Adaptability in Herpesviruses: Glycoprotein D-Independent Infectivity of Pseudorabies Virus

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Initial contact between herpesviruses and host cells is mediated by virion envelope glycoproteins which bind to cellular receptors. In several alphaherpesviruses, the nonessential glycoprotein gC has been found to interact with cell surface proteoglycans, whereas the essential glycoprotein gD is involved in stable secondary attachment. In addition, gD is necessary for penetration, which involves fusion between virion envelope and cellular cytoplasmic membrane. As opposed to other alphaherpesvirus gD homologs, pseudorabies virus (PrV) gD is not required for direct viral cell-to-cell spread. Therefore, gD− PrV can be passaged in noncomplementing cells by cocultivating infected and noninfected cells. Whereas infectivity was found to be strictly cell associated in early passages, repeated passaging resulted in the appearance of infectivity in the supernatant, finally reaching titers as high as 10⁶ PFU/ml (PrV gD− Pass). Filtration experiments indicated that this infectivity was not due to the presence of infected cells, and the absence of gD was verified by Southern and Western blotting and by virus neutralization. Infection of bovine kidney cells constitutively expressing PrV gD interfered with the infectivity of wild-type PrV but did not inhibit that of PrV gD−. Similar results were obtained after passaging of a second PrV mutant, PrV-376, which in addition to gD also lacks gE, gI, and gJ. Penetration assays demonstrated that PrV gD− Pass entered cells much more slowly than wild-type PrV. In summary, our data demonstrate the existence of a gD-independent mode of initiation of infection in PrV and indicate that the essential function(s) that gD performs in wild-type PrV infection can be compensated for after passaging. Therefore, regarding the requirement for gD, PrV seems to be intermediate between herpes simplex virus type 1, in which gD is necessary for penetration and cell-to-cell spread, and varicella-zoster virus (VZV), which lacks a gD gene. Our data show that the relevance of an essential protein can change under selective pressure and thus demonstrate a way in which VZV could have evolved from a PrV-like ancestor.

Envelope glycoproteins execute important functions in attachment and penetration of herpesviruses (45). In several alphaherpesviruses, initial interaction between free virions and target cells is mediated by interaction of glycoprotein C (gC) homologs with cell surface proteoglycans, in particular those carrying heparan sulfate glycosaminoglycans (reviewed in references 33 and 45). This primary interaction is sensitive to competition by exogenous heparin and converts to stable secondary attachment (19, 30). Secondary binding requires the presence of gD in the virion envelope (19). Evidence in support of the hypothesis that gD binds a secondary cellular virus receptor has been presented (15), and gD of herpes simplex virus type 1 (HSV-1) was recently shown to bind to the mannose-6-phosphate receptor on target cells (1). However, this interaction is not required for virion infectivity, and direct proof for a gD-receptor interaction necessary for productive infection of target cells is lacking. Following attachment, penetration is initiated by fusion of the virion lipid envelope with the cytoplasmic membrane of the target cell. For this process, which occurs at neutral pH, gD, the gH-gL complex, and gB have been found to be essential (33, 45). Since gD is involved in attachment and penetration, it might link these two initial events in alphaherpesvirus infection. Besides their role in penetration, gH-gL and gB are also involved in direct transmission of infectivity from primary infected to adjacent noninfected cells, a process called direct cell-to-cell spread (33, 45). Whereas gD is required for direct cell-to-cell spread in HSV-1 (28) and bovine herpesvirus 1 (BHV-1) (9), pseudorabies virus (PrV) gD appears to be unique in that it is dispensable for this process (39, 41), although in its absence plaque size decreases (36). It has therefore been postulated that like PrV gE, PrV gD plays a modulating role in cell-to-cell spread (36).

