Glycoproteins Required for Entry Are Not Necessary for Egress of Pseudorabies Virus

Barbara Klupp, Jan Altenschmidt, Harald Granzow, Walter Fuchs, and Thomas C. Mettenleiter

Institutes of Molecular Biology and Infectology, Friedrich-Loeffler-Institut, Südufer 10, 17493 Greifswald-Insel Riems, Germany

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Membrane fusion is an essential process for cell development and physiology as well as for the replication of enveloped viruses (reviewed in reference 35). The herpesvirus replication cycle is thought to comprise two distinct fusion processes between a viral envelope and a cellular membrane (reviewed in reference 41). During entry, the viral envelope fuses with the plasma membrane. For this fusion process, viral glycoprotein B (gB), gH, and gL are essential, forming the conserved core fusion machinery (reviewed in reference 59). gD, which is expressed only in several members of the Alphaherpesvirinae, is a receptor-binding protein and functions as a trigger of fusion. Interactions with one of its cellular receptors, herpes simplex virus type 1 (HSV-1) gD undergoes conformational changes (36), which signal to gB and gHgL to form a multi-protein fusion complex (2, 3). Recent studies (61) uncovered the presence of different phases in herpesvirus fusion as described previously for other fusion processes (reviewed in reference 55). In phase I, the interaction of gD with its cellular receptor brings the two membranes in close proximity, while in phase II, lipid mixing between the two adjacent membranes can be observed, dependent on the presence of gHgL. Hemi-fusion is resolved into full fusion by gHgL and gB (61). The contribution of gL, which forms a complex with and is anchored via gH, is still enigmatic.

Capsids that have been formed in the nucleus acquire a primary envelope by budding at the inner nuclear membrane (INM). They then have to gain access to the cytoplasm for final tegumentation and envelopment. This translocation has been proposed to occur by the fusion of the primary envelope with the outer nuclear membrane (ONM), which constitutes a second fusion process between a viral envelope and a cellular membrane (41, 57). However, protein requirements for this proposed fusion during nuclear egress are still a matter of debate. Recently, we showed that the simultaneous expression of two conserved herpesvirus proteins, pseudorabies virus (PrV) pUL31 and pUL34, is sufficient to form primary envelope-like vesicles in the perinuclear space, indicating that these two proteins are sufficient for budding at and fusion from the INM (29). These vesicles accumulate in the perinuclear space similarly to primary enveloped particles of mutants lacking the alphaherpesvirus-specific kinase pUS3 (28, 53, 56, 64). However, so far, the fusion competence of these vesicles with the ONM could not be shown unequivocally.

Several studies reported accumulations of perinuclear virions in the absence of different viral glycoproteins, such as gB in PrV strain NIA-3 (49) or gK in HSV-1 (13), indicating that they might be involved in fusion with the ONM. However, these observations could not be confirmed (19, 38), arguing against an essential involvement in deenvelopment at the ONM. Recently, an HSV-1 mutant simultaneously lacking gB and gH has been shown to be deficient in nuclear egress, resulting in the accumulation of primary enveloped particles in the perinuclear space. This indicates that the presence of either gB or gH may be sufficient to promote the fusion of the primary envelope with the ONM (12). Although these data suggest that fusion-active herpesvirus glycoproteins take part in fusion during entry and nuclear egress, they also indicate that the molecular mechanism of these fusion events is different, since the deletion of either one is sufficient to completely block viral entry, whereas only the absence of both affects nuclear egress. If these viral envelope...
glycoproteins indeed play a role in nuclear egress, they should be targeted to the inner nuclear membrane to become incorporated into the primary envelope. Contributing to this model, several studies showed the presence of glycoproteins such as gB of HSV-1, human cytomegalovirus (HCMV), or Epstein-Barr virus (1, 9, 15, 16, 17, 18, 52, 60, 62) or HSV-1 gC (9, 22), gD (9, 60, 62), and gM (5) in the nuclear membrane and/or in the primary envelope either by immunoelectron microscopy or subcellular fractionation with subsequent immunoblotting.

