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Cell Surface Proteoglycans Are Not Essential for Infection by Pseudorabies Virus

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Cell surface proteoglycans, in particular those carrying heparan sulfate glycosaminoglycans, play a major role in primary attachment of herpesviruses to target cells. In pseudorabies virus (PrV), glycoprotein gC has been shown to represent the major heparan sulfate-binding virion envelope protein (T. C. Mettenleiter, L. Zsak, F. Zuckermann, N. Sugg, H. Kern, and T. Ben-Porat, J. Virol. 64:278–286, 1990). Since PrV gC is nonessential for viral infectivity in vitro and in vivo, either the interaction between virion envelope and cellular heparan sulfate is not necessary to mediate infection or other virion envelope proteins can substitute as heparan sulfate-binding components in the absence of gC. To answer these questions, we analyzed the infectivity of isogenic gC^+ and gC^- PrV on mouse L-cell derivatives with defects in glycosaminoglycan biosynthesis, using a rapid and sensitive fluorescence-based β-galactosidase assay and single-cell counting in a fluorescence-activated cell sorter. Our data show that (i) in the virion, glycoprotein gC represents the only proteoglycan-binding envelope protein, and (ii) cellular proteoglycans are not essential for infectivity of PrV. Attachment studies using radiolabeled virions lacking either gC or the essential gD confirmed these results and demonstrated that PrV gD mainly contributes to binding of Pr virions to cell surface components other than proteoglycans. These data demonstrate the presence of a proteoglycan-independent mode of attachment for Pr virions leading to infectious entry into target cells.

Attachment of free infectious virions to target cells is mediated by the interaction of virion proteins with cell surface molecules acting as virus receptors (31). For several viruses, such as human immunodeficiency virus (4, 13, 29, 61), rabies virus (11, 55), or poliovirus (59), more than one cellular receptor has been identified or postulated. In herpesviruses, the gammaherpesvirus Epstein-Barr virus has been shown to bind to complement receptor 2, also designated CD21, on susceptible B lymphocytes (42). The betaherpesvirus human cytomegalovirus (HCMV) apparently interacts with aminopeptidase N, also designated CD13 (52), although other cellular proteins have also been implicated in HCMV binding to target cells (23, 56). The fibroblast growth factor receptor has been proposed as a receptor for the alphaherpesvirus herpes simplex virus type 1 (HSV-1) (19), although its role in viral infection is disputed (40, 41, 53). In the alphaherpesviruses, it has been shown that HSV-1 and HSV-2 interact with cell surface glycosaminoglycans (26, 30, 64), in particular the highly sulfated and negatively charged heparan sulfate (HS) (8, 51). The alphaherpesvirus pseudorabies virus (PrV) also binds to cell surface HS, and glycoprotein gC has been demonstrated to represent the major HS-binding PrV envelope protein (39, 65). A similar function has subsequently been reported for HSV-1 gC (15). In addition to HSV and PrV, the alphaherpesvirus bovine herpesvirus 1 (BHV-1 [27, 28, 43]), the betaherpesvirus HCMV (6, 22), and the gammaherpesvirus BHV-4 (60) also interact with cell surface HS. Therefore, binding to cellular proteoglycans appears to be a relatively common mode of attachment for herpesviruses in general.

Attachment studies of radiolabeled Pr virions to target cells by using different incubation and wash conditions demonstrated that PrV binding to cells is a biphasic process (21). An initial interaction, which is sensitive toward competition by exogenous heparin and has therefore been designated heparin-sensitive binding, is mediated by gC. This primary binding converts in a time-dependent reaction into a secondary attachment which is resistant against heparin competition. For this heparin-resistant binding, presence of gD in the virion is essential (21). Similar biphasic attachment processes have been observed in HCMV (6) and HSV-1 (32). In HSV-1, gD has also been shown to play a prominent role in stable attachment to target cells (32).

gCs of HSV-1, BHV-1, and PrV are nonessential for viral infectivity in cell culture and in the animal (27, 34, 53). Therefore, a mode of attachment leading to infectious entry of the virion which is independent of gC must exist. In HSV-1, gC-deleted virus mutants still rely on HS for binding to target cells, and the essential gB has been shown to mediate this interaction in the absence of gC (14). Therefore, gC^- HSV-1 still exhibits a marked sensitivity toward inhibition by exogenous heparin, an HS-related glycosaminoglycan. In contrast, gC^- PrV mutants appear to be quite insensitive to inhibition by exogenous heparin or to pretreatment of cells with heparinase (39). However, by using virion lysates, other PrV envelope glycoproteins, especially gB, have also been observed to bind to immobilized heparin (49). Therefore, the question remained as to whether gC^- PrV infects cells via an HS-dependent pathway. To answer this question, we analyzed the infectivity of isogenic gC^+ and gC^- PrV mutants on mouse L-cell derivatives with different defects in biosynthesis of glycosaminoglycans (1, 10). Our data show that in the virion, gC represents the only HS-binding PrV protein and that the presence of cell surface proteoglycans is not necessary for infection by PrV. In the case of PrV,

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TABLE 1. PrV mutants and phenotypes

Virus designation	Phenotype[a]
PrV-Ka	β-Gal$^-$gC$^+$gG$^+$
PrV-17	β-Gal$^-$gC$^-$gG$^+$
PrV-1112	β-Gal$^+$gC$^+$gG$^-$
PrV-8411	β-Gal$^+$gC$^-$gG$^-$

[a] β-Gal, β-galactosidase.

therefore, a proteoglycan-independent attachment and entry pathway has to be postulated (39). The presence of both virion gC and cell surface proteoglycans, however, increases infectivity of PrV. In addition, our results corroborate earlier findings (21) implicating virion gD in attachment of PrV to a different cellular virus receptor.

MATERIALS AND METHODS

Viruses. PrV strain Ka (20) was used as the prototypic wild-type strain in all experiments. Mutant 1112 was derived from Ka by insertion of a gG (previously designated gX) β-galactosidase expression cassette into the nonessential gG gene (36). Interruption of this gene has previously been shown not to interfere with any biological parameter of the virus analyzed either in vitro or in vivo (24, 35). Mutant 17 carries a deletion of a 1.4-kb *Xho*I fragment eliminating ca. 80% of the gC gene and part of the 3′ end of the upstream prv43 gene (39, 45). Mutant 8411 was derived from mutant 17 by insertion of the gG–β-galactosidase (gG-βgal) cassette into the gG gene. Properties of these viral mutants are summarized in Table 1. The gD$^-$ PrV mutant was propagated on complementing, gD-expressing MDBK cells as described (46). Virions lacking gD were produced after infection of normal MDBK cells with phenotypically complemented gD$^-$ PrV mutant stock.