Recently it was demonstrated that BHV-1 gD was not absolutely required for direct cell-to-cell spread (27), although the reason for the differing results remains unclear. These authors also reported that BHV-1 virions lacking gD were able to infect bovine kidney (MDBK) cells expressing a genetically altered form of gD in which the normal membrane anchor had been substituted by a glycosylphosphatidylinositol (GPI) anchor. However, gD− BHV-1 was unable to infect normal MDBK cells, indicating that the presence of gD, whether in the virion or in the target cell membrane, was required for infection. Since PrV efficiently spreads directly from cell to cell in the absence of gD, it is possible to passage gD− PrV-infected or transfected cells by cocoeeding with noninfected cells. After repeated passaging, we observed in the cell culture supernatant PrV virions capable of infecting target cells efficiently in the absence of gD. The data thus show that the presence of gD is not necessary for initiation of PrV infection and that the roles that gD plays in attachment and penetration either are dispensable or can be compensated for by other virion constituents.

MATERIALS AND METHODS

Viruses and cells. Virus mutants are based on the Kaplan strain of PrV (17). The gD− PrV mutant 133 (PrV gD−) has been described elsewhere (41). It
lacks the 5’-terminal half of the gD gene as well as the gene encoding the nonessential gG and, instead, contains a gG–β-galactosidase expression cassette (35). PrV-376 carries a 5.5-kbp deletion in the genome eliminating the genes encoding gG, gD, and gI and most of the gE gene (36). Genotypes of virus mutants are shown in Fig. 1. PrV mutants lacking gD were propagated on gD-expressing MT50-3 cells as reported previously (41). MDBK cells constitutively express low (MT50-3) and high (MT50-5) levels of PrV gD as well as PrV gB-expressing MT-3 cells have been described elsewhere (41, 42).

**Passaging of gD- PrV.** DNA was isolated from PrV gD- and PrV-376 and transfected by the calcium phosphate-coprecipitation technique (11) into African green monkey (Vero) cells in tissue culture plates (diameter, 6 cm; ca. 10^6 cells per plate). Since transfection of DNA overcomes the defect in infectivity of noncomplemented gD-PrV, cytopathic effect (CPE) developed. After nearly complete CPE was observed, residual cells were trypsinized and reseeded with ca. 10^7 noninfected Vero cells in 75-cm^2 tissue culture flasks in 15 ml of medium until complete CPE developed. Residual cells were again trypsinized and reseeded with noninfected cells. After brief centrifugation to sediment cells and cellular debris, supernatants were titrated on Vero cell monolayers.

**Antibodies and antisera.** Monoclonal antibodies (MAbs) directed against PrV gH, gD, and gE were prepared according to standard procedures (21, 29). Neutralization assays were performed as described previously (42). Whereas the anti-gD MAb neutralized virion infectivity complement independent, the anti-gB and anti-gE MAbs required complement for neutralization. Preparation of a gH-specific antipeptide serum has been described elsewhere (24).

**Western blots.** Virions were purified through a discontinuous 30, 40, and 50% sucrose gradient (24, 25). For protein analysis, PrV virions were boiled in lysis buffer and proteins were separated in a discontinuous sodium dodecyl sulfate–10% polyacrylamide gel (26). After electrotransfer to nitrocellulose membranes (46), they were probed with MAbs as described previously (23).

**Filtration.** Supernatant of infected cells exhibiting complete CPE was clarified by low-speed centrifugation and subsequently filtered under pressure through a 0.2-μm-pore-size syringe-mounted filtration unit (Schleicher & Schuell, Dassel, Germany). Virus titers were determined before and after filtration.

**One-step growth analysis.** One-step growth curves were analyzed as described previously (10). Intracellular and extracellular virus titers were determined and plotted separately.