The INM of uninfected cells contains a unique subset of integral membrane proteins, while the ONM shares many integral membrane proteins with the endoplasmic reticulum, consistent with the continuity between the endoplasmic reticulum and the ONM. To reach the INM, integral membrane proteins must move past the nuclear pore complex, which, for larger proteins that cannot diffuse through the nuclear pore, seems to require a specific targeting motif (24, 37). Such a specific INM-targeting motif has been described for HCMV gB (45, 52). Arguing against an abundant presence of viral envelope glycoproteins in the INM and the primary virion, electron microscopic images showed that the primary envelopes of PrV, HSV-1, equine herpesvirus 1, and infectious laryngotracheitis virus lack the typical spike structure of mature virions (4, 19, 20).

To shed more light on the role of viral glycoproteins in the fusion of the primary envelope with the ONM, we isolated PrV mutants simultaneously lacking two of the essential glycoproteins in different combinations. In addition, we scanned PrV-infected cells for the presence of viral glycoproteins in the nuclear membrane or in the primary envelope. Since biochemical approaches to study the protein composition of perinuclear virosomes are still hampered by the lack of reliable methods for particle preparation, we used immunoelectron microscopy with monoclonal or polyclonal antibodies against the major PrV glycoproteins. Furthermore, after plasmid transfection, cells coexpressing pUL31, pUL34, gB, and gH were investigated for the colocalization of these proteins by laser scanning microscopy.

MATERIALS AND METHODS

Viruses and cells. All mutants were derived from PrV strain Kaplan (PrV-Ka) (23). Virus stocks were propagated on pig kidney (PSEK or SK6) or rabbit kidney (RK13) cells or on complementing RK13 cell lines as summarized in Table 1. Briefly, PrV-gB was propagated on RK13-gB cells expressing gB under the control of the HCMV immediate-early 1 (IE1) enhancer/promoter complex (46). PrV-ΔgD (47) was grown on RK13-gD cells (48), which express gD under the control of the HCMV IE1 promoter/enhancer complex or, since cells constitutively expressing gD are partially resistant against infection (51), on RK13-Bam7 cells obtained after the cotransfection of RK13 cells with the genomic BamHI-fragment 7 comprising the gD open reading frame and pSV2neo (58). RK13-Nde cells containing a genomic fragment comprising the gD hybrid gene isolated from PrV-ΔgLPass (25) were used for the generation of mutants lacking gH and gL. After the restoration of the gB gene by cotransfection with a plasmid carrying a β-galactosidase expression cassette under the control of the PrV gG promoter (42), Kan’, kanamycin resistance cassette; GFP, expression cassette under the control of the HCMV IE1 promoter/enhancer.

<table>
<thead>
<tr>
<th>Reference or Mutation</th>
<th>Insertion in:</th>
<th>Complementing cell line</th>
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<tbody>
<tr>
<td>PrV-ΔgB</td>
<td>gD</td>
<td>RK13-gB</td>
</tr>
<tr>
<td>PrV-ΔgD</td>
<td>gF</td>
<td>RK13-gD</td>
</tr>
<tr>
<td>PrV-ΔgH</td>
<td>FRT</td>
<td>RK13-gH</td>
</tr>
<tr>
<td>PrV-ΔgL</td>
<td>gD</td>
<td>RK13-gD/gH</td>
</tr>
<tr>
<td>PrV-ΔgB/D</td>
<td>lacZ, GFP</td>
<td>Kan’</td>
</tr>
<tr>
<td>PrV-ΔgB/H</td>
<td>GFP</td>
<td>FRT</td>
</tr>
<tr>
<td>PrV-ΔgL/H</td>
<td>GFP</td>
<td>FRT</td>
</tr>
<tr>
<td>PrV-ΔgL/L</td>
<td>Kan’</td>
<td>51</td>
</tr>
</tbody>
</table>