Cells. As the parental cell line, the clone 1D line of LMtk$^-$ murine fibroblasts was used. The L-cell derivatives gro2C, deficient in the biosynthesis of HS but carrying chondroitin sulfate (CS), and sog9, unable to synthesize either HS or CS, were selected by their relative resistance against infection with HSV-1 (1, 10) (Table 2). Lack of the respective cell surface proteoglycan was verified by anion-exchange high-performance liquid chromatography of metabolically radiolabeled glycosaminoglycans (1, 10). Cells were grown in minimal essential medium–10% fetal calf serum.

Southern blotting. Virion DNA was isolated as described previously (36). After cleavage with *Bam*HI, resulting fragments were separated in a 0.8% agarose gel. Transfer to nylon membranes and hybridization using [^{32}P]dCTP-labeled nick-translated probes followed standard procedures (48).

Infectivity assays. Confluent cells in six-well plastic dishes were infected with serial dilutions of purified gC$^+$ or gC$^-$ PrV for 1 h at 37°C. Thereafter, the inoculum was replaced with fresh medium, and the cells were further incubated for 5 h at 37°C. Fluorescence-activated cell sorting (FACS) analysis was performed essentially as described previously (47), with minor modifications. Briefly, cells were detached from the plate in 1 ml of 5 mM EDTA in phosphate-buffered saline (PBS) lacking divalent cations, transferred to sterile Eppendorf tubes, and sedimented by brief low-speed centrifugation at 4°C. The cell pellet was resuspended in 50 μl of prewarmed (37°C) PBS–2% fetal calf serum and labeled by addition of an equal volume of prewarmed 2 mM fluorescein-β-D-galactopyranoside (Molecular Probes, Eugene, Ore.) in distilled water. The suspension was mixed and incubated for 1 min at 37°C. Thereafter, 2 ml of ice-cold PBS–2% fetal calf serum was added, and samples were kept on ice until analysis on a FACStar plus (Becton Dickinson, Mountain View, Calif.) equipped with a 150-mW argon laser (Omnichrome 543/150BS). Per assay, 10^4 cells were analyzed. Analysis of viable cells was ascertained by electronic gating in the forward scatter versus side scatter mode. For evaluation, data were processed by using the PC-Lysis software (Becton Dickinson). Cell numbers were determined individually for each cell line, using the counting mode of the FACS. These numbers served as basis for the particle-per-cell calculations. Percentages of infected cells were determined by using the statistics option of the PC-Lysis software. Differentiation between infected and noninfected cells yielded overlaps of a maximum of 5%. At least three independent experiments using different purified virion preparations were performed, yielding qualitatively similar results. For Fig. 3 to 5, data from one representative assay each are shown.

TABLE 2. L-cell mutants and phenotypes

Cell line	HS	CS
L	+	+
gro2C	−	+
sog9	−	−

The number of virus particles required for infection of 50% of the cells under the experimental conditions used, the 50% infectious dose (ID_{50}), was calculated from the plotted titration curves. Relative infectivity of the virus mutants was calculated as the ratio of ID_{50} values on the respective mutant cell line compared with the ID_{50} on L cells, which was assigned a value of 1.

For analysis of heparin sensitivity of gC$^+$ and gC$^-$ PrV, plaque titrations were performed on MDBK and L cells in the presence or absence of 50 μg of heparin per ml as described previously (39).

Determination of particle number. Virions were purified by centrifugation through a step gradient of 30, 40, and 50% sucrose as described previously (21). Purity of the virus preparation was assessed by electron microscopy (data not shown). To determine particle numbers, the DNA content of the purified virus suspension was assayed by using a colorimetric test (2). From the DNA content, particle numbers were calculated, assuming a molecular weight of 90×10^6 for the PrV genome (21, 66). To ascertain that the PrV-containing fraction of the gradient did not contain contaminating cellular DNA, cell homogenates of non-infected cells were processed by the same procedure. Only negligible traces of cellular DNA could be detected (data not shown). Purified virion suspensions were regularly titrated on bovine kidney (MDBK) and L cells. Titers on MDBK cells were approximately 10-fold higher than those determined on murine L cells, as has been observed before (63).

Attachment assays. For attachment assays, virions were labeled with [*methyl*-^{3}H]thymidine and purified as described previously (21). Pr virions lacking gD were obtained by infection of noncomplementing cells with phenotypically complemented virus after propagation on gD-expressing cells (46). To analyze binding of the virus mutants, L and gro2C cells were inoculated at 4°C for 2 h with the purified radiolabeled virion preparation. Thereafter, the inoculum was removed and the monolayer was washed with either PBS (to estimate total binding) or PBS supplemented with 50 μg of heparin per ml (to differentiate heparin-sensitive from heparin-resistant binding [21]). Cells were lysed by addition of 1% sodium dodecyl sulfate, and cell-associated radioactivity was determined. Values are plotted as percent adsorption, i.e., percentage of input radioactivity that remained cell associated after the different wash procedures. At least three independent experiments were evaluated.

RESULTS

Characterization of virus mutants. To isolate a β-galactosidase-expressing gC-negative PrV mutant, virion DNA from the gC-deletion mutant 17 (39) was cotransfected with plasmid TT-80 containing the gG-βgal expression cassette inserted into the gG gene locus (36). Transfection progeny was harvested and titrated under an agarose overlay containing the chromogenic β-galactosidase substrate Bluo-Gal (Gibco-BRL, Bethesda, Md.). Blue-staining plaques were picked and purified three times. One isolate, designated 8411, was arbitrarily chosen for further investigation. For genotypic analysis, virion DNA from wild-type strain Ka (Fig. 1, lanes 1), the gG-βgal mutant 1112 (Fig. 1, lanes 2), the gC-deletion mutant 17 (Fig. 1, lanes 3), and the gG-βgal-positive gC-deletion mutant 8411 (Fig. 1, lanes 4) was analyzed in a 0.8% agarose gel after *Bam*HI cleavage. Figure 1A shows the ethidium bromide-stained gel. It is evident that in both gC$^-$ mutants 17 and 8411, *Bam*HI fragment 2, which encompasses most of the gC gene, comigrated with *Bam*HI fragment 3 as a result of the 1.4-kb deletion in the gC gene. In mutants 1112 and 8411, the novel gG-βgal fragment created by fusion of the gG-βgal cassette to the viral *Bam*HI fragment 10 is visible migrating between *Bam*HI fragments 6 and 7 (35). Concomitantly, fragment 10 is lost. Hybridization with *Bam*HI fragment 2 (Fig. 1B), with a gC-specific probe (Fig. 1C), and with a β-galactosidase-specific probe (Fig. 1D) confirmed that mutant 8411 contains the gG-βgal expression cassette in the gG locus and a deletion in the gC gene.