**Attachment assays.** Analysis of total, heparin-resistant, and heparin-independent binding was performed as reported previously (18, 19). Briefly, cells were infected at a multiplicity of infection of 5 with wild-type PrV, PrV gD- PrV gD- Pass (see Results), or phenotypically complemented PrV gD-, and progeny virions were labeled with [methyl-^3H]thymidine (Amersham-Buchler, Braunschweig, Germany). They were purified by centrifugation at 22,000 rpm for 2 h through a discontinuous gradient consisting of 2 ml each of 50, 40, and 30% sucrose in a Beckman SW41 rotor. Virions accumulating at the interphase between the 40 and 50% sucrose layers were aspirated, diluted 1:4 in PBS, pelleted by ultracentrifugation, and resuspended in PBS. Routine screening in an electron microscope demonstrated the presence of highly pure preparations of enveloped virions. Particle-to-cpm ratios were calculated based on the determination of DNA content in the virion preparations (18, 19, 47). They varied between ca. 200 and 1,000 for all virus strains and mutants. For the attachment studies, 2 × 10^7 cells per well were incubated with 50,000 cpm of labeled virus for 2 h on ice. They were then either washed with PBS supplemented with 1% bovine serum albumin.
(PBS-A) to assay total binding or incubated with PBS-A containing 50 μg of heparin per ml to assay heparin-resistant binding. Heparin-independent attachment was assayed by adding 50 μg of heparin per ml during the adsorption phase (18, 19).

**RESULTS**

**Selection of infectious gD<sup>-</sup> PrV.** In PrV, gD has been found to be required for virion penetration into target cells but not for direct viral cell-to-cell spread (39, 41). Therefore, by passing infected cells together with noninfected cells, a strictly cell-bound infection should result. To eliminate the possibility of residual input gD from gD-transcomplemented virions, we started infection by transfecting gD<sup>-</sup> PrV virion DNA into Vero cells. After CPE had developed, cells were trypsinized and reseeded with noninfected Vero cells. After every fifth passage, the presence of infectious virus in the supernatant was analyzed by titration on Vero cells (Fig. 2). Whereas in the first passages, despite the presence of a complete CPE, no infectious virus could be recovered from the supernatant, starting at passage 10 infectivity appeared in the supernatant. Titers gradually increased during further passaging until reaching ca. 10<sup>7</sup> PFU/ml at passage 50. This virus population was designated PrV gD<sup>-</sup> Pass. Additional passaging did not lead to a further increase in viral titers.

Several explanations for this unexpected observation are possible. (i) The DNA preparation used for transfection could have contained viral genomes in which the gD gene had been restored by marker rescue after propagation of the gD<sup>-</sup> PrV mutant on complementing cells. (ii) Infectivity in the supernatant could be cell associated, leading to infection by adhesion of free-floating infected cells to noninfected cells and direct cell-to-cell transmission of infectivity (12). (iii) A gD-independent mode of infection of free PrV virions could exist, and this phenotype might be selected for by repeated passaging. To differentiate between these possibilities, the following experiments were performed.

**Absence of gD in PrV gD<sup>-</sup> Pass.** To prove that infectivity in PrV gD<sup>-</sup> Pass was not due to the inadvertent presence of gD, e.g., by rescue of the gD deletion in PrV gD<sup>-</sup> after propagation on gD-expressing complementing cells, supernatants from cells infected with either wild-type PrV or PrV gD<sup>-</sup> Pass were incubated with MAbs against gB, gD, and gE in the presence or absence of complement. As shown in Fig. 3, all antibodies neutralized wild-type PrV in the presence of complement. The anti-gD MAb also exhibited complement-independent neutralizing activity against wild-type PrV. In contrast, the anti-gD MAb failed to neutralize infectivity of PrV gD<sup>-</sup> Pass without or with complement. In addition, Southern blot hybridizations gave no indication of rescue (data not shown). Together these data indicate gD-independent infectivity in PrV gD<sup>-</sup> Pass.