Generation of double deletion mutants. For the generation of double deletion mutants, we used a combination of bacterial artificial chromosome (BAC) mutagenesis and classical homologous recombination. To isolate PrV-ΔgB/H, PrV-ΔgL/D, and PrV-ΔgL/H, a gH deletion mutant was constructed on the basis of the recently described BAC clone prvV-ΔgB (35). To this end, plasmid pTNA7-Pvu, containing a 4,351-bp PvuI fragment of the PrV genome (nucleotides 85989 to 63349; GenBank accession no. BK001744) (26), was cleaved with SanD1 and BstXI, thereby deleting 1,853 bp of the gH open reading frame (Fig. 1). After a fill-in of the 5′ overhang, a 1,258-bp BstXI fragment of pPrK1 containing a kanamycin resistance gene flanked by flp recombinase recognition (FRT) sites was inserted after blunt ending (10). The insert of the resulting plasmid was amplified using primers DH3 and DHREV (25), and the PCR product was used for the mutagenesis of the BAC clone prvV-ΔgB/H (35), followed by the flp recombinase-mediated deletion of the kanamycin resistance gene, resulting in prvV-ΔgL/H after PCR to yield the double mutant PrV-ΔgB/H LF4. After the restoration of the gB gene by cotransfection with the authentic gB DNA, PrV-ΔgL/H could be reconstituted on RK13-gH cells. For the generation of a mutant lacking gH and gL, the BAC clone prvV-ΔgL/H could be rescued on RK13-gH/L cells (see above). A mutant simultaneously lacking gB and gH was isolated after the cotransfection of PrV-ΔgL gDNA (58) and a 3.5-kbp EcoRI fragment containing the BAC clone pPrV-ΔgL/H gDNA with plasmid pDHDgfp (47) into RK13-gB/gH cells. Genomic DNA of all mutants was tested for the correct deletion and insertion by restriction enzyme analysis and Southern blotting (data not shown). For a summary, see Table 1.

Generation of a gD-specific rabbit antiserum. The ectodomain of gD (corresponding to amino acids 25 to 343) was amplified using primers GEX FW D (5′-CACAGAATTCGCCGCCACCCTCCCCCGGCCC-3′) and GEX REV D (5′-CACACTCGGCGGCCGGGTCCCGCTTGGAAA-3′) and the cloned BamHI-fragment 7 as a template. The PCR product was cloned into pGEX-t1-F1 (Amerham Biosciences, Freiburg, Germany) using restriction enzyme sites added by the primers (EcoRI and Xhol [shown in italics above]). Bacterial expression, purification of the glutathione S-transferase fusion protein, and immunization were done as described previously (27).

Western blotting. RK13 cells grown in six-well tissue culture dishes were infected at a multiplicity of infection (MOI) of 5 with the different transcomplemented single and double glycoprotein deletion mutants and harvested after overnight incubation by scraping into the medium. Cells were collected by centrifugation at 13,000 rpm for 2 min in an Eppendorf centrifuge, washed twice with phosphate-buffered saline (PBS), and resuspended in 100 μl

a lacZ, β-galactosidase expression cassette under the control of the PrV gG promoter (42); Kan’, kanamycin resistance cassette; GFP, expression cassette under the control of the HCMV IE1 promoter/enhancer.

Western blotting. RK13 cells grown in six-well tissue culture dishes were infected at a multiplicity of infection (MOI) of 5 with the different transcomplemented single and double glycoprotein deletion mutants and harvested after overnight incubation by scraping into the medium. Cells were collected by centrifugation at 13,000 rpm for 2 min in an Eppendorf centrifuge, washed twice with phosphate-buffered saline (PBS), and resuspended in 100 μl
of PBS and the same volume of sample buffer. After polyacrylamide gel electrophoresis, proteins were electrotransferred onto nitrocellulose membranes and incubated with antisera against gB (1:200,000) (35), gD (1:10,000) (this study), gH (1:100,000) (25), or gL (1:10,000) (33) or a monoclonal antibody specific for gE (A9-b15) (25). After incubation with peroxidase-conjugated secondary antibodies (Dianova, Hamburg, Germany), bound antibody was detected by enhanced chemiluminescence (Super Signal; Pierce, Bonn, Germany) and recorded on X-ray film.

**RESULTS**

Isolation and in vitro characterization of PrV mutants. None of the PrV-Ka single or multiple glycoprotein gene deletion mutants tested so far on various cell lines, i.e., PrV-ΔgB (19), PrV-ΔgE/I (7), PrV-ΔgH (19, 50), PrV-ΔgK (31), PrV-ΔgL (H. Granzow, personal communication), PrV-ΔgM (7), PrV-ΔgN (H. Granzow, personal communication), or PrV-ΔgE/I/M (8), showed a defect in nuclear egress, indicating that none of these essential or nonessential glycoproteins alone or in the tested combinations is necessary for this process. Since it is known that important steps during herpesvirus replication may be executed by more than one protein possessing redundant functions, we isolated double deletion mutants simultaneously lacking two of the essential glycoproteins. Thus, we generated mutants lacking gB and gD (PrV-ΔgB/D), gB and gH (PrV-ΔgB/H), gD and gH (PrV-ΔgD/H), or gH and gL (PrV-ΔgH/L) (Fig. 1 and Table 1).

