to intact biotinylated PrV virions, although the significance of these interactions remains unknown. In the VOPBA, the original conformation of the virion envelope is retained to a large degree, as shown by only a very modest reduction in viral infectivity after biotinylation. In contrast, fractionation of cell membranes leads to disruption of higher-order structure between cell membrane components. However, the availability of virus mutants devoid of either essential or nonessential virion envelope glycoproteins allows one to assay the contribution of single viral surface

proteins to the first steps of virus infection, and a combination of genetical approaches (1, 12) with the biochemical techniques described here will help to characterize the respective cellular reaction partners.

ACKNOWLEDGMENTS

This study was supported by grant Me 854/2-3 from the Deutsche Forschungsgemeinschaft and grant ERB-CHRX-CT92-0029 from the EEC.

REFERENCES

1. **Banfield, B., Y. Leduc, L. Esford, K. Schubert, and F. Tufaro.** 1995. Sequential isolation of proteoglycan synthesis mutants by using herpes simplex virus as a selective agent: evidence for a proteoglycan-independent virus entry pathway. J. Virol. **69:**3482–3489.
2. **Boyle, J., D. Weismiller, and K. Holmes.** 1987. Genetic resistance to mouse hepatitis virus correlates with absence of virus-binding activity on target tissues. J. Virol. **61:**185–189.
3. **Brown, D., and J. K. Rose.** 1992. Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. Cell **68:**533–544.
4. **Brunetti, C. R., R. L. Burke, B. Hoflack, T. Ludwig, K. Dingwell, and D. Johnson.** 1995. Role of mannose 6-phosphate receptors in herpes simplex virus entry into cells and cell-to-cell transmission. J. Virol. **69:**3517–3528.
5. **Brunetti, C. R., R. L. Burke, S. Kornfeld, W. Gregory, F. R. Masiarz, K. S. Dingwell, and D. C. Johnson.** 1994. Herpes simplex virus glycoprotein D acquires mannose 6-phosphate residues and binds to mannose 6-phosphate receptors. J. Biol. Chem. **269:**17067–17074.
6. **Byrne, K. M., D. Horohov, and K. Kousoulas.** 1995. Glycoprotein B of bovine herpesvirus-1 binds heparin. Virology **209:**230–235.
7. **Compton, T., D. Nowlin, and N. R. Cooper.** 1993. Initiation of human cytomegalovirus infection requires initial interaction with cell surface heparan sulfate. Virology **193:**834–841.
8. **Crane, S., J. Buzy, and J. Clements.** 1991. Identification of cell membrane proteins that bind visna virus. J. Virol. **65:**6137–6143.
9. **Edge, A. S., C. R. Faltynek, L. Hof, L. E. Reichert, and P. Weber.** 1981. Deglycosylation of glycoproteins by trifluoromethanesulfonic acid. Anal. Biochem. **118:**131–137.
10. **Flynn, S., B. Burgett, D. Stein, K. Williamson, and P. Ryan.** 1993. The amino-terminal one-third of pseudorabies virus glycoprotein gIII contains a functional attachment domain, but this domain is not required for the efficient penetration of Vero cells. J. Virol. **67:**2646–2654.
11. **Fuller, A. O., and W.-C. Lee.** 1992. Herpes simplex virus type 1 entry through a cascade of virus-cell interactions requires different roles of gD and gH in penetration. J. Virol. **66:**5002–5012.
12. **Gruenheid, S., L. Gatzke, H. Meadows, and F. Tufaro.** 1993. Herpes simplex virus infection and propagation in a mouse L cell mutant lacking heparan sulfate proteoglycans. J. Virol. **67:**93–100.
13. **Hascall, V. C., and J. H. Kimura.** 1982. Proteoglycans: isolation and characterisation. Methods Enzymol. **82:**769–800.
14. **Herold, B. C., R. Visalli, N. Susmarski, C. Brandt, and P. G. Spear.** 1994. Glycoprotein C-independent binding of herpes simplex virus to cells requires cell surface heparan sulphate and glycoprotein B. J. Gen. Virol. **75:**1211–1222.
15. **Herold, B. C., D. WuDunn, N. Soltys, and P. G. Spear.** 1991. Glycoprotein C of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and in infectivity. J. Virol. **65:**1090–1098.
16. **Höök, M.** 1984. Cell-surface glycosaminoglycans. Annu. Rev. Biochem. **53:**847–869.
17. **Inghirami, G., M. Nakamura, J. Balow, A. Notkins, and P. Casali.** 1988. Model for studying virus attachment: identification and quantitation of Epstein-Barr virus-binding cells by using biotinylated virus in flow cytometry. J. Virol. **62:**2453–2463.
18. **Johnson, D., R. L. Burke, and T. Gregory.** 1990. Soluble forms of herpes simplex virus glycoprotein D bind to a limited number of cell surface receptors and inhibit virus entry into cells. J. Virol. **64:**2569–2576.
19. **Kaner, R. J., A. Baird, A. Mansukhani, C. Basilico, B. Summers, R. Florkiewicz, and D. Hajjar.** 1990. Fibroblast growth factor receptor is a portal of cellular entry for herpes simplex virus type 1. Science **248:**1410–1413.
20. **Kaplan, A. S., and T. Ben-Porat.** 1961. The action of 5-fluorouracil on the nucleic acid metabolism of pseudorabies virus-infected and non-infected rabbit kidney cells. Virology **13:**78–92.
21. **Kaplan, A. S., and A. Vatter.** 1959. A comparison of herpes simplex and pseudorabies viruses. Virology **7:**394–407.
22. **Karger, A., and T. C. Mettenleiter.** 1993. Glycoproteins gIII and gp50 play dominant roles in the biphasic attachment of pseudorabies virus. Virology **194:**654–664.
