Role of the cytoplasmic tails of pseudorabies virus glycoproteins B, E and M in intracellular localization and virion incorporation

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The cytoplasmic domains of several herpesviral glycoproteins encompass potential intracellular sorting signals. To analyse the function of the cytoplasmic domains of different pseudorabies virus (PrV) glycoproteins, hybrid proteins were constructed consisting of the extracellular and transmembrane domains of envelope glycoprotein D (gD) fused to the cytoplasmic tails of gB, gE or gM (designated gDB, gDE and gDM), all of which contain putative endocytosis motifs. gD is a type I membrane protein required for binding to and entry into target cells. Localization of hybrid proteins compared to full-length gB, gE and gM as well as carboxy-terminally truncated variants of gD was studied by confocal laser scanning microscopy. The function of gD hybrids was assayed by trans-complementation of a gD-negative PrV mutant. The carboxy-terminal domains of gB and gM directed a predominantly intracellular localization of gDB and gDM, while full-length gD and a tail-less gD mutant (gDc) were preferentially expressed on the cell surface. In contrast gDE, and a gDB lacking the putative gB endocytosis signal (gDB△29), were predominantly located in the plasma membrane. Despite the different intracellular localization, all tested proteins were able to complement infectivity of a PrV gD~ mutant. Cells which stably express full-length gD and plasma-membrane-associated gD hybrids exhibit a significant resistance to PrV infection, while cells expressing predominantly intracellularly located forms do not. This suggests that the assumed sequestration of receptors by gD, which is supposed to be responsible for the interference phenomenon, occurs at the cell surface.

Introduction

Herpesviral glycoproteins are involved in attachment and entry of free virions, in virus maturation and egress, and in modulation of direct viral cell-to-cell spread. Although the roles of several glycoprotein homologues within the family Herpesviridae have been at least partially identified, the functions of most glycoproteins are not well understood in detail (Mettenleiter, 2000; Steven & Spear, 1997). Several herpesviral glycoproteins are internalized from the cell surface by endocytosis (Tirabassi & Enquist, 1998). The carboxy-terminal regions of nearly all known glycoproteins of the alphaherpesvirus pseudorabies virus (PrV) encompass putative endocytosis signals, i.e. YxxL and dileucine motifs. These motifs are thought to mediate retrieval of proteins into the cytoplasm by interaction with adaptor complexes (Marsh & McMahon, 1999; Trowbridge et al., 1993). Generally, endocytosis has been shown to be important for receptor regulation, antigen presentation and protein recycling (Marks et al., 1996; Trowbridge et al., 1993; Trowbridge, 1991). Although the purpose of endocytosis of virally encoded glycoproteins has not been clarified, it was proposed as a mechanism to target membrane proteins to the site of final envelopment, i.e. the trans-Golgi network (Zhu, 1995). Recently, the intracellular targeting of several herpesviral glycoproteins has been analysed. Best studied are pseudorabies virus glycoproteins E and I (Tirabassi & Enquist, 2000, 1999, 1998), varicella-zoster virus (VZV) gE (Olson et al., 1998; Olson & Grose, 1997) and gB of human cytomegalovirus (HCMV; Meyer & Radsak, 2000; Radsak et al., 1996; Reschke et al., 1995).

HCMV gB was shown to be internalized prior to incorporation into the viral envelope (Radsak et al., 1996). Recent studies suggest an acidic cluster within the carboxy...
terminus of gB as the key determinant for cellular trafficking, which includes recycling via the early endocytic pathway and finally leads to an apical sorting of gB (Tugzov et al., 1999, 1998). In the case of VZV gE the tyrosine residue in the YxxL motive was determined to be important for internalization of the protein followed by recycling to the cell surface or targeting either to endosomal compartments or to the trans-Golgi network, which is the assumed site of envelopment (Alconada et al., 1999; Gershon et al., 1994; Olson & Grose, 1997).

PrV gE is efficiently endocytosed for the first 6 h post-infection only (Tirabassi & Enquist, 1998). However, biotinylation experiments showed that retrieved gE was not directly targeted into viral particles, and efficient endocytosis of gE was not required for gE incorporation into virions, nor for virulence or spread in the rat central nervous system (Tirabassi & Enquist, 2000, 1999, 1998). Thus, the importance of endocytosis for virion morphogenesis remains unclear.