The productive replication of all these mutants was dependent on transgenic cell lines (Table 2). In RK13-gB/gD cells, both proteins are expressed under HCMV IE1 promoter/enhancer control. To avoid any fusogenic effects that may occur by the constitutive coexpression of gB and gH, RK13-gB/gH cells constitutively expressed only gB, whereas the gH open reading frame was under the control of its authentic promoter and required transactivation during viral infection. To allow the simultaneous constitutive expression of gH and gL, both

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**FIG. 1.** Diagram of PrV glycoprotein deletion mutants. (A) Schematic map of the PrV genome. It consists of a unique long (UL) and a unique short (US) region, which is bracketed by inverted repeats (IR, internal repeat; TR, terminal repeat). (B) Locations of BamHI restriction sites are given. Fragments are numbered according to their sizes. The locations of the essential glycoprotein genes are indicated by boxes. (C) Relevant parts of the genome are magnified to show the deletions (hatched boxes) introduced into genes encoding gB, gD, gH, and gL. Arrows indicate transcriptional direction. R, region of reiterated repeats; UNG, uracil DNA-glycosylase; TK, thymidine kinase.

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Electron microscopy. For ultrathin sectioning, RK13 or SK6 cells were infected at an MOI of 1 and fixed approximately 14 h postinfection (p.i.). Fixation and embedding for routine microscopy and for intracellular immune labeling of viral proteins were done as described previously (19). Immunolabeling was performed with anti-gB polyclonal serum (35), anti-gC monoclonal antibody (B16-c8) (25), anti-gH serum (25), anti-gD serum (this paper), anti-gM serum (35), or anti-UL34 serum (27) and gold-labeled anti-rabbit or anti-mouse secondary antibodies. The ultrathin sections were analyzed with an electron microscope (EM400T, Tecnai 12; Phillips, Eindhoven, The Netherlands).

**Transfection and immunofluorescence.** RK13 cells were cotransfected with plasmid p3ieUL31/UL34gfp, coexpressing pUL31 and a GFP-tagged version of pUL34, and pcDNA-gB(009) (46), comprising full-length gB, together with pcDNA-gH (32). Alternatively, an expression plasmid for the C-terminally truncated and more fusogenic gB(008) (32, 46) was cotransfected with pcDNA-gH (32). Cells were fixed 2 days posttransfection with 3% paraformaldehyde, permeablized with 3% paraformaldehyde-0.3% Triton X-100, and incubated with a monoclonal antibody against gB (A20-c26) (47) or the polyclonal anti-gH serum (25). Bound antibodies were detected with Alexa-555 goat anti-mouse and Alexa-647 goat anti-rabbit secondary antibodies (Invitrogen, Molecular Probes, Karlsruhe, Germany). Fluorescence was preserved with a 9:1 mixture of glycerol in PBS containing 25 mg of 1,4-diazabicyclooctane per milliliter and analyzed with a confocal laser scanning microscope (LSM510; Zeiss, Oberkochen, Germany).

The productive replication of all these mutants was dependent on transgenic cell lines (Table 2). In RK13-gB/gD cells, both proteins are expressed under HCMV IE1 promoter/enhancer control. To avoid any fusogenic effects that may occur by the constitutive coexpression of gB and gH, RK13-gB/gH cells constitutively expressed only gB, whereas the gH open reading frame was under the control of its authentic promoter and required transactivation during viral infection. To allow the simultaneous constitutive expression of gH and gL, both
Glycoproteins necessary for entry are dispensable for nuclear egress of PrV. To test whether any of the double deletions of essential glycoprotein genes caused a defect in nuclear egress or release from infected cells, RK13 cells were infected at an MOI of 1 with the phenotypically complemented virus mutants, fixed approximately 14 h p.i., and processed for electron microscopy. As demonstrated in Fig. 3 and 4, all of the tested double deletion mutants showed normal virion morphogenesis, and none of them displayed any impairment of nuclear egress. In contrast to the situation in HSV-1 (12), neither accumulations of primary virions in the perinuclear space nor large invaginations of the INM were observed (Fig. 3A and C and 4A). In the cytoplasm, capsids were present in the process of secondary envelopment (Fig. 3B and D and 4B), or enveloped virions within vesicles were found (Fig. 3B and D and 4B and D). Moreover, numerous virus particles lined the cell surface (Fig. 3 and 4). Thus, our data confirm that glycoproteins gB, gD, gh, and gL in the tested combinations are not required for the release of primary virions from the perinuclear space or for virion maturation in and release from the cytoplasm. Normal virion maturation was observed not only in rabbit kidney cells (RK13) but also in swine kidney cells (SK6) (data not shown), indicating that cell origin has no influence on the observed phenotype.