To check for heparin sensitivity, virus suspensions were titrated on MDBK cells in the absence and in the presence of 50 μg of heparin per ml. As shown in Fig. 2, the presence of heparin during the attachment process inhibited the gC$^+$ strains Ka and 1112 by more than 90%, whereas both gC$^-$ mutants 17 and 8411 were essentially not affected. The rather limited but still measurable effect of heparin on attachment of

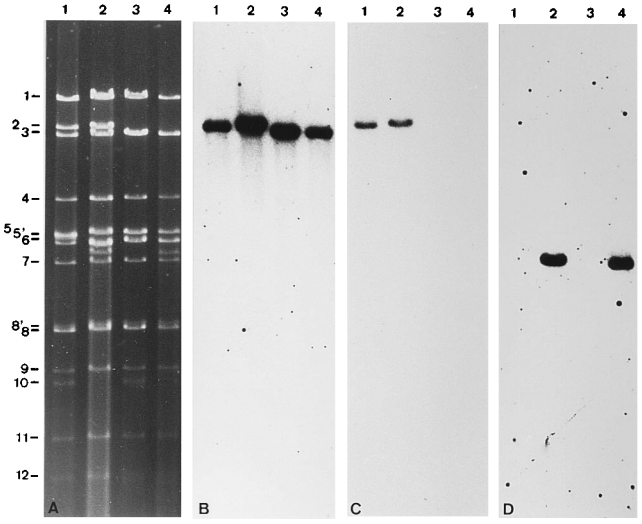

FIG. 1. Genomic characterization of PrV mutant 8411. Genomic viral DNA of wild-type PrV Ka (lanes 1), gG-βgal PrV mutant 1112 (lanes 2), gC$^-$ deletion mutant 17 (lanes 3), and gG-βgal gC$^-$ deletion mutant 8411 (lanes 4) was cleaved with *Bam*HI, and resulting fragments were separated in a 0.8% agarose gel. (A) Ethidium bromide-stained gel. After transfer, nylon membranes were probed with *Bam*HI fragment 2 encompassing most of the gC gene (B), a gC-specific probe (C), and a β-galactosidase-specific probe (D). On the left, positions of *Bam*HI fragments of wild-type PrV DNA are indicated.

a gC$^-$ PrV mutant described before (21) could not be observed when other preparations of heparin were used and thus may be attributed to impurities in the heparin (data not shown). These results demonstrate that infection of cells by mutant 8411 is not sensitive to the presence of exogenous heparin and that, therefore, mutant 8411 behaves like mutant 17 in this respect (39). Similar results were obtained when virus was titrated on murine L cells (data not shown).

Infectivity of isogenic gC$^+$ and gC$^-$ PrV on murine L cells. To assess the importance of gC for PrV infectivity, murine L cells were infected with serial dilutions of β-galactosidase-expressing gC$^+$ PrV 1112 and β-galactosidase-expressing gC$^-$ PrV 8411 and analyzed by FACS. Data are shown in Fig. 3. Both viruses were able to infect murine L cells to a level of 100%. However, the specific infectivity of gC$^-$ PrV was approximately 10-fold lower than that of gC$^+$ PrV; i.e., 10 times more particles of gC$^-$ PrV than of gC$^+$ PrV were needed to infect 50% of the L cells (see also Table 3). This result is in agreement with previous data for rabbit, bovine, or porcine kidney cells which indicated that the presence of gC enhances infectivity of PrV on these cells (39, 62, 66).

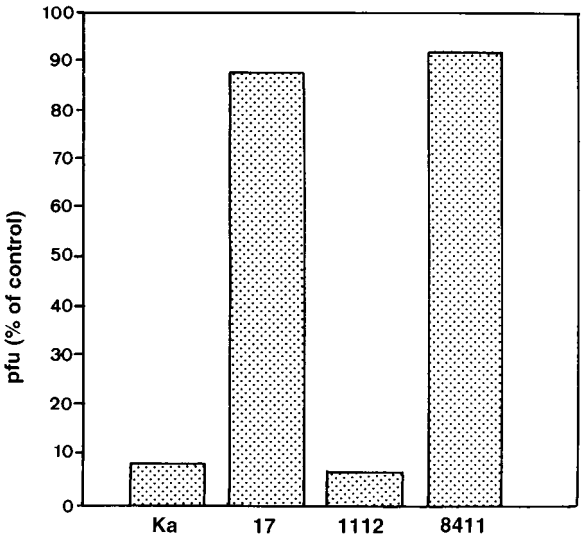

FIG. 2. Heparin sensitivity of gC$^+$ and gC$^-$ PrV. The gC$^+$ Pr viruses Ka and 1112 and the gC$^-$ mutants 17 and 8411 were titrated on MDBK cells in the presence of 50 μg of heparin per ml. Titers were compared with those determined without heparin. Relative percentages of titers with heparin compared with those without heparin are indicated for each virus.

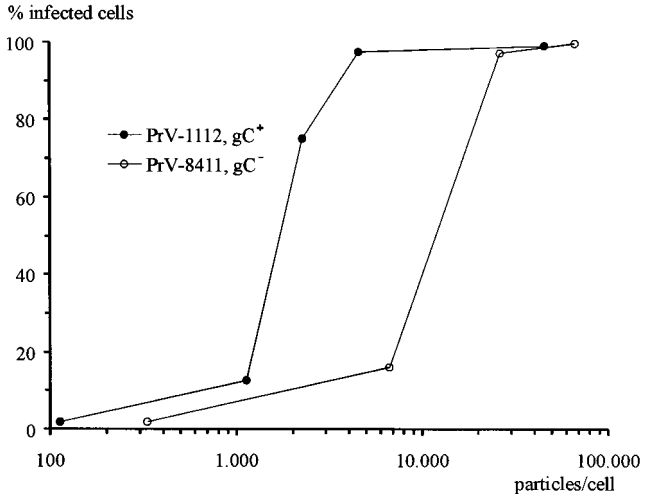

FIG. 3. Infectivity of gC$^+$ and gC$^-$ PrV on L cells. L cells were infected with β-galactosidase-expressing gC$^+$ PrV 1112 and gC$^-$ PrV 8411 and analyzed as described in Materials and Methods. Plotted is the percentage of infected cells against the number of particles per cell used for infection.

Infectivity of gC$^+$ PrV on L cells mutated in glycosaminoglycan biosynthesis. Interaction between virion gC and cell surface glycosaminoglycan is a major step in the primary attachment of PrV to target cells (39). To assess the importance of HS and/or CS in this process, mutant L cells deficient in synthesis of HS (gro2C [10]) or HS and CS (sog9 [1]) were infected with serial dilutions of β-galactosidase-expressing gC$^+$ PrV 1112 and analyzed by FACS. Results of a representative experiment are shown in Fig. 4. It is evident that gro2C and sog9 cells proved to be more resistant to PrV infection than parental L cells. At a given amount of input virus, significantly fewer gro2C or sog9 cells than wild-type L cells became infected. ID_{50} values and relative infectivities were calculated from the diagram (Fig. 4) and are presented in Table 3. The infectivity of gC$^+$ PrV on gro2C cells was approximately 22%, and on sog9 cells approximately 17%, of that found on parental L cells. At least three independent experiments using different virion preparations were performed. All of them yielded qualitatively similar results, although, as expected, ID_{50} values varied somewhat between different virus preparations. These data show that the absence of HS decreases the infectivity of gC$^+$ PrV to approximately the same level as observed for gC$^-$ PrV on HS-positive cells (Fig. 3 and 5). Additional absence of CS only slightly decreases the infectivity further, indicating that CS in gro2C cells cannot efficiently substitute for HS in PrV attachment.