Previously, we reported that PrV gB is efficiently endocytosed from the plasma membrane of transfected rabbit kidney cells. This process required the presence of a portion of the cytoplasmic tail containing dileucine and YxxL endocytosis signals. Other domains within the gB tail seem to be responsible for efficient incorporation into virion particles or for modulation of direct cell-to-cell spread. However, efficient endocytosis did not appear to be essential for any of these functions (Nixdorf et al., 2000).

gD of herpes simplex virus 1 (HSV-1) is an envelope component essential for binding to cellular gD receptors (Cocchi et al., 1998; Geraghty et al., 1998; Krummenacher et al., 1998; Lopez et al., 2000; Whitbeck et al., 1999, 1997) and virus entry. Similar functions have been described for PrV and bovine herpesvirus (BHV)-I gD (Geraghty et al., 1998), although a gD-independent entry pathway could be demonstrated for PrV and BHV-1 mutants (Schmidt et al., 1997; Schröder et al., 1997). Binding of exogenously added soluble gD as well as intracellular expression of gD in stably transfected cells was shown to inhibit infection of otherwise susceptible cells. This phenomenon is thought to be caused by sequestration of gD receptors (Dasika & Letchworth, 2000; Johnson et al., 1990; Petrovskis et al., 1988). For HSV-1 gD not only the cytoplasmic tail but also the transmembrane domain directed intracellular targeting including retention in the endoplasmic reticulum and the Golgi complex (Ghosh & Ghosh, 1999).

PrV gD is detectable at the cell surface despite the presence of a YxxL motif in the carboxy-terminal region (our unpublished observations). To test for function of this cytoplasmic domain and to study potential sorting capacities of other glycoprotein tails, we constructed truncated and chimeric forms of PrV gD. Hybrids were expressed in rabbit kidney cells (RK13), and intracellular localization as well as functional complementation of a gD-deficient virus mutant were analysed.

### Methods

#### Viruses and cells.

All virus mutants used in this study are based on PrV strain Kaplan (PrV-Ka) (Kaplan & Vatter, 1959). PrV-1112 carries a lacZ insertion cassette in the nonessential gG locus (Mettlenleiter & Raul, 1990) and behaves in a multitude of *in vitro* and *in vivo* experiments like wild-type PrV. PrV-gD- Pass was derived from a noninfectious gD- PrV mutant by serial passaging in cell culture (Schmidt et al., 1997).

For transient or stable expression, rabbit kidney cells (RK13) were transfected with 5 µg of plasmid DNA by using SuperFect reagent (Qiagen) under transfection conditions suggested by the manufacturer. To establish stable recombinants, cells were selected in medium containing 0.5 mg/ml G418 (Life Technologies).

#### Immunodetection.

Western blotting (immunoblotting), radioimmunoprecipitation and immunofluorescence analyses were performed as described previously (Lukács et al., 1985; Klupp et al., 1997) using monoclonal antibodies (MAbs) directed against PrV gD (b51-c5-1 or c14-c27), PrV gE (A9-b15-26), PrV gB (a80-c16) and a peptide antiserum recognizing the carboxy-terminal portion of PrV gM (Dijkstra et al., 1996). The antisera specific for the cytoplasmic tail of PrV gE was kindly provided by L. W. Enquist (Tirabassi & Enquist, 2000).

#### Confocal laser scanning microscopy.

For indirect immunofluorescence analysis, cells were seeded onto coverslips in six-well culture dishes, grown to confluency, and fixed with 3% paraformaldehyde for 20 min followed by permeabilization with 3% paraformaldehyde–0.3% Triton X-100 for 15 min. Cells were washed twice with PBS, and incubated with appropriate MAbs or antisera diluted in PBS for 1 h. After thorough washing, anti-mouse or anti-rabbit IgG–fluorescein isothiocyanate (FITC) conjugate (DAKO) was added for 45 min. Monolayers were then washed twice and counterstained with 10-6 M propidium iodide in PBS–10% glycerol. Finally, samples were analysed by confocal laser scanning microscopy (LSM510; Zeiss).

#### In vitro transcription and translation.

For determination of apparent molecular masses of the polypeptides, an *in vitro* transcription/translation assay using the Tnt coupled Reticulocyte Lysate System (Promega) was performed. Translation products were separated by SDS–PAGE (7.5% polyacrylamide) and visualized by autoradiography.