In vitro replication. To confirm the replication defects, RK13 cells and cell lines expressing the respective glycoproteins were infected in parallel with PrV-Ka and the corresponding glycoprotein deletion mutants. As shown in Table 2, infectious progeny exceeding 100 PFU/ml could not be found after the infection of RK13 cells or single-glycoprotein-expressing cells with transcomplemented PrV-DgB/D, PrV-DgB/H, or PrV-DgD/H, whereas on complementing cells, titers reached between 10^5 and 10^6 PFU/ml. The ghgL double deletion mutant showed slightly increased titers on the single-gh- or single-gL-expressing cells, but only infection of doubly ghgL-expressing cells resulted in wild-type-like growth.

![FIG. 2. Western blot analysis of glycoprotein deletion mutants.](image)

**TABLE 2. In vitro replication**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Titer (PFU/ml)</th>
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<tbody>
<tr>
<td>PrV-Ka</td>
<td>3 × 10^6</td>
</tr>
<tr>
<td>PrV-DgB/D</td>
<td>10^5</td>
</tr>
<tr>
<td>PrV-Ka</td>
<td>8 × 10^6</td>
</tr>
<tr>
<td>PrV-DgB/H</td>
<td>&lt;10^5</td>
</tr>
<tr>
<td>PrV-Ka</td>
<td>6 × 10^6</td>
</tr>
<tr>
<td>PrV-DgD/H</td>
<td>&lt;10^5</td>
</tr>
<tr>
<td>PrV-Ka</td>
<td>10^5</td>
</tr>
<tr>
<td>PrV-DgL/L</td>
<td>&lt;10^5</td>
</tr>
<tr>
<td>PrV-Ka</td>
<td>10^5</td>
</tr>
<tr>
<td>PrV-DgH/L</td>
<td>10^5</td>
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</tbody>
</table>

a RK13 or the indicated glycoprotein-expressing cell lines were infected at an MOI of 5 by PrV-Ka or phenotypically complemented double mutant viruses and harvested approximately 20 h p.i., and viral progeny was titrated on complementing cells. Given are the values of a representative experiment.

Genes were inserted into plasmid p3ie (29) under the control of the murine and HCMV IE1 promoter/enhancer complex, respectively. To complement the gD/gH double mutant, we used RK13-Nde cells expressing a gDH hybrid protein, which was previously shown to efficiently complement gh-, gl-, and gd-negative PrV mutants (25). A summary of the generated mutants with the introduced deletions and insertions of foreign sequences as well as the cell lines used for complementation is given in Fig. 1 and Table 1.

To verify protein expression patterns of the generated virus recombinants, RK13 cells were infected with phenotypically complemented mutants or PrV-Ka and harvested approximately 20 h p.i. Parallel Western blots were incubated with sera directed against gB, gD, gh, or gl or with a monoclonal antibody specific for PrV gE. As shown in Table 2, no gB signal could be detected in lysates of cells infected by mutant PrV-DgB, PrV-DgB/D, or PrV-DgB/H, while gh was not found in PrV-DgH, PrV-DgB/H, or PrV-DgD/H, or PrV-DgH/L-infected cells, nor was gD found in cells infected with any of the gd deletion mutants. gl, the complex partner of gh, was not present in lysates of cells infected with the gL deletion mutants but also not in those devoid of gh (PrV-DgH, PrV-DgB/H, and PrV-DgD/H), indicating that gl is either unstable or secreted into the cell culture supernatant in the absence of gh. Incubation with the anti-gE monoclonal antibody served as a loading control.

### Western blot analysis of glycoprotein deletion mutants.

![FIG. 2. Western blot analysis of glycoprotein deletion mutants.](image)
Accumulations of primary enveloped virus particles have been described to occur after the infection of swine kidney cells with a gB deletion mutant of PrV strain NIA-3 (49). Since we were unable to verify this finding in our gB mutant of PrV-Ka (19), we constructed a matching gB deletion mutant of PrV strain NIA-3. In ultrastructural analyses, no accumulations of primary virions were detectable in either RK13 or swine kidney cells (data not shown).