Infectivity of gC$^-$ PrV on L cells mutated in glycosaminoglycan biosynthesis. It was now of interest to analyze the specific infectivity of gC$^-$ PrV on L-cell mutants in glycosaminoglycan biosynthesis. Therefore, in an experiment similar to that

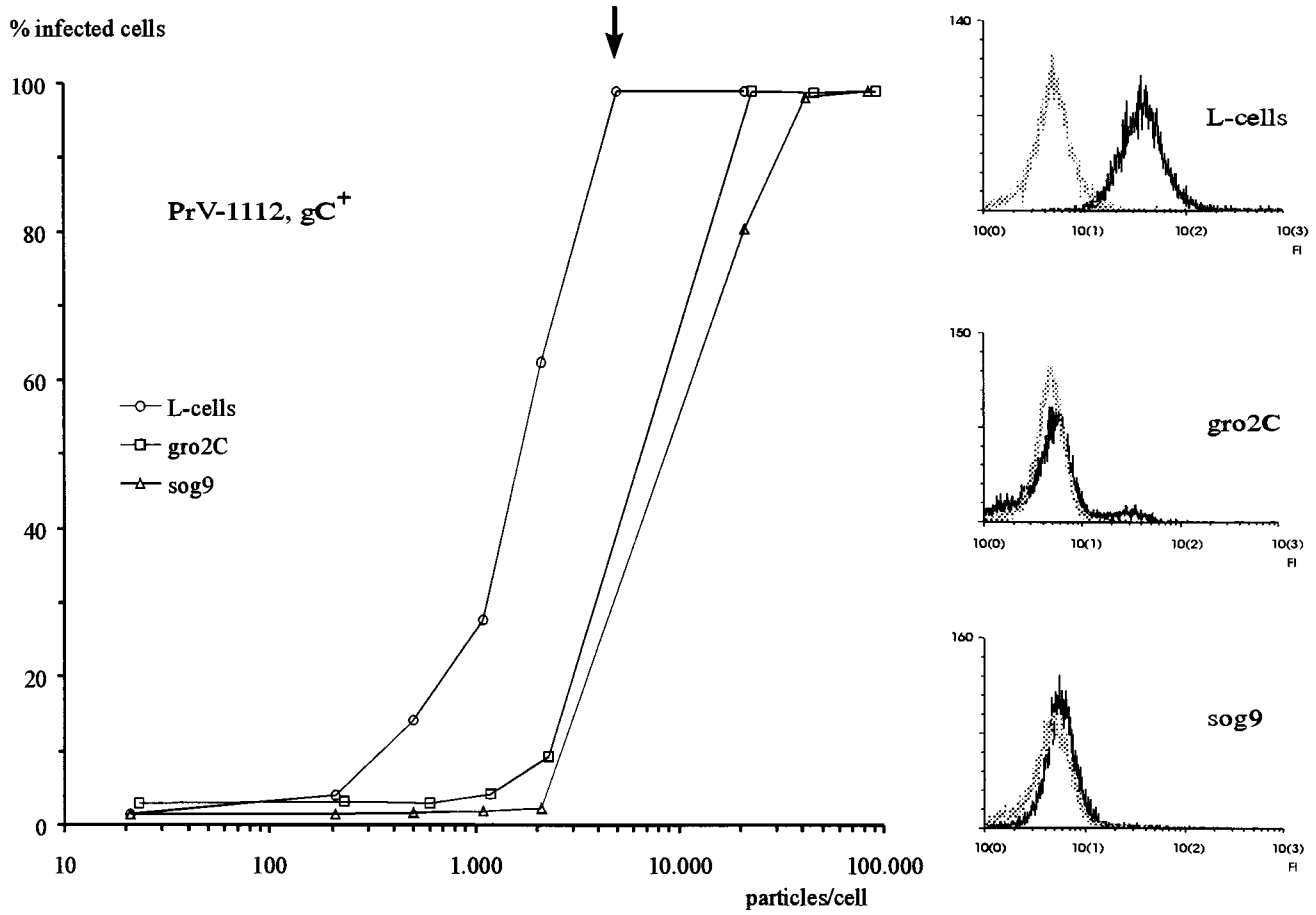

FIG. 4. Infectivity of gC^+ PrV on L, gro2C, and sog9 cells. L, gro2C, and sog9 cells were infected with β-galactosidase-expressing gC^+ PrV 1112 and assayed by FACS analysis. Plotted in the diagram is the percentage of infected cells against the number of particles per cell used for infection. The histograms show the distribution of fluorescence intensities (dark lines) at the particle number per cell indicated by the arrow. Shaded lines show fluorescence of noninfected control cells. The *x* axis indicates fluorescence intensity; the *y* axis shows number of cells (47).

described above, parental L cells and proteoglycan mutants gro2C and sog9 were infected with serial dilutions of gC^- PrV 8411 and analyzed. Data from a representative experiment are shown in Fig. 5 and Table 3. The infectivity of PrV 8411 was similar on all three cell lines tested. The absence of either HS in gro2C cells or HS and CS in sog9 cells did not significantly alter the infectivity of the virus compared with parental L cells. Therefore, in the absence of the virion envelope glycoprotein gC, the presence or absence of cell surface proteoglycans did not influence PrV infectivity. These data show that gC is the only virion envelope glycoprotein interacting productively with cell surface proteoglycans. In the absence of gC, cell surface proteoglycans apparently play no important role in initiation of infection.

TABLE 3. Infectivity of gC^+ and gC^- PrV on L cells and mutant cells[a]

Cell line	gC^+ PrV 1112		gC^- PrV 8411	
	ID_{50} (10^3)	Relative infectivity	ID_{50} (10^4)	Relative infectivity
L	1.5	1.0	1.3	1.0
gro2C	6.9	0.22	1.0	1.3
sog9	8.8	0.17	1.4	0.9

[a] ID_{50} values were calculated from the data shown in Fig. 4 and 5. The number of particles necessary for infection of 50% of L cells was assigned a relative infectivity of 1, and relative infectivities of both PrV mutants on gro2C and sog9 cells were calculated accordingly.