#### Construction of a gD-PrV-mutant.

To generate a gD- PrV mutant, a recombinant vector was created with upstream sequences comprising the U4,g (gE) gene and downstream U7,g (gB) sequences which were obtained by two-step-PCR from cloned BanHl fragment 7 of viral DNA. The primers for the upstream sequences were 5′ CACA-GCATGCCGAGCTATCGTCGACCTGGAGGTGATGTATGGGA 3′ and 5′ CACAGTGGACCTGCCCAGTGATGTACCAGCAGATCAATTAGGGAG 3′ [nucleotides 449–469 and 1900–1881 of GenBank accession no. M10996 (Rea et al., 1985) respectively]; for downstream sequences: 5′CACAGATCCGCGGGAAGCGTACACCGGGAATAGCGCAGTAAAAGCGCAG 3′ and 5′ CACAGTGGACGAGCTATCGTCGACCTGGAGGTGATGTATGGGA 3′ [nucleotides 1214–1233 of GenBank accession no. M144336 (Petrovskis et al., 1986), respectively]. Splh, Sull, BanHl and Knl restriction sites which were introduced for convenient cloning are underlined. Fragments were amplified using Pfx-Platinum polymerase (Life Technologies) and directly cloned into appropriately cleaved pUC19. The resulting plasmid was digested with BanHl and Sull, and lacZ or GFP marker genes were inserted. The resulting plasmids, designated pD0lacZ and pD0GFP respectively, were transfected with PrV-Ka DNA into RK13 cells which carry BanHl fragment 7 of PrV-Ka (ab7-13; A. Brack, unpublished data). Marker gene-expressing mutants were isolated. Correct deletion of gD sequences and insertion of marker
Table 1. Primers used for fusion PCR

<table>
<thead>
<tr>
<th>Mutant ORF</th>
<th>5' end primer*</th>
<th>Fusion primer†</th>
<th>3' end primer</th>
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<tr>
<td>DM</td>
<td>ATATGGATCCATGCTGCTGCC-AGGCTATT [nt 43–62 of GenBank accession no. AJ271966 (Brack et al., 2000)]</td>
<td>GTGTCGCTACATCTTCTTCCGCC-TGGTCGCGCTGGCATC [nt 1144–1164 of GenBank accession no. AJ271966 (Brack et al., 2000) and nt 2610–2590 of GenBank accession no. X97257 (Dijkstra et al., 1996)]</td>
<td>CACAGAATCTTATCCAAAGCCGAG-GTTTCGTCAC [nt 2404–2430 of GenBank accession no. X97257 (Dijkstra et al., 1996)]</td>
</tr>
<tr>
<td>DE</td>
<td>See above</td>
<td>GTGTCGCTACATCTTCTTCCGCC-TGGTCGCGCTGGCATC [nt 1144–1164 of GenBank accession no. AJ271966 (Brack et al., 2000) and nt 2610–2590 of GenBank accession no. X97257 (Dijkstra et al., 1996)]</td>
<td>CACACTCGAGACCACCTCGTGCCAG-GCCCGGG [nt 611–588 of GenBank accession no. D10452 (van Zijl et al., 1990)]</td>
</tr>
<tr>
<td>DB</td>
<td>See above</td>
<td>GTGTCGCTACATCTTCTTCCGCC-TGGTCGCGCTGGCATC [nt 1144–1164 of GenBank accession no. AJ271966 (Brack et al., 2000) and nt 2610–2590 of GenBank accession no. X97257 (Dijkstra et al., 1996)]</td>
<td>CACAGAATCTTATCCAAAGCCGAG-GTTTCGTCAC [nt 2404–2430 of GenBank accession no. X97257 (Dijkstra et al., 1996)]</td>
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<tr>
<td>DBA29</td>
<td>See above</td>
<td>See above</td>
<td>CCGAAATCTTACCGCGAGATGTGCAG-GTGGGC [nt 2730–2710 of GenBank accession no. M17321 (Robbins et al., 1987)]</td>
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* Restriction sites introduced for convenient cloning are underlined.
† Only nucleotide sequence of coding strand is shown.

Fig. 1. Predicted amino acid sequence of mutated gD. Carboxy-terminal amino acid sequences of gD, truncated gD and hybrid gD molecules are shown. The predicted transmembrane domain is shaded grey; YxxL and dileucine endocytosis motifs are underlined. The in-frame fusion sites are marked by arrows.

genes was verified by Southern- and Western blot analysis. In mutant PrV-gD0β gG sequences were also deleted due to homologous recombination of the duplicate gG promoter sequences, as has also been described for our previous gD−PrV mutant (Rauh & Mettenleiter, 1991).