**PrV glycoproteins are not present in detectable amounts in perinuclear virions.** A thorough investigation of the protein composition of perinuclear virions is still hampered by a lack of a reliable purification protocol. Therefore, we performed immunoelectron microscopy on RK13 cells fixed 12 h after infection with PrV-Ka. Ultrathin sections were incubated with monoclonal or monospecific polyclonal antibodies against the essential glycoproteins gB (Fig. 5A and B), gD (Fig. 5E and F), and gH (Fig. 5G and H) as well as against gC (Fig. 5C and D) or gM (Fig. 5I and J). Whereas all of them reacted well with extracellular virions (Fig. 5B, D, F, H, and J), virions located in the perinuclear space were not decorated by gold label (Fig. 5A, C, E, G, and I). In contrast, as described previously (27), an antiserum against pUL34 (Fig. 5K and L) labeled only perinuclear virions (Fig. 5K) but not mature virus particles (Fig. 5L). Furthermore, unlike the pUL34-specific serum, none
of the glycoprotein-specific antibodies labeled the nuclear envelope.

PrV gB and gH do not colocalize to perinuclear vesicles formed after coexpression of pUL31 and pUL34. For an additional test of whether PrV glycoproteins gB and gH are targeted to the INM and may be included in primary virions, we used the capability of PrV pUL31 and pUL34 to form vesicles at the INM after simultaneous expression in RK13 cells (29). Vesicles formed after the transfection of expression plasmid p3ieUL31/UL34gfp were easily detectable by fluorescence microscopy, since pUL34 had been C-terminally tagged with GFP (Fig. 6, green fluorescence). After additional cotransfection with pcDNA-gH or pcDNA-gDH (25) and either a plasmid expressing full-length gB [gB(009)] or a C-terminally truncated gB [gB(008)] with enhanced fusogenicity (32, 46), the localization of the glycoproteins was determined by indirect immunofluorescence reactions with a monoclonal anti-gB antibody (A20-c26) or with rabbit anti-gH serum (Fig. 6, blue fluorescence). A significant colocalization of the immunofluorescence signals with the punctate GFP autofluorescence signal was not observed in any of the assays (Fig. 6), indicating that PrV gB,
DISCUSSION

The salient results of this study are as follows: (i) none of the tested PrV double deletion mutants in essential glycoproteins showed any obvious defect in nuclear egress, indicating that viral glycoproteins essential for virus entry play no role in the fusion of the primary envelope with the ONM in PrV; (ii) none of the tested envelope glycoproteins was detected by immunogold labeling in the inner nuclear envelope of PrV-infected rabbit kidney cells or in primary virions; (iii) neither PrV gB nor gH localizes to membranous vesicles induced by pUL31/pUL34 coexpression; and (iv) none of the tested PrV double deletion mutants showed an impairment of secondary envelopment or release of enveloped virions, demonstrating that viral glycoproteins essential for entry are not required for egress.

The mechanism of virion formation and egress in herpesviruses has been a long standing matter of debate (reviewed in references 40, 41, and 43). In an earlier model, it was speculated that capsids, which are formed in the nucleus, gain all tegument and envelope proteins of mature virions during budding at the INM. These perinuclear virions were then suggested to be transported from the perinuclear space via the lumen of the endoplasmic reticulum and the Golgi apparatus toward the plasma membrane by the cellular secretory pathway (reviewed in reference 54). For this model, it has to be postulated that all tegument proteins are actually imported into the nucleus and that all viral envelope proteins are targeted to the INM to become incorporated into the perinuclear virion. Moreover, this model supposes continuity between the perinuclear and the mature extracellular virion translating into identical protein composition. In recent years, increasing biochemical, genetic, and morphological data argued for the alternative envelopment-deenvelopment-reenvelopment model (57; reviewed in reference 43). This scenario suggests that perinu-
clear virions gain an envelope from the INM, which is subsequently lost by fusion with the ONM, thereby releasing the nucleocapsids into the cytoplasm for final tegumentation and envelopment. In this case, the composition of perinuclear (primary) virions and mature virus particles can differ drastically. The demonstration of proteins present in primary but not in mature virions and vice versa (27) is among the strongest evidence for this now widely accepted model (43).