Attachment of gC^- and gD^- PrV to L and gro2C cells. To supplement our findings on infectivity of the virus mutants with physical binding studies, radiolabeled wild-type, gC^-, and gD^- Pr virions were incubated with L-cell and gro2C cell monolayers for 2 h at 4°C. Both gC and gD have previously been found to be involved in different steps of the biphasic attachment process of PrV (21). The fraction of virus that remained stably bound after thorough washing with PBS was determined (total binding; Fig. 6A). Total binding was further differentiated into heparin-sensitive and heparin-resistant binding by competition with exogenous heparin after washing with PBS supplemented with 50 μg of heparin per ml. Bound virus that could be displaced by exogenous heparin represented the heparin-sensitive binding fraction (Fig. 6B), whereas virus that remained stably bound after the wash with PBS-heparin comprised the heparin-resistant binding fraction (Fig. 6C). As regards total binding, attachment of wild-type Pr virions to gro2C cells was significantly lower than attachment to L cells, which reflects the observed difference in infectivity. Total binding of purified gD^- Pr virions was also significantly lower on gro2C than on L cells. In addition, in agreement with previous results (21), gD^-

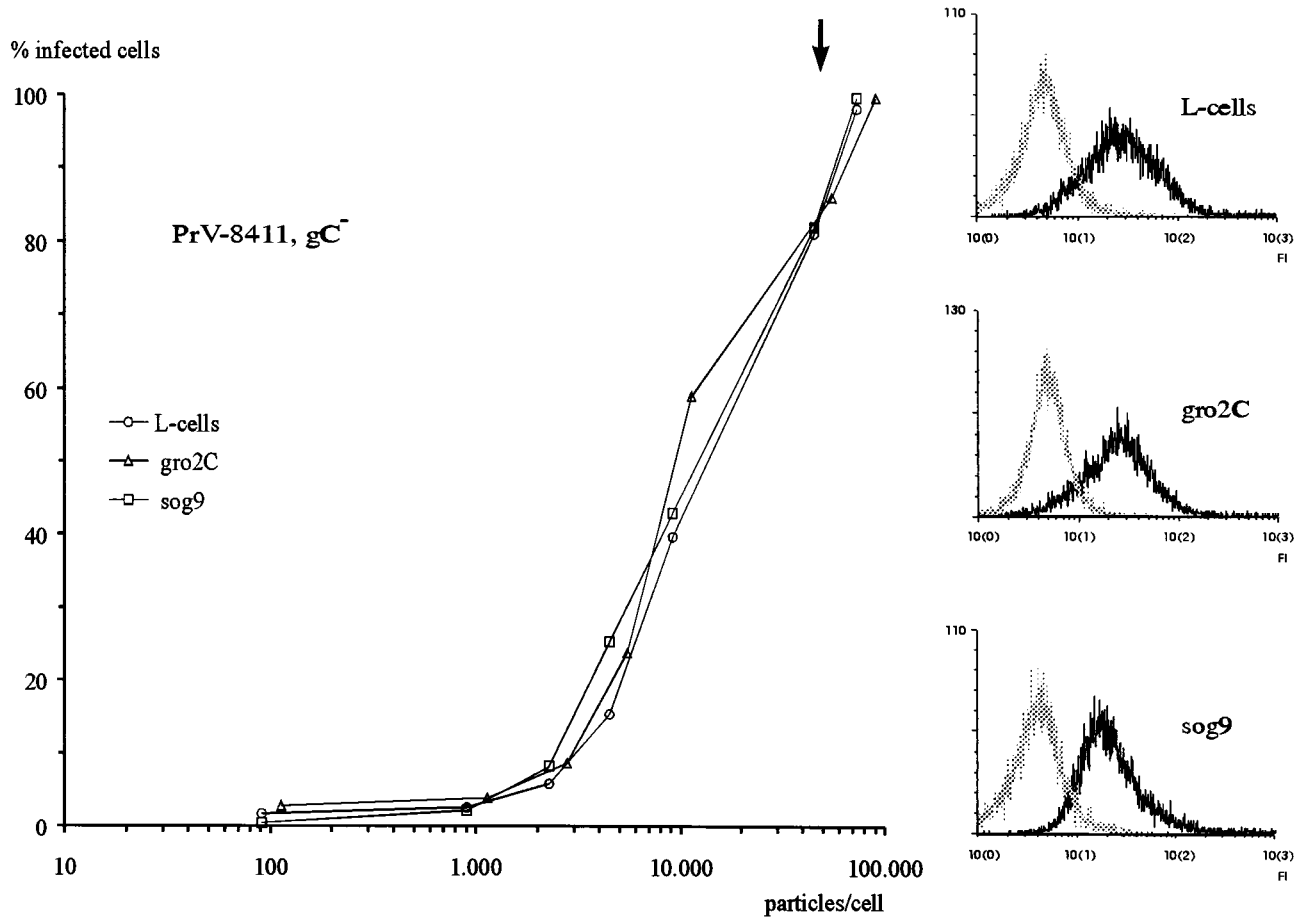

FIG. 5. Infectivity of gC⁻ PrV on L, gro2C, and sog9 cells. L, gro2C, and sog9 cells were infected with β-galactosidase-expressing gC⁻ PrV 8411 and assayed by FACS analysis. Data were plotted as indicated in the legend to Fig. 4. Histograms correspond to the particle number per cell indicated by the arrow. Shaded lines show fluorescence of noninfected control cells. Note that the particle numbers required to infect the same percentage of L cells was more than 10 times higher for gC⁻ PrV than for gC⁺ PrV (see Fig. 3 and 4 for comparison).

Pr virions did not attach as efficiently to L cells as did wild-type Pr virions. In gC⁻ PrV, total binding to L cells was lowest of all mutants tested and only slightly decreased on gro2C cells. These results show that the absence of HS proteoglycans significantly decreased total binding of wild-type and gD⁻ PrV, whereas the overall much lower attachment of gC⁻ PrV was only slightly affected by the absence of HS.

Differentiation into the heparin-resistant and heparin-sensitive binding fractions showed that wild-type and gD⁻ PrV relied on HS proteoglycans for the heparin-sensitive binding (Fig. 6B), since heparin-sensitive attachment was essentially abolished on gro2C cells compared with L cells. In contrast, the low level of heparin-sensitive binding of gC⁻ PrV was not affected by the presence or absence of HS. (It is important to note in this context that levels of adsorption attained by gC⁻ PrV on both cell lines and by gD⁻ and wildtype PrV on gro2C cells were near background levels.)

Heparin-resistant binding of wild-type PrV was slightly lower on gro2C cells than on L cells. A similar result was seen with gC⁻ and gD⁻ PrV, albeit at very low levels. Notably, gD⁻ PrV was unable to mediate significant heparin-resistant binding on either cell line. All attachment of gD⁻ PrV appeared to be heparin sensitive, which corresponds well with previous findings showing an involvement of gD in the heparin-resistant attachment of PrV (21). For all three binding assays, results for sog9 cells were similar to those for gro2C cells (data not shown).