23. **Karger, A., A. Saalmüller, F. Tufaro, B. W. Banfield, and T. C. Mettenleiter.** 1995. Cell surface proteoglycans are not essential for infection by pseudorabies virus. J. Virol. **69:**3482–3489.
24. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
25. **Langeland, N., and L. J. Moore.** 1990. Reduction of HSV-1 binding to BHK cells after treatment with phosphatidylinositol-specific phospholipase C. FEBS Lett. **277:**253–256.
26. **Lee, W.-C., and A. O. Fuller.** 1993. Herpes simplex virus type 1 and pseudorabies virus bind to a common saturable receptor on Vero cells that is not heparan sulfate. J. Virol. **67:**5088–5097.
27. **Li, Y., S. van Drunen Littel-van den Hurk, L. Babiuk, and X. Liang.** 1995. Characterization of cell binding properties of bovine herpesvirus 1 glycoproteins B, C, and D: identification of a dual cell-binding function of gB. J. Virol. **69:**4758–4768.
28. **Liang, X., L. Babiuk, and T. Zamb.** 1993. Mapping of heparin-binding structures on bovine herpesvirus 1 and pseudorabies virus gIII glycoproteins. Virology **194:**233–243.
29. **Liang, X., L. A. Babiuk, S. van Drunen Littel-van den Hurk, D. R. Fitzpatrick, and T. J. Zamb.** 1991. Bovine herpesvirus 1 attachment to permissive cells is mediated by its major glycoproteins gI, gIII, and gIV. J. Virol. **65:**1124–1132.
30. **Ligas, M. W., and D. C. Johnson.** 1988. A herpes simplex virus mutant in which glycoprotein D sequences are replaced by β-galactosidase sequences binds to but is inable to penetrate into cells. J. Virol. **62:**1486–1494.
31. **Lycke, E., M. Johansson, B. Svennerholm, and U. Lindahl.** 1991. Binding of herpes simplex virus to cellular heparan sulphate, an initial step in the adsorption process. J. Gen. Virol. **72:**1131–1137.
32. **Maisner, A., J. Schneider-Schaulies, K. Liszewski, J. Atkinson, and G. Herrler.** 1994. Binding of measles virus to membrane cofactor protein (CD46): importance of disulfide bonds and *N*-glycans for the receptor function. J. Virol. **68:**6299–6304.
33. **McClain, D., and A. O. Fuller.** 1994. Cell-specific kinetics and efficiency of herpes simplex virus type 1 entry are determined by two distinct phases of attachment. Virology **198:**690–702.
34. **Meier, T., S. Arni, S. Malarkannan, M. Poincelet, and D. Hoessli.** 1992. Immunodetection of biotinylated lymphocyte-surface proteins by enhanced chemiluminescense: a nonradioactive method for cell-surface protein analysis. Anal. Biochem. **204:**220–226.
35. **Mettenleiter, T. C.** 1994. Initiation and spread of α-herpesvirus infections. Trends Microbiol. **2:**2–4.
36. **Mettenleiter, T. C., L. Zsak, F. Zuckermann, N. Sugg, H. Kern, and T. Ben-Porat.** 1990. Interaction of glycoprotein gIII with a cellular heparinlike substance mediates adsorption of pseudorabies virus. J. Virol. **64:**278–286.
37. **Mirda, D. P., D. Navarro, P. Paz, P. L. Lee, L. Pereira, and L. T. Williams.** 1992. The fibroblast growth factor receptor is not required for herpes simplex virus type 1 infection. J. Virol. **66:**448–457.
38. **Muggeridge, M., G. Cohen, and R. Eisenberg.** 1992. Herpes simplex virus infection can occur without involvement of the fibroblast growth factor receptor. J. Virol. **66:**824–830.
39. **Okazaki, K., T. Matsuzaki, Y. Sugahara, J. Okada, M. Hasebe, Y. Iwamura, M. Ohnishi, T. Kanno, M. Shimizu, E. Honda, and Y. Kono.** 1991. BHV-1 adsorption is mediated by the interaction of glycoprotein gIII with heparin-like moiety on the cell surface. Virology **181:**666–670.
40. **Ravindranath, R., and M. Graves.** 1990. Attenuated murine cytomegalovirus binds to N-acetylglucosamine, and shift to virulence may involve recognition of sialic acids. J. Virol. **64:**5430–5440.
41. **Roderiquez, G., T. Oravecz, M. Yanagishita, D. Chequer Bou-Habib, H. Mostowski, and M. A. Norcross.** 1995. Mediation of human immunodeficiency virus type 1 binding by interaction of cell surface heparan sulfate proteoglycans with the V3 region of envelope gp120-gp41. J. Virol. **69:**2233–2239.
42. **Sawitzky, D., H. Hampl, and K.-O. Habermehl.** 1990. Comparison of heparin-sensitive attachment of pseudorabies virus (PRV) and herpes simplex virus type 1 and identification of heparin-binding PRV glycoproteins. J. Gen. Virol. **71:**1221–1225.
43. **Schreurs, C., T. C. Mettenleiter, F. Zuckermann, N. Sugg, and T. Ben-Porat.** 1988. Glycoprotein gIII of pseudorabies virus is multifunctional. J. Virol. **62:**2251–2257.
44. **Shieh, M.-T., and P. G. Spear.** 1991. Fibroblast growth factor receptor: does it have a role in the binding of herpes simplex virus? Science **253:**208–209.
45. **Shieh, M.-T., D. WuDunn, R. Montgomery, J. Esko, and P. G. Spear.** 1992. Cell surface receptors for herpes simplex virus are heparan sulfate proteoglycans. J. Cell Biol. **116:**1273–1281.
46. **Spear, P. G.** 1993. Entry of alphaherpesviruses into cells. Semin. Virol. **4:**167–180.
47. **Springer, B. A., M. W. Pantoliano, F. A. Barbera, P. L. Gunyuzlu, L. D. Thompson, W. F. Herblin, S. A. Rosenfeld, and G. W. Book.** 1994. Identification and concerted function of two receptor binding surfaces on basic fibroblast growth factor required for mitogenesis. J. Biol. Chem. **269:**26879–26884.
48. **Thaker, S. R., D. L. Stine, T. J. Zamb, and S. Srikumaran.** 1994. Identification of a putative cellular receptor for bovine herpesvirus 1. J. Gen. Virol. **75:**2303–2309.

49. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
50. **Trybala, E., T. Bergström, D. Spillmann, B. Svennerholm, S. Olofsson, S. Flynn, and P. Ryan.** 1995. Mode of interaction between pseudorabies virus and heparan sulfate/heparin, abstr. 78. *In* Abstracts of the 20th International Herpesvirus Workshop, Groningen, The Netherlands.
51. **Vanderplasschen, A., M. Bublot, J. Dubuisson, P.-P. Pastoret, and E. Thiry.** 1993. Attachment of the gammaherpesvirus bovine herpesvirus 4 is mediated by the interaction of gp8 glycoprotein with heparinlike moieties on the cell surface. Virology **196:**232–240.
52. **WuDunn, D., and P. G. Spear.** 1989. Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. J. Virol. **63:**52–58.
53. **Yanagishita, M., R. J. Midura, and V. C. Hascall.** 1987. Proteoglycans: isolation and purification from tissue culture. Methods Enzymol. **138:**279–289.
54. **Zhu, Z., M. Gershon, R. Ambron, C. Gabel, and A. Gershon.** 1995. Infection of cells by varicella zoster virus: inhibition of viral entry by mannose 6-phosphate and heparin. Proc. Natl. Acad. Sci. USA **92:**3546–3550.
55. **Zsak, L., N. Sugg, T. Ben-Porat, A. K. Robbins, M. E. Whealy, and L. W. Enquist.** 1991. The gIII glycoprotein of pseudorabies virus is involved in two distinct steps of virus attachment. J. Virol. **65:**4317–4324.