This assumed fusion process was suggested to be mediated by a mechanism similar to that used during entry. In support of this, various glycoproteins of different herpesviruses were detected in the nuclear membranes of infected or transfected cells, e.g., gB of varicella-zoster virus (21), HSV-1 (1, 9, 15, 60, 62), Epstein-Barr virus (17, 18, 63), and HCMV (45, 52) as well as gC, gD, and gM of HSV-1 (5, 9, 60, 62).

Interestingly, PrV and HSV-1 deletion mutants lacking either gB, gD, gH, or gL, which are unable to fuse at the plasma membrane during entry, are apparently not impaired in nuclear egress (12, 19, 50), already indicating different requirements for the two fusion processes. Since it had become clear that functional redundancy may exist at various stages of virion formation (8, 11, 34), HSV-1 mutants that lack more than one of the viral glycoproteins required for entry have been constructed. Indeed, a recent report showed that perinuclear virions accumulated in the simultaneous absence of gB and gH (12), implying that gB and gH fulfill redundant functions in fusion with the ONM, whereas either one is essential for penetration.

In light of these results, we investigated the effect of different double deletion mutants in PrV lacking either gB and gD, gB and gH, gD and gH, or gH and gL. These viruses could be propagated only on cell lines providing both deleted gene products in trans, demonstrating that the growth defect on noncomplementing cells is indeed due only to the introduced deletions. Surprisingly, although as expected all mutants did not produce significant amounts of infectious progeny on noncomplementing cells, in ultrastructural studies, none of these mutants showed an obvious defect in nuclear egress. In fact, we were unable to detect any difference in the steps of virion morphogenesis in cells infected by wild-type PrV or the different glycoprotein mutants. Nucleocapsids were formed normally in infected nuclei and were found in the cytoplasm in close proximity to membranes and undergoing secondary envelopment. Moreover, numerous virus particles lining the plasma membrane could be detected, indicating that not only nuclear egress but also virion morphogenesis of PrV in the cytoplasm proceeds in the absence of the different glycoproteins. Due to the complexity of the system, we were so far unable to isolate a mutant virus simultaneously lacking all four essential glycoproteins (gB, gD, gH, and gL), which requires quadruple complementing cell lines, to test whether the presence of any of the essential proteins is necessary at all for virion morphogenesis. However, our data on the double mutants make it unlikely that this mutant would present with a drastically different phenotype during nuclear egress. Thus, at least in PrV, glycoproteins essential for penetration are neither

FIG. 6. gB and gH do not colocalize with vesicles formed by pUL31 and pUL34. RK13 cells were transfected with p3ieUL31/UL34gfp and plasmids expressing either full-length gB(009) (46) and gH or C-terminally truncated gB(008) (46) and gDH. Cells were fixed 2 days after transfection and incubated with a monoclonal antibody against gB (A20-c26) (red) and the monospecific serum against gH (blue). Autofluorescence and indirect immunofluorescence were detected by laser scanning confocal microscopy.
singly nor in combination required for nuclear egress or virion morphogenesis in and release from the cytoplasm.

These results differ from data on an HSV-1 mutant simultaneously lacking gB and gH (12). In HSV-1-deleted HaCaT and Vero cells, a significant amount of primary enveloped virions accumulated in the perinuclear space. This is a clear difference from our results with PrV. However, the block was not complete, and intracytoplasmic nucleocapsids and enveloped virions as well as virions on the cell surface could be observed. Taken together, both studies demonstrated that neither gH nor gB, either alone or in combination, is essential for nuclear egress, demonstrating that different fusion mechanisms have to exist for entry and egress.

Most of our studies on PrV morphogenesis were performed on rabbit kidney cells, which support virus replication to high titers but are not derived from the natural host. To test for the influence of the host cell, we also analyzed the various single and double glycoprotein deletion mutants on swine kidney cells (data not shown). Although in this cell system the absence of gB alone has been reported to result in accumulations of primary enveloped virions (49), we were unable to observe any differences in nuclear egress or virion morphogenesis from the rabbit kidney cells. The reason for this discrepancy is unclear at present.