Construction of truncated and hybrid gD open reading frames. The plasmid encoding the soluble form of gD, designated gDs, was generated by insertion of a BamH1–BstBI fragment excised from gDgl-CMV (Gerds et al., 1999) into BamH1/XbaI-cleaved pRc/CMV.
Fig. 2. For legend see facing page.
cells, since about half of the U₆ gene (Rauh & Mettenleiter, 1991). The rescue frequency of this gene (Rauh & Mettenleiter, 1991), which constitutively expresses the U₆ (gD) gene under control of the HCMV immediate-early promoter-enhancer region (gDs; see Fig. 1). Both were cloned into mammalian expression vectors under the control of the HCMV immediate-early promoter-enhancer complex. After transfection into RK13 cells, stably expressing cell lines were established and analysed.

In indirect immunofluorescence with an anti-gD MAb, cells expressing gDc showed a bright staining near the plasma membrane in addition to weaker intracellular spotted signals (Fig. 2C). Surface expression of gDc could be verified by immunofluorescence assay on non-permeabilized cells (Fig. 3C). Thus, deletion of the intracytoplasmic domain of gD had no drastic effect on subcellular localization. As expected gDs, which lacks the complete cytoplasmic tail and the predicted membrane spanning domain, is secreted in large amounts (not shown). In addition, a distinct membrane-associated staining was detectable in immunofluorescence on non-permeabilized (Fig. 3D) and permeabilized (Fig. 2D) cells.

Construction of cell lines expressing hybrid gD

Intracytoplasmic domains of different PrV glycoproteins are involved in intracellular targeting and localization (Nixdorf et al., 2000; Tirabassi & Enquist, 1999). To test whether the sorting signals also function in a heterologous context, hybrid proteins consisting of the amino-terminal portion of PrV gD, including its own transmembrane domain, and the cytoplasmic tail of gB or gE were constructed and designated gDB and gDE, respectively. In addition, a gD hybrid containing the carboxy-terminal part of gM (gDM) was engineered. So far, membrane localization and topology of PrV gM has not yet been determined in similar experiments. However, in the context of a hybrid protein containing the transmembrane and cytoplasmic domains of gE, the membrane localization and topology of this protein has been described (Nixdorf et al., 1999).

Role of cytoplasmic tails of PrV glycoproteins

Fig. 2. Intracellular localization of PrV glycoproteins. Normal RK13 and RK13 cells stably expressing wild-type or mutant glycoproteins as indicated were grown to confluency, fixed and permeabilized with 3% paraformaldehyde–0.3% Triton X-100, and incubated with the appropriate antisera or Mabs. Confocal laser scan microscopy was performed after incubation with FITC-conjugated secondary antibodies. Nuclear DNA was stained with propidium iodide.
Fig. 3. For legend see facing page.
been analysed. For gB, the carboxy-terminal 29 aa are responsible for efficient endocytosis from the cell surface (Nixdorf et al., 2000). Therefore, another hybrid gD protein was constructed carrying a truncated gB tail, here designated gDB29. It lacks the carboxy-terminal 29 aa of gB including the putative endocytosis signals, and is therefore identical to the carboxy-terminal domain of the previously described gB-008 (Nixdorf et al., 2000), which is predominantly associated with the plasma membrane. All hybrid ORFs were established by fusion-PCR and inserted into pcDNA3 for constitutive expression. Resulting plasmids were transfected into RK13 cells, and stably expressing cell lines were selected and analysed.

In indirect immunofluorescence using a gD-specific MAb, cells expressing gDE or gDBA29 showed a bright membrane-associated fluorescence in permeabilized cells (Fig. 2J, L), while gDM and gDB are found preferentially in the cytoplasm (Fig. 2I, K). On non-permeabilized cells gDM, gDE and gDBA29 were detected (Fig. 3I, J, L), although the signal on gDM-expressing cells was relatively weak. Non-permeabilized RK13-gDB cells failed to react (Fig. 2K). To determine expression and membrane orientation of gDE and gDM, polyclonal antisera specific for the corresponding carboxy-terminal portions were used in the immunofluorescence assay. Both antisera reacted only with permeabilized cells (not shown), indicating an intracellular localization of the gE and gM tails.