DISCUSSION

Primary attachment to cell surface proteoglycans has been reported for members of all three subfamilies of herpesviruses (reviewed in references 33 and 53). However, so far it has been unclear whether interaction of virion components with cellular surface glycosaminoglycans is necessary to mediate subsequent steps in the initiation of virus infection leading to infectious entry of the virion into the target cell. In the absence of the major HS-binding glycoprotein of HSV-1, the nonessential glycoprotein gC, it is the essential gB which binds HS (14). Therefore, gC⁻ HSV-1 virions are still sensitive to heparin in attachment and infectivity. Since gB is indispensable for HSV-1 infectivity by playing an essential role during fusion between virion envelope and cellular cytoplasmic membrane (3), it is unclear whether the HS-binding function and the role that gB plays in membrane fusion are related and whether binding of gB to HS is necessary for initiation of the infectious process. We show here that in the absence of gC, specific infectivity of PrV for normal L cells is reduced to levels similar to those observed on proteoglycan-deficient cell mutants independent of the presence or absence of gC. PrV virion gB,

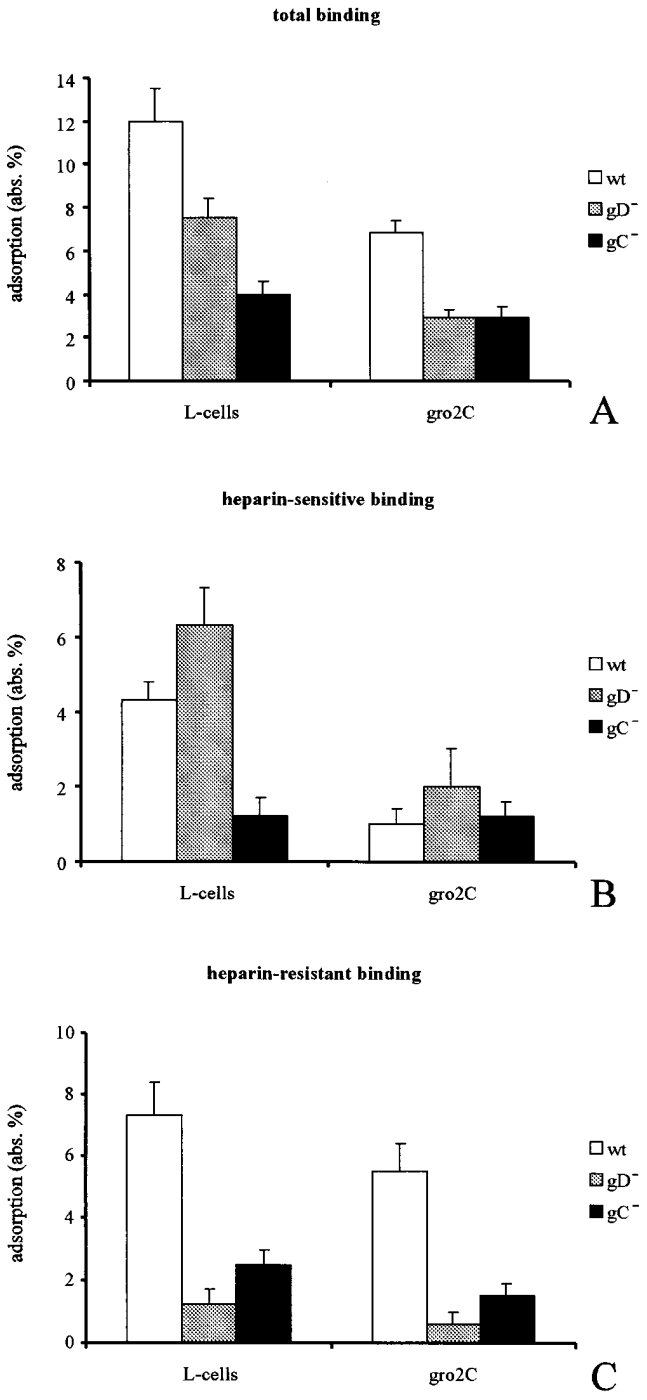

FIG. 6. Differentiation between total, heparin-sensitive, and heparin-resistant binding of gC^- and gD^- PrV on L and gro2C cells. Radiolabeled wild-type (wt) PrV or virions lacking glycoprotein gC or gD were adsorbed to wild-type L cells or HS-deficient gro2C cells for 1 h at 4°C. Thereafter, total binding was determined after washing with PBS (A). Heparin-sensitive (B) and heparin-resistant (C) binding were differentiated by washing with PBS supplemented with 50 μg of heparin per ml. Binding is indicated as percentage of input radioactive material belonging to each binding fraction, i.e., that remained bound (total binding and heparin-resistant binding) or could be displaced (heparin-sensitive binding) after the different wash procedures. Data for sog9 cells did not differ significantly from those for gro2C cells (not shown).

therefore, apparently does not bind productively to HS, at least not in the absence of gC, although PrV gB is probably capable of binding heparin in in vitro assays using solubilized glycoproteins (49). This observation corroborates earlier results (39) (see also Fig. 2) that gC^- PrV is resistant against inhibition of infection by soluble heparin. It appears, therefore, that in the virion, gC represents the only HS-binding PrV protein and that gC^- PrV enters murine L cells by a proteoglycan-independent pathway. gC has previously been shown to be involved in two distinct steps of attachment of PrV (66). Since gC^- PrV is still infectious, both of these steps apparently are not necessary for successful initiation of infection.

It is noteworthy that PrV gB can functionally substitute for HSV-1 gB in infectivity of HSV-1 virions (38). This could be explained either by PrV gB, in the context of the HSV-1 virion envelope, acquiring HS-binding activity or by the fact that HS binding is not required for gB function in HSV-1. Analyses differentiating between the two possibilities are currently under way. Interestingly, in HSV-1, gC appears to be the only virion glycoprotein capable of inducing hemagglutination by binding to HS in erythrocyte membranes. Deletion of gC abrogates the hemagglutinating ability of HSV-1, whereas restoration of gC expression restores hemagglutination (58). Although this might be interpreted as gB playing no role in binding of HS in this assay, it appears also conceivable that erythrocyte HS differs from that in other cell types and that HSV-1 gB is more restricted in its interaction with particular forms of HS than is gC.

Although gC is clearly dispensable for infectivity of PrV, it enhances virion infectivity in the presence of cell surface proteoglycans. In their absence, lack of gC did not significantly affect the infectivity of PrV for L cells, indicated by the result that the relative infectivity of gC^- PrV was similar on L, gro2C, and sog9 cells. We noted in this context that the specific infectivity of PrV for murine L cells was approximately 10-fold less than observed on bovine kidney (MDBK) cells (data not shown). The reason for this is unclear at present.