![FIG. 2. Selection of infectious PrV gD<sup>-</sup> by passaging in cell culture. DNA isolated from PrV gD<sup>-</sup> and PrV-376 was transfected into Vero cells. After development of CPE, cells were trypsinized and reseeded with uninfected cells. After every fifth passage, cleared supernatants were titrated on Vero cells to check for the presence of extracellular infectious virus.](http://jvi.asm.org/)

![FIG. 3. Neutralization of PrV gD<sup>-</sup> Pass. Wild-type PrV (WT), PrV gD<sup>-</sup> Pass (gD<sup>-</sup> Pass), and PrV gD<sup>-</sup> Pass phenotypically gD complemented by growth on gD-expressing MT50-3 cells (gD<sup>-</sup> Pass gD<sup>+</sup>) (ca. 200 PFU of each) were incubated with a MAB directed against gB, gD, or gE for 1 h at 37°C either with (+) or without (−) addition of 5% normal rabbit serum as a source of complement. Thereafter, infectivity was determined by titration on Vero cells. Indicated is the percent relative infectivity compared to that in assays with negative control serum.](http://jvi.asm.org/)
Infectivity of PrV gD− Pass is associated with virions lacking gD. The propensity of gD− PrV-infected cells to transmit infectivity to noninfected cells is well known (12, 39, 41). Therefore, it was necessary to exclude the possibility that infectivity in PrV gD− Pass was mediated by infected cells and not free virions. To this end, filtration experiments were performed. Supernatants from cells infected with wild-type PrV or PrV gD− Pass were harvested after cells exhibited complete CPE, clarified by centrifugation, and filtered through a syringe-mounted 0.2-μm-pore-size filter. Virus titers were determined before and after filtration. Filtration reduced titers of wild-type PrV by ca. 90%, indicating that viral aggregates or cell-bound infectivity accounted for most of the viral titer. Alternatively, mechanical shearing could have resulted in this drop in infectivity. In comparison, PrV gD− Pass showed a similar reduction in titer and, as observed with wild-type PrV, retained significant infectivity even after filtration (data not shown).

To further analyze whether infectivity of PrV gD− Pass is associated with virions, wild-type PrV (Fig. 4, lanes 1) and PrV gD− Pass (Fig. 4, lanes 2) virions were purified through a sucrose gradient, which efficiently separates cells and cellular debris from virions. Virion fractions were analyzed by Western blotting using anti-gD (Fig. 4A), anti-gB (Fig. 4B), anti-gC (Fig. 4C), anti-gE (Fig. 4D), and anti-gH (Fig. 4E) antibodies. Since electrophoresis was performed under reducing conditions, the disulfide-linked gB complex dissociated (29) and only the smaller subunit was recognized by the MAb. It is evident that the glycoprotein profiles of wild-type PrV and PrV gD− Pass were identical with the exception of the absence of gD in PrV gD− Pass. The same result was observed when a polyclonal anti-vaccinia virus gD serum was used (data not shown). Immunoelectron microscopy confirmed the absence of gD and the presence of the other glycoproteins in purified virion preparations (data not shown). Thus, in PrV gD− Pass, infectivity is transmitted by virions lacking gD, indicating the existence of a gD-independent mode of initiation of infection by extracellular virions. No overt alteration in any of the other glycoproteins tested was observed.

Specific infectivity of PrV gD− Pass. Herpesvirus proteins have been differentiated into essential and nonessential polypeptides on the basis of their requirement for in vitro viral replication. Nonessential glycoproteins of alphaherpesviruses include gC, gE, gG, gI, gJ, gM, and gN. Though these proteins are nonessential, their absence may under certain conditions reduce specific viral infectivity. For example, deletion of the nonessential glycoprotein gC, which mediates primary attachment of PrV to cell surface proteoglycans (18), leads to a ca. 50-fold decrease in specific infectivity (Table 1). Determination of specific infectivity of PrV gD− Pass resulted in numbers similar to those observed for PrV gC−, with a ca. 50-fold reduction compared to wild-type PrV (Table 1). In contrast, specific infectivity of PrV gD− was ca. 103-fold lower than that of wild-type PrV. These data show that lack of gD in PrV gD− Pass decreased infectivity to a similar degree as absence of gC does in wild-type PrV. Therefore, it is reasonable to state that gD is not required more for PrV gD− Pass infection than gC is for PrV wild-type infection. By this definition, gD has to be regarded as a nonessential glycoprotein in PrV gD− Pass.