In contrast to previous reports of other herpesviruses, we were unable to detect viral glycoproteins either in the nuclear envelope or in primary virions. Since there is still no reliable protocol for the purification of primary virions, we used immunoelectron microscopy on ultrathin sections, which, in our hands, has proven to be a powerful tool for the direct visualization of virion morphology and the presence of specific virion constituents (19). Since monoclonal antibodies might not react with presumably immature glycoprotein forms potentially present in the nuclear envelope, we used potent polyclonal antisera raised against the different glycoproteins. Our polyclonal gB serum is directed against gB purified from virions (35). It reacts with the uncleaved precursor as well as with the cleaved subunits in all tested applications and at very high dilutions (Fig. 2) and efficiently decorated intracytoplasmic and extracellular virions. However, no labeling was found on primary virions or at the nuclear membrane. Similar results were obtained with monospecific sera against gD, gH, or gM, which have been generated by immunization with bacterially expressed glutathione S-transferase fusion proteins comprising either the complete extraluminal domain (gH or gD) or the C-terminal tail (gM). It appears very unlikely that neither of these sera would react with the putative immature glycoprotein forms in the nuclear membrane or primary virions if they are indeed present. However, we cannot exclude the possibility that the amount of these proteins in the primary virions or in the nuclear envelopes is too low to be detected. In contrast, primary virions and the nuclear membrane were labeled by a pUL34-specific antiserum (27), showing that primary envelopes are accessible to antibodies in our assay.

In an alternative, independent approach, the coexpression of either gB and gH or gB and gDH with pUL31 and pUL34gfp had no detectable influence on vesicle formation from the INM induced by pUL31 and pUL34 (29), and immunofluorescence analysis showed no colocalization of gB, gH, or gDH with the punctate GFP signal. As shown previously, the expression of gB results in the formation of intracytoplasmic inclusions (46), which were separated from the nuclear rim by staining and perinuclear speckles of pUL34gfp, as was the gH-specific signal.

In our studies on PrV, none of single-deletion mutants lacking gB (19), gE (8), gH (19), gK (31), gL (H. Granzow, personal communication), gM (7), or gN (H. Granzow, personal communication) showed a defect in the nuclear egress of primary enveloped virus particles. Also, a variety of multiple-deletion mutants lacking, e.g., gE/gI (7), gE/gI/gM (8), gE/gI/gG (8), gG/gD/gE/gI (44), UL34/gK (30), or UL34/gM (30) showed no accumulation of virions in the perinuclear space, indicating that these proteins, singly or in the tested combinations, are also not required for this process. Of all the proteins tested, only the absence of the alphaherpesvirus-specific protein kinase pUS3 has so far been shown to result in a consistent impairment of release of primary virions from different alphaherpesviruses, e.g., PrV, HSV-1, or Marek’s disease virus (28, 53, 56, 64), from the perinuclear space. However, the molecular basis for this phenotype is still unclear.

Taken together, our data favor a model in which viral glycoproteins that are relevant for entry are not required for the egress of PrV from either the nucleus or the cytoplasm. The absence of PrV glycoproteins from the nuclear membrane and their dispensability for nuclear egress contrast findings from other herpesviruses. Thus, it is conceivable that PrV has evolved a mechanism for nuclear egress different from those of other herpesviruses. On the other hand, the demonstration of herpesvirus glycoproteins in the INM and in primary virions by primarily immunological detection is difficult to reconcile with the clear difference in morphology between primary enveloped particles, which lack the characteristic spikes, and mature intracytoplasmic and extracellular enveloped virions. Concerning the nuclear egress of PrV, it is clear that pUL31 and pUL34 are sufficient to form perinuclear virus-like vesicles, although it remains unclear whether these vesicles have the capacity to fuse with the ONM. Since viral fusion-active glycoproteins apparently do not play a role in the nuclear egress of PrV, our results suggest that cellular proteins normally required for intracellular membrane fusion (or the fusion of nuclear envelope vesicles after mitosis) either may be hijacked from cytoplasmic membranes to the inner and/or outer nuclear membrane or are already present and are involved in a hitherto-recognized fusion process affecting the nuclear membranes. For further investigation on the molecular mechanism of nuclear egress, a reliable protocol for the purification of primary virions to allow an analysis of their proteome would be extremely helpful. In addition, small interfering RNA knockdown experiments targeting genes encoding proteins known to be present in nuclear membranes should shed more light on cellular proteins involved in vesicle formation, fission, and probably fusion with the ONM.

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