Since infection of target cells by PrV is dependent on neither gC nor cellular proteoglycans, it is still unclear which envelope glycoprotein mediates virion attachment in the absence of gC and which cellular surface molecule acts as the receptor. Previous studies indicated a biphasic attachment process of wild-type PrV in which a heparin-sensitive primary interaction which is mediated by gC converts to a heparin-resistant binding involving gD (21). Similar findings have subsequently been reported for HSV-1 (32). A biphasic attachment process obviously also takes place in the attachment of HCMV (6). Several studies yielded circumstantial evidence that gD might be a receptor-binding glycoprotein in HSV (9, 17), PrV (21), and BHV-1 (21). However, the respective cellular receptors are still unknown. Results presented here show that in the absence of gD, PrV is able to attach to target cells in a heparin-sensitive way only. Heparin-resistant binding of PrV to parental and mutated L cells is, therefore, dependent on the presence of gD. Since gC^- gD^+ PrV can still infect gro2C and sog9 cells in the absence of cell surface glycosaminoglycans, these mutant cells appear to be promising starting material with which to search for secondary PrV receptors. By using attachment of PrV-infected cells as a model system, it was demonstrated that in the absence of gC, antibodies against gD were most effective in inhibiting attachment (12). As has been described for gB, gD is an essential glycoprotein which precludes infectivity assays with gD^- mutant viruses (44, 46). However, PrV gD is not necessary for direct cell-to-cell transmission of infectivity, which might make mutant virus-infected cells a good system to study this mode of interaction.

Results presented here also show that CS is unable to substitute for HS in mediating attachment of PrV via gC. Infectivity of gC$^-$ PrV was similar on cell lines gro2C, which lacks HS but carries normal amounts of CS, and sog9, which lacks both HS and CS. Therefore, nonspecific electrostatic interactions between the negatively charged proteoglycans (8, 16) and the, under physiological conditions, most probably positively charged gC HS-binding domains (7, 57) appear unlikely. Rather, a specific gC-HS interaction involving recognition of critical patterns in both HS and gC appears to occur during PrV infection. In HSV-1, it has been demonstrated that infectivity on gro2C cells was approximately 10-fold less than that on L cells (10). This finding parallels our results for PrV. In contrast, infectivity of HSV-1 on sog9 cells was further decreased more than 10-fold (1), which indicated that CS was able to function as a cellular attachment molecule for HSV-1 in the absence of HS. Whether this difference can be attributed to the proteoglycan-binding function of HSV-1 gB or whether HSV-1 and PrV gC exhibit different affinities for CS remains to be established.

The role that gC plays in a natural infection is still unclear. In infection of mice by HSV-1 or HSV-2, gC did not appear to play a significant role, since respective gC$^-$ mutants were as virulent as the wild-type viruses (18, 54). After intranasal infection of pigs with PrV, which most closely mimics a natural infection in a natural virus-host system, it was demonstrated that gC$^-$ PrV, in contrast to PrV mutants deleted of gE or gI, was fully capable of neuroinvasion and neural spread and exhibited a wild-type-like virulent phenotype (25). However, gC appears to significantly influence virulence in the absence of other virion glycoproteins, such as gE (37). Since PrV gE has been implicated in direct cell-to-cell spread (67), it has been hypothesized that under conditions in which cell-to-cell spread is limited, as is the case for gE$^-$ PrV, reinfection of neighboring cells by free virions released from infected cells which attach via the gC-dependent pathway may be the main mode of viral spread (67). Our experimental system assays infectivity on a single-cell basis before viral spread by either means can occur (FACS) or measures only physical attachment of radiolabeled virions. Therefore, any possible influence on cell-to-cell spread, as analyzed by plaque titration, for example, is excluded from the analysis.

The demonstration that a gC- and cellular proteoglycan-independent mode of attachment leading to productive infection occurs in PrV indicates the presence of additional or alternative cellular alphaherpesvirus receptors besides glycosaminoglycans (5, 50). Further work will focus on the identification and characterization of these receptor molecules, which will also increase our understanding of the initial events in alphaherpesvirus infections.