Attachment of PrV gD− Pass. PrV, BHV-1, and HSV-1 gD homologs have been implicated in a secondary attachment step mediating stable binding of virion to host cell in a heparin-resistant manner (19, 30). In HSV-1, the mannose-6-phosphate receptor has been shown to bind gD (1). However, the importance of this interaction for HSV-1 infectivity is unclear. To assay attachment of PrV gD− Pass, labeled virions were incubated for 1 h at 4°C with Vero cells. Thereafter, total binding was determined after washing with PBS, and heparin-resistant binding was analyzed after washing with PBS supplemented with 50 μg of heparin per ml (19). As shown in Fig. 5, compared to wild-type PrV, PrV gD− virions exhibited similar total binding but were significantly impaired in heparin-resistant binding (19). Attachment of PrV gD− Pass virions proved to be similar to that of PrV gD− virions. Therefore, attachment of PrV gD− Pass resembles that of PrV gD−, which indicates that the function of PrV gD in stable attachment is not critical for initiation of a productive virus infection and is not compensated for in PrV gD− Pass.

Penetration kinetics of PrV gD− Pass. PrV gD has been implicated in attachment and penetration. To analyze penetration of PrV gD− Pass, the rate of entry into Vero cells was determined. As shown in Fig. 6, wild-type PrV entered cells rapidly and 50% of PFU became resistant to low-pH inactivation at 6 min after the temperature shift. In contrast, PrV gD− Pass entered cells much more slowly, and 50% penetration was reached only 53 min after the temperature shift. This result shows that penetration of PrV gD− Pass is delayed compared to that of wild-type PrV, which highlights the contribution of gD for efficient penetration. However, these data also show

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**TABLE 1. Specific infectivities of PrV mutants**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Specific infectivity (particles/PFU)</th>
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<tbody>
<tr>
<td></td>
<td>Expt 1</td>
</tr>
<tr>
<td>Wild-type PrV</td>
<td>$2.1 \times 10^5$</td>
</tr>
<tr>
<td>PrV gD</td>
<td>$2.0 \times 10^7$</td>
</tr>
<tr>
<td>PrV gD− Pass</td>
<td>$1.3 \times 10^7$</td>
</tr>
<tr>
<td>PrV gC−</td>
<td>$0.9 \times 10^8$</td>
</tr>
</tbody>
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* Determined by calculating particle numbers in preparations of gradient-purified virions obtained from supernatants of infected cells, based on their DNA content (18, 47). Virion preparations were routinely assayed by electron microscopy for purity and presence of enveloped virions.
that in PrV gD\(^{-}\) Pass, penetration does occur in the absence of gD.

**One-step replication of PrV gD\(^{-}\) Pass.** To assay replication of PrV gD\(^{-}\) Pass in more detail, one-step growth curves were established (Fig. 7). The results showed a delay in replication of PrV gD\(^{-}\) Pass compared to wild-type PrV due to an extended eclipse phase which is most likely due to the delay in penetration. However, at later times after infection, the overall shapes of the growth curves were similar in wild-type PrV and PrV gD\(^{-}\) Pass, although the latter reached only ca. 10-fold-lower titers. This finding correlates with the decreased specific infectivity of PrV gD\(^{-}\) Pass virions. Significant differences between wild-type PrV and PrV gD\(^{-}\) Pass in the proportion of cell-associated and released infectivity were not observed. Together these data show that like wild-type PrV, PrV gD\(^{-}\) Pass is able to complete a full replicative cycle, which supports the finding that gD is not required for productive replication of PrV gD\(^{-}\) Pass.