Characterization of gD derivatives

Correct expression of recombinant proteins was first analysed in an in vitro-coupled transcription/translation assay in the absence of microsomal membranes. Translation products were separated by SDS–PAGE, and labelled proteins were visualized by autoradiography. All truncated and hybrid forms of gD, including gDB (see below), migrated exactly as expected from the calculated molecular masses (data not shown). To determine proper intracellular processing, RK13 cells were metabolically radiolabelled, lysed, and precipitated with gD-specific MAb c14-c27. Precipitates were separated in SDS–PAGE under reducing conditions, and labelled proteins were visualized by autoradiography.

Entry of PrV into recombinant RK13 cells

RK13 cells are fully susceptible to infection by wild-type PrV, but constitutive expression of gD leads to partial resistance to infection (Petrovskis et al., 1988). This interference had been explained by intracellular sequestration of gD receptors, which constituted one of the first pieces of evidence for interaction of gD with cellular receptors (Johnson et al., 1990). To test whether gD with a mutated carboxy-terminal domain still caused interference indicative of interaction with cellular receptors, RK13 cells as well as recombinant RK13 expressing truncated and hybrid gD molecules were infected with serial dilutions of wild-type-like PrV-1112. Two days post-infection, cells were fixed, stained with X-Gal, and plaques and foci were counted. As demonstrated in Fig. 5(A), compared to RK13 cells infectivity of PrV-1112 was reduced at least 10-fold on RK13-gD, RK13-gDC, RK13-gDE and RK13-gDBA29 cells. Titration on RK13-gDM led to only a ca. 2-fold reduction of infectivity. No titre reduction was observed on RK13 cells expressing gDs and gDB. In summary, cell lines expressing gD forms with a preferential (gDM) or exclusive intracellular localization (gDB) exhibited a decreased interference (gDM) or no interference at all (gDB). Cells expressing a preferentially secreted gD (gDs) also do not exhibit interference.

To exclude fortuitous natural resistance of selected cell clones against PrV infection, an infectious gDΔ− PrV mutant (PrV-gDΔ−Pass) was titrated on these cells. PrV-gDΔ−Pass does not bind to known gD receptors, and, therefore, is resistant to gD-mediated interference (Nixdorf et al., 1999; Schmidt et al., 1997). As expected, PrV-gDΔ−Pass infected all recombinant RK13 cells with equal efficiency (Fig. 5B).

Incorporation of mutant gD into virions

gD is an important component of the virion envelope. To test for incorporation of mutated gD into virion particles, cell
Fig. 5. Titration of PrV on cells expressing wild-type and mutant gD. Wild-type-like PrV-1112 (A) and PrV-gD'Pass (B) were titrated in parallel on normal RK13 and RK13 cells stably expressing wild-type or mutant gD proteins. Data are averages of three independent experiments; vertical lines indicate standard deviations.

Fig. 6. Incorporation of wild-type and mutant gD into virions. RK13 cells and recombinant RK13 cells expressing wild-type or mutant gD proteins were infected with phenotypically complemented PrV-gD0β at an m.o.i. of 0.1. Virus progeny was purified by sucrose-gradient centrifugation, lysed, proteins separated in SDS–PAGE and transferred onto nitrocellulose; blots were probed with gD-specific MAb b51-c5-1 (A) or an anti-gE MAb A9-b15-26 (B).

lines expressing wild-type or mutant gD were infected with phenotypically complemented PrV-gD0β for 2 days at an m.o.i. of 0.1. Progeny virions were purified by sucrose-gradient centrifugation, and analysed by Western blot. As shown in Fig. 6(A), wild-type gD, gDc, gDM, gDE, gDB and gDBΔ29 were all detectable in purified virion preparations, although the amount of gDB in virions appeared to be less than that of the other gD mutants. As expected, in virus progeny derived from RK13 or RK13-gDs, which expresses soluble gD, no gD was detectable. For control, gE was present in comparable amounts...
in all preparations (Fig. 6B). To verify absence of non-structural proteins, parallel blots were probed with a U1,50-specific antiserum, which did not yield any signal (data not shown).