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REFERENCES

1. **Banfield, B. W., 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. **Burton, K.** 1956. A study on the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of DNA. Biochem. J. **62:**315–323.
3. **Cai, W., B. Gu, and S. Person.** 1988. Role of glycoprotein B of herpes simplex virus type 1 in viral entry and cell fusion. J. Virol. **62:**2596–2604.
4. **Callebaut, C., B. Krust, E. Jacotot, and A. G. Hovanessian.** 1993. T cell activation antigen CD26 as cofactor for entry of HIV in CD4$^+$ cells. Science **262:**2045–2050.
5. **Campadelli-Fiume, G., D. Stirpe, A. Boscaro, E. Avitabile, L. Foa-Tomasi, L. Barker, and B. Roizman.** 1990. Glycoprotein C-dependent attachment of herpes simplex virus to susceptible cells leading to productive infection. Virology **178:**213–222.
6. **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.
7. **Flynn, S. J., B. L. Burgett, D. S. Stein, K. S. Wilkinson, 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.
8. **Fransson, L.-A.** 1987. Structure and function of cell-associated proteoglycans. Trends Biochem. Sci. **12:**406–411.
9. **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.
10. **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.
11. **Hanham, C., F. Zhao, and G. Tignor.** 1993. Evidence from the anti-idiotypic network that the acetylcholine receptor is a rabies virus receptor. J. Virol. **67:**530–542.
12. **Hanssens, F., H. Nauwynck, and M. Pensaert.** 1993. Involvement of membrane-bound viral glycoproteins in adhesion of pseudorabies virus-infected cells. J. Virol. **67:**4492–4496.
13. **Harouse, J. M., S. Bhat, S. L. Spitalnik, M. Laughlin, K. Stefano, D. H. Silberberg, and F. Gonzales-Scarano.** 1991. Inhibition of entry of HIV-1 in neural cell lines by antibodies against galactosyl ceramide. Science **253:**320–323.
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. **Johnson, D. C., 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.
18. **Johnson, D. C., M. McDermott, C. Chrisp, and J. Glorioso.** 1986. Pathogenicity in mice of herpes simplex virus type 2 mutants unable to express glycoprotein C. J. Virol. **58:**36–42.
19. **Kaner, R., 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 A. Vatter.** 1959. A comparison of herpes simplex and pseudorabies viruses. Virology **7:**394–407.
21. **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.
22. **Kari, B., and R. Gehrz.** 1992. A human cytomegalovirus glycoprotein complex designated gC-II is a major heparin-binding component of the envelope. J. Virol. **66:**1761–1764.
23. **Keay, S., T. Merigan, and L. Rasmussen.** 1989. Identification of cell surface receptors for the 86-kilodalton glycoprotein of human cytomegalovirus. Proc. Natl. Acad. Sci. USA **86:**10100–10103.
24. **Kimman, T., N. de Wind, N. Oei-Lie, J. Pol, A. Berns, and A. Gielkens.** 1992. Contribution of single genes within the unique short region of Aujeszky's disease virus (suid herpesvirus type 1) to virulence, pathogenesis and immunogenicity. J. Gen. Virol. **73:**243–251.
25. **Kritas, S. K., M. B. Pensaert, and T. C. Mettenleiter.** 1994. Role of gI, gp63, and gIII in the invasion and spread of Aujeszky's disease virus (ADV) in the olfactory nervous pathway of the pig. J. Gen. Virol. **75:**2319–2327.
26. **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.
27. **Liang, X., L. Babiuk, and T. Zamb.** 1991. Pseudorabies virus gIII and bovine herpesvirus 1 gIII share complementary functions. J. Virol. **65:**5553–5557.
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. **Long, D., J. Berson, D. Cook, and R. Doms.** 1994. Characterization of human immunodeficiency virus type 1 gp120 binding to liposomes containing galactosylceramide. J. Virol. **68:**5890–5898.
30. **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.
31. **Marsh, M., and A. Helenius.** 1989. Virus entry into animal cells. Adv. Virus Res. **36:**107–151.
32. **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.
33. **Mettenleiter, T. C.** 1994. Initiation and spread of α-herpesvirus infections. Trends Microbiol. **2:**2–4.
34. **Mettenleiter, T. C.** 1994. Pseudorabies (Aujeszky's disease) virus: state of the art. Acta Vet. Hung. **42:**153–177.
35. **Mettenleiter, T. C., H. Kern, and I. Rauh.** 1990. Isolation of a viable herpesvirus (pseudorabies virus) mutant specifically lacking all four known nonessential glycoproteins. Virology **179:**498–503.
36. **Mettenleiter, T. C., and I. Rauh.** 1990. A glycoprotein gX-β-galactosidase fusion gene as insertional marker for rapid identification of pseudorabies virus mutants. J. Virol. Methods **30:**55–66.
37. **Mettenleiter, T. C., C. Schreurs, F. Zuckermann, T. Ben-Porat, and A. Kaplan.** 1988. Role of glycoprotein gIII of pseudorabies virus in virulence. J. Virol. **62:**4622–4626.
38. **Mettenleiter, T. C., and P. G. Spear.** 1994. Glycoprotein gB (gII) of pseudorabies virus can functionally substitute for glycoprotein gB in herpes simplex virus type 1. J. Virol. **68:**500–504.
39. **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.
40. **Mirda, D., D. Navarro, P. Paz, P. Lee, L. Pereira, and L. Williams.** 1992. The fibroblast growth factor receptor is not required for herpes simplex virus type 1 infection. J. Virol. **66:**448–457.
41. **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.
42. **Nemerow, G. R., and N. Cooper.** 1992. CR2 (CD21) mediated infection of B-lymphocytes by Epstein-Barr virus. Semin. Virol. **3:**117–124.
43. **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 heparinlike moiety on the cell surface. Virology **181:**666–670.
44. **Peeters, B., N. de Wind, M. Hooisma, F. Wagenaar, A. Gielkens, and R. Moormann.** 1992. Pseudorabies virus envelope glycoproteins gp50 and gII are essential for virus penetration, but only gII is involved in membrane fusion. J. Virol. **66:**894–905.
45. **Powers, L., K. Wilkinson, and P. Ryan.** 1994. Characterization of the prv43 gene of pseudorabies virus and demonstration that it is not required for virus growth in cell culture. Virology **199:**81–88.
46. **Rauh, I., and T. C. Mettenleiter.** 1991. Pseudorabies virus glycoproteins gII and gp50 are essential for virus penetration. J. Virol. **65:**5348–5356.
47. **Saalmüller, A., and T. C. Mettenleiter.** 1993. Rapid identification and quantitation of cells infected by recombinant herpesvirus (pseudorabies virus) using a fluorescence-based β-galactosidase assay and flow cytometry. J. Virol. Methods **44:**99–108.
48. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
49. **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.
50. **Sears, A., B. McGwire, and B. Roizman.** 1991. Infection of polarized MDCK cells with herpes simplex virus 1: two asymmetrically distributed cell receptors interact with different viral proteins. Proc. Natl. Acad. Sci. USA **88:**5087–5091.
51. **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.
52. **Söderberg, C., T. Giugni, J. Zaia, S. Larsson, J. Wahlberg, and E. Möller.** 1993. CD13 (human aminopeptidase N) mediates human cytomegalovirus infection. J. Virol. **67:**6476–6585.
53. **Spear, P. G.** 1993. Entry of alphaherpesviruses into cells. Semin. Virol. **4:**167–180.
54. **Sunstrum, J. C., C. E. Chrisp, M. Levine, and J. Glorioso.** 1988. Pathogenicity of glycoprotein C negative mutants of herpes simplex virus type 1 for the mouse central nervous system. Virus Res. **11:**17–32.
55. **Superti, F., B. Hauttecoeur, M. Morelec, P. Goldoni, B. Bizzini, and H. Tsiang.** 1986. Involvement of gangliosides in rabies virus infection. J. Gen. Virol. **67:**47–56.
56. **Taylor, H., and N. Cooper.** 1990. The human cytomegalovirus receptor on fibroblasts is a 30-kilodalton membrane protein. J. Virol. **64:**2484–2490.
57. **Trybala, E., T. Bergström, B. Svennerholm, S. Jeansson, J. Glorioso, and S. Olofsson.** 1994. Localization of a functional site on herpes simplex virus type 1 glycoprotein C involved in binding to cell surface heparan sulphate. J. Gen. Virol. **75:**743–752.
58. **Trybala, E., B. Svennerholm, T. Bergström, S. Olofsson, S. Jeansson, and J. Goodman.** 1993. Herpes simplex virus type 1-induced hemagglutination: glycoprotein C mediates virus binding to erythrocyte surface heparan sulfate. J. Virol. **67:**1278–1285.
59. **Tucker, S., C. Thornton, E. Wimmer, and R. Compans.** 1993. Bidirectional entry of poliovirus into polarized epithelial cells. J. Virol. **67:**29–38.
60. **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.
61. **Weiss, R.** 1992. Human immunodeficiency virus receptors. Semin. Virol. **3:**79–84.
62. **Whealy, M., A. Robbins, and L. W. Enquist.** 1988. Pseudorabies virus glycoprotein gIII is required for efficient virus growth in tissue culture. J. Virol. **62:**2512–2515.
63. **Whealy, M., A. Robbins, F. Tufaro, and L. W. Enquist.** 1992. A cellular function is required for pseudorabies virus envelope glycoprotein processing and virus egress. J. Virol. **66:**3803–3810.
64. **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.
65. **Zsak, L., N. Sugg, and T. Ben-Porat.** 1992. The different interactions of a gIII mutant of pseudorabies virus with several different cell types. J. Gen. Virol. **73:**821–827.
66. **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.
67. **Zsak, L., F. Zuckermann, N. Sugg, and T. Ben-Porat.** 1992. Glycoprotein gI of pseudorabies virus promotes cell fusion and virus spread via direct cell-to-cell transmission. J. Virol. **66:**2316–2325.