**Infection by PrV gD\(^{-}\) Pass is not sensitive to gD-mediated interference.** Cells expressing homologous gD have been reported to exhibit an increased resistance to infection by several alphaherpesviruses, e.g., PrV (40), BHV-1 (5), and HSV-1 (16), and this resistance is dependent on the presence of gD in both virion and cytoplasmic membrane (3, 4, 7, 8). To test for sensitivity of PrV gD\(^{-}\) Pass to interference, MT50-3 cells (constitutively expressing low amounts of PrV gD) and MT50-5 cells (expressing high amounts of PrV gD) were infected with wild-type PrV, PrV gD\(^{-}\) Pass, and gD-complemented PrV gD\(^{-}\) Pass. As demonstrated in Fig. 8, wild-type PrV was most efficient in infection of parental MDBK cells, whereas infectivity was strikingly lower on MT50-3 cells and decreased even further on MT50-5 cells. In contrast, there was no significant difference in the ability of PrV gD\(^{-}\) Pass to infect the different cell lines. However, when PrV gD\(^{-}\) Pass was phenotypically gD complemented after propagation on MT50-3 cells, interference similar to that observed with wild-type PrV was detected. All viruses were able to infect gB-expressing MT-3 cells to similar extents.

**Infectivity of PrV-376 Pass lacking gG, gD, gI, and gE.** Genes for gG, gD, gI, and gE are located in the U\(_S\) region in alphaherpesvirus genomes in conserved collinear positions. On the basis of conservation of structural features in the protein products, it has been proposed that this gene cluster evolved by duplication and mutation of a single primordial gene (31). To assay whether any of these related glycoproteins might be necessary for the gD\(^{-}\) Pass phenotype, passaging experiments in Vero cells were performed with a virus mutant, PrV-376, lacking all of the U\(_S\)-encoded glycoproteins (36). Results pre-
US-encoded proteins except gD are viable in vitro, it has been, coproteins, gI and gE (6). Since deletion mutants in any of the
despite a striking conservation of two other US-encoded gly-
the alphaherpesviruses and belonging to the same genus,
Varicellovirus (PrV) and BHV-1, lacks a gD-homologous gene
despite a striking conservation of two other US-encoded gly-
could have evolved from a primordial nonessential gene (31).
This implies that an alphaherpesvirus lacking the U\textsubscript{c} region
genomes indicated that gG, gD, and gI are related and thus could have evolved by gene duplication and divergence. In addition,
gE shows signs of a distant relationship to gD (31). It could, therefore, be imagined that any one of these glycoproteins
acquired functions by an evolutionary process in vitro as might have happened in vivo. However, our results for the gG, gD, gl, and gE
deletion mutant PrV-376 argue against this hypothesis. Even in the absence of all U\textsubscript{c}-encoded glycoproteins, gD-independent infectivity of PrV developed during passaging.
In PrV gD\textsuperscript{−}, the 3\textsuperscript{−}-terminal half of the gD gene is still present (Fig. 1) and might theoretically be expressed, although we never observed any protein product related to gD, using different MAbs or monospecific antisera in a variety of tests (22, 41). However, any influence of this hypothetically expressed gD fragment on the results presented here is highly unlikely, since a similar phenotype was obtained for PrV-376 Pass, which lacks all gD gene sequences.

DISCUSSION

Herpesvirus glycoproteins involved in penetration of free virions include the essential gB, gH, and gL, all of which are conserved throughout the herpesvirus family. In contrast, gD has been found to be required for penetration in the alphaherpesviruses HSV-1 (28), PrV (39, 41), and BHV-1 (9), but a homologous protein is not present in beta- and gammaherpes-
ities. In addition, varicella-zoster virus (VZV), also a member of the alphaherpesviruses and belonging to the same genus, Varicellovirus, as PrV and BHV-1, lacks a gD-homologous gene despite a striking conservation of two other U\textsubscript{c}-encoded glycoproteins, gI and gE (6). Since deletion mutants in any of the
U\textsubscript{c}-encoded proteins except gD are viable in vitro, it has been hypothesized that this cluster of dispensable genes might have been acquired during evolution of herpesviruses (43) and/or
the same protocol as reported here. However, the mutation leading to infectivity of free gD\textsuperscript{−} BHV-1 virions in addition to the capacity for direct cell-to-cell spread has not been identified so far. In this context, it is interesting that a GPI-anchored BHV-1 gD was able to complement a gD\textsuperscript{−} BHV-1 mutant (27) and that this GPI-anchored gD in the cellular membrane rendered cells sensitive to infection by gD-negative BHV-1. However, gD\textsuperscript{−} BHV-1 was not able to infect normal cells in these assays (27).