**Complementation of infectivity of PrV-gD<sup>−</sup> by gD derivatives**

gD is required for penetration of wild-type PrV but is not essential for direct cell-to-cell spread. Absence of gD results in drastically reduced virus titres by loss of the ability to enter target cells efficiently unless membrane fusion is induced experimentally, e.g. with polyethylene glycol (Rauh & Mettenleiter, 1991). To determine whether truncated gD or hybrid gD were able to complement the entry defect of a gD<sup>−</sup> PrV-mutant, RK13 cells expressing wild-type or mutated gD were infected with phenotypically complemented PrV-gD<sup>0</sup>β for 2 h at an m.o.i. of 0.1. Thereafter, residual extracellular input virus was inactivated by low-pH treatment (Mettenleiter, 1989) and cells were further incubated until complete cytopathic effect was observed. Virus progeny was then harvested, and titrated in parallel on RK13 cells expressing wild-type or mutant gD and RK13-gDc cells. After complete CPE had developed virus progeny was harvested, and titrated in parallel on RK13 cells (open bars) and RK13-gD cells (shaded bars). Data represent averages of three independent experiments; vertical lines indicate standard deviations.

**Discussion**

These studies were initiated to investigate the role of intracytoplasmic domains in subcellular localization and function of PrV glycoproteins. By confocal laser scanning microscopy gD and gE were easily detectable at the cell surface, while gB and gM could not be detected on non-permeabilized cells. Since the carboxy-terminal portions of all four proteins contain putative endocytosis motifs, we assumed that the carboxy-terminal domains have different properties which may result in distinct localization. The intracytoplasmic portions of several herpesviral glycoproteins comprise functional domains which are responsible for intracellular targeting. For example it has been shown that PrV gB and gE, as well as VZV gE and HCMV gB are internalized from the plasma membrane, and efficient endocytosis requires carboxy-terminally located sequence motifs (Nixdorf et al., 2000; Olson & Grose, 1997; Tirabassi & Enquist, 1999; Tugizov et al., 1999). To test whether the carboxy-terminal domains of gB, gE and gM are sufficient to induce endocytosis of a preferentially membrane-associated heterologous protein, carboxy-terminally truncated derivatives of the envelope glycoprotein gD, which is also present in the plasma membrane of infected cells, as well as hybrid proteins consisting of the extracellular and transmembrane region of gD fused to the carboxy-terminal domains of gB, gE and gM, were constructed and analysed.

Surprisingly, absence of the complete intracytoplasmic tail of gD (gDc) had no obvious effect on intracellular localization, incorporation into virions or recovery of infectivity. Therefore, this region of gD is apparently not essential for its function, at least in cultured cells. The only detectable difference compared to full-length gD was a slightly reduced interference after infection with wild-type PrV. In contrast, absence of the complete intracytoplasmic domain drastically decreased or abolished incorporation into virions of PrV gB and gE (Nixdorf et al., 2000; Tirabassi et al., 1997). However, it was recently suggested for HSV-1 gD that either the cytoplasmic tail or the
transmembrane domain are sufficient for targeting to the nuclear envelope, the predicted site for primary envelopment (Ghosh & Ghosh, 1999). Thus, the transmembrane region may also be sufficient for intracellular targeting and function of PrV gD.

As expected, a gD lacking the carboxy-terminal portion including the predicted transmembrane domain (gDs) is secreted, and is therefore not available for incorporation and restoration of infectivity. Though, by immunofluorescence, PrV gDs was shown to be present at the plasma membrane, RK13-gDs cells did not exhibit interference with wild-type PrV infection. It has previously been reported that interference could be induced by exogenous addition of soluble forms of HSV-1 (Johnson et al., 1990) and BHV-1 gD (Dasika & Letchworth, 2000). This difference can be explained by the high amount of soluble gD, at least 100 μg of purified protein/ml, which had to be added before infection to block virus entry. Thus, the amount of secreted gDs may be insufficient to mediate interference exogenously. Apparently, intracellular sequestration of gD receptors by gDs does also not occur in RK13-gDs cells.