Marek’s disease virus (MDV), a herpesvirus initially classified as belonging to the \textit{Gammaherpesvirinae} based on its oncogenicity but recently shown to be more closely related to alphaherpesviruses in gene content (2), contains a gD-homologous gene which is apparently not expressed during infection, at least in cell culture, and no mature protein has so far been observed either in vitro or in vivo. Mutants with insertions in the MDV gD gene have been isolated either after targeted insertional mutagenesis (37) or after integration of retroviral sequences (14). Thus, in MDV, gD is apparently not necessary for replication. However, it is noted that MDV in culture remains highly cell associated and titers of free infectious virus are extremely low. In contrast, high-titered cell-free virus can be isolated from feather follicles of infected chickens (37). Whether MDV gD is expressed in feather follicle epithelium and whether this leads to efficient release of virions from infected cells and/or enhances the infectivity of cell-free virions is unclear. Interestingly, VZV, which is also highly cell associated in vitro, lacks any gD homolog. Nevertheless, varicella blisters contain large amounts of cell-free infectious virus. However, a more thorough examination of the apparently gD-independent infectivity of these alphaherpesviruses has not yet been performed. It should be noted, however, that in PrV gD\textsuperscript{−} Pass and PrV-376 Pass, free virions could be isolated with significant titers, and no obvious increased cell association of infectivity was detected in the absence of gD.

The peculiar phenotype of gD-negative PrV, i.e., its ability to directly spread from cell to cell but inability to infect cells from the outside, led to the development of a novel type of attenuated live vaccine which is naturally biologically contained within the vaccinated animal (13, 38). This virus is particularly attractive as a safe virus vector. Our finding that infectivity of PrV can develop in the absence of gD could question the safety of PrV gD\textsuperscript{−}-based live vaccines. However, in several experiments in vivo, we (13) and others (38) were unable to detect gD-independent infectivity in animals vaccinated with gD\textsuperscript{−} PrV mutants, which may argue against a significant influence of the in vivo process on selection of infectious gD\textsuperscript{−} PrV mutants. We are currently analyzing the growth properties of PrV gD\textsuperscript{−} Pass in mice and pigs to estimate the pathogenic power of any possibly selected virus.

Finally, our finding that gD, in an evolutionary process, changes from essential for infectivity to nonessential might give a clue to the evolution of viruses lacking gD, such as VZV. In HSV-1, gD has been shown to be required for penetration and direct cell-to-cell spread (28). In PrV, gD is dispensable for direct cell-to-cell spread but still required for penetration. gD is not present in VZV, which correlates with the cell-associated character of this virus, at least in cell culture. PrV gD\textsuperscript{−} Pass is infectious even in the absence of gD, serving as an example of how a VZV-like virus could have evolved from a PrV-like ancestor. Of course, we realize that these scenarios are highly speculative, and it is not clear whether VZV-like viruses lost a gD gene or whether the others gained a gD gene. In either case, our experimental findings could help to explain a “missing link.”

In summary, we show that PrV can evolve in a way such that it does not require gD for infectivity. Therefore, in PrV, every U\textsubscript{3}-encoded gene has been demonstrated to be dispensable for viral replication in cell culture. Based on our infectious quadruple glycoprotein deletion mutant PrV-376 Pass, we are now attempting to isolate a U\textsubscript{3}-less PrV mutant to test the hypothesis that the U\textsubscript{3} region-encoded genes are in toto nonessential.

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