The deduced amino acid sequence of PrV gM predicts eight hydrophobic stretches of sufficient length to span the lipid bilayer (Dijkstra et al., 1997). In PrV gM, dileucine and YxxL motives are found in the extreme carboxy-terminal hydrophilic domain but subcellular location of gM has not been analysed so far. Confocal microscopy suggests an intracellular localization of gM when it is expressed in RK13 cells. A similar, predominantly intracellular localization was observed (using a gD-specific MAb) in RK13 cells expressing a chimeric protein consisting of the extracellular and transmembrane portions of gD fused to the carboxy terminus of gM (gDM). This suggests that the carboxy-terminal portion of gM functions in a heterologous context to direct a membrane protein to the cytoplasm. If the described targeting capability of the transmembrane portion of HSV gD is also valid for PrV, there may thus be two competitive targeting domains within gDM. The weak membrane fluorescence observed in RK13-gDM cells may be a result of inefficient endocytosis, caused either by interaction of the gD portion with its membrane-associated receptor or by the suggested sorting signals in the membrane-spanning domain of gD. In contrast, the gB tail totally precluded surface localization of gD (see below). Unfortunately, the gM-specific antisera is directed against the extreme carboxy-terminal portion, one of the very few regions of this protein with a high antigenicity index, and hence absence of surface fluorescence on non permeabilized RK13-gM cells could be due to intracellular localization of the gM tail, despite membrane association of gM. Therefore, our studies do not prove absence of gM from the plasma membrane of RK13-gM cells. However, they clearly show the capacity of the gM tail to redirect a heterologous protein from a predominantly membrane-associated to a predominantly intracellular localization. In spite of the predominant intracytoplasmic localization gDM is efficiently incorporated into mature virion particles and supports recovery of infectivity. Interestingly, RK13-gDM cells show a reduced interference which correlates with the decreased surface expression compared to wild-type gD or gDc.

PrV gE is internalized from the plasma membrane, and the functional involvement of the cytoplasmic tail within the endocytosis process has been analysed in detail (Tirabassi & Enquist, 1999, 1998). However, retrieval of gE is not necessary for incorporation into virions nor for infectivity (Tirabassi & Enquist, 1999). In RK13-gE cells gE shows a distinct membrane localization in addition to an intracellular localization. This contrasting result may be due to the different assay systems. Previously, gE endocytosis was detected in infected cells during the first 6 h after infection (Tirabassi & Enquist, 1998). We analysed a stably gE-expressing cell line which presumably leads to a steady-state production level. Correlating with the surface expression of gE, gDE is also detected on the cell surface and causes interference. In addition, gDE is fully able to complement the entry defect of gD− PrV.

PrV gB expressed in RK13 cells is endocytosed from the plasma membrane (Nixdorf et al., 2000). In contrast to a more uniform intracellular distribution of gE, in RK13-gB (Nixdorf et al., 2000) or MT-3 MDBK cells (Rauh & Mettenleiter, 1991) gB is present in intracytoplasmic vesicles (unpublished). Thus, gB was not detectable on the cell surface, except when at least the carboxy-terminal 29 amino acids, which include putative endocytosis signals, are deleted. This localization pattern is reflected by hybrid proteins consisting of the extracellular and transmembrane portions of gD and either the complete gB tail or a gB tail lacking the putative endocytosis signals. As demonstrated by confocal laser scanning microscopy, FACS analysis (not shown) and immunofluorescence on non-permeabilized cells, the full-length gB tail seems to be capable of mediating efficient retrieval of a heterologous protein, since gDB is only detected intracellularly, and not on the cell surface. In contrast, gDBΔ29 is preferentially detected in the plasma membrane. However, interpretation of these results is complicated due to the aberrant migration of gDB in gel electrophoresis. Neither in RK13-gDB nor in trans-complemented virions was a protein detectable which migrated as expected. Sequencing as well as in vitro translation of the recombinant ORF verified correct construction and proper expression of gDB, but the intracellularly expressed hybrid protein migrated with an apparent molecular mass ca. 10 kDa smaller than expected. This might be an artefact caused by unusual folding of gDB, but considering the normal migration of gDBΔ29 we think it more likely that an altered processing of gDB occurs after retrieval from the plasma membrane, for which the full-length tail of gB is responsible. However, gDB as well as gDBA29 were incorporated into virions and complemented infectivity of gD− PrV despite their different cellular localization. As observed with gDs and partially reflected by RK13-gDM, no interference was detected on
RK13-gDB cells, which again correlates with the absence of gDB from the plasma membrane.

In summary, we report distinct roles of carboxy-terminal portions of PrV glycoproteins for subcellular targeting of hybrid proteins. This altered localization does not strictly correlate with presence or absence of endocytosis motifs within the carboxy-terminal domain, suggesting additional properties in the cytoplasmic portion or other parts of the proteins which are responsible for localization. Surprisingly, gD function in virus entry is not or only slightly reduced in all hybrid proteins irrespective of their different intracellular localization. In addition, surface localization of gD and gD hybrids correlated with resistance against superinfection by gD-positive PrV, which indicates that the assumed sequestration of gD receptor(s) by celluarily expressed gD may occur at the cell surface.

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References


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