Unidirectional complementation between glycoprotein B homologues of pseudorabies virus and bovine herpesvirus 1 is determined by the carboxy-terminal part of the molecule

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The most highly conserved glycoproteins in herpesviruses, homologues of glycoprotein B (gB) of herpes simplex virus, have been shown to play essential roles in membrane fusion during penetration and direct cell-to-cell spread of herpes virions. In studies aimed at assessing whether sequence conservation is reflected in the conservation of functional properties, we previously showed that bovine herpesvirus 1 (BHV-1) gB was able to functionally complement a gB- PrV mutant. To analyse in detail the function of gB in BHV-1, and to be able to test for reciprocal complementation between pseudorabies virus (PrV) and BHV-1 gB, we isolated a gB- BHV-1 mutant on a cell line stably expressing BHV-1 gB. Functional analysis showed that BHV-1 gB was essential for penetration as well as for direct cell-to-cell spread of BHV-1 gB. However, PrV gB was unable to complement plaque formation, i.e. direct cell-to-cell spread, or penetration of gB- BHV-1 virions despite its incorporation into the virion envelope. Analysis of cell lines expressing chimeric gB molecules composed of PrV and BHV-1 gB showed that plaque formation of both gB- mutants was complemented when the carboxy-terminal half of the chimeric gB was derived from BHV-1 gB and the amino-terminal half from PrV gB. In the opposite case, unidirectional complementation occurred. Although the chimeric molecules were generally less efficient in complementing infectivity of free virions, a similar complementation pattern was observed. In summary, our data show a unidirectional pattern of transcomplementation between the gB glycoproteins of PrV and BHV-1. This indicates that these proteins are functionally related but not identical. The unidirectional transcomplementation pattern was determined by the provenance of the carboxy-terminal half in chimeric gB proteins indicating that regions which are important for gB function but differ between PrV and BHV-1 reside in this part of gB.

Introduction

Herpesviruses encode a large number of glycoproteins which are involved in several steps during virus replication including attachment, penetration, release, direct cell-to-cell spread and syncytia formation. The most highly conserved herpesvirus glycoproteins are homologous to the gB glycoprotein of herpes simplex virus type 1 (HSV-1). In all cases analysed, gB homologues have been shown to be essential for penetration of free virions involving fusion between the virion envelope and the cellular cytoplasmic membrane, as well as direct transmission of infectivity from primary infected cells to neighbouring noninfected cells, designated as direct viral cell-to-cell spread (Spear, 1993). Therefore, gB- virus mutants could only be isolated on transgenic cell lines that provide the missing glycoprotein in trans and phenotypically complement the respective virus mutant (Cai et al., 1988; Peeters et al., 1992; Rauh et al., 1991).

The high conservation of primary structure of herpesvirus gB homologues as well as their apparently related functions prompted us to analyse their trans-complementation capabilities, i.e. whether different gB homologues are able to functionally complement defects associated with the lack of gB in heterologous herpesviruses. To this end, genetically engineered pseudorabies virus (PrV) and HSV-1 mutants that lacked gB and corresponding complementing cell lines expressing PrV and HSV-1 gB, respectively, were isolated. Cross-complementation studies showed that whereas PrV was able to complement a gB- HSV-1 mutant, complementation of gB- PrV by HSV-1 gB was not observed (Mettenleiter & Spear, 1994). PrV and HSV-1 gB share 50% identical amino acids (Bzik et al., 1984; Pellet et al., 1985; Robbins et al., 1987). Also, whereas PrV gB is
post-translationally cleaved by a protease located in the trans-Golgi into two subunits that remain linked by disulphide bonds (Hampl et al., 1984; Lukács et al., 1985; Whealy et al., 1990). HSV-1 gB is not cleaved (Claesson-Welsh & Spear, 1986). Therefore, the observed unidirectional complementation could be due to the lower homology as compared to other gB proteins (see below), or the difference in post-translational modification.

BHV-1 and PrV are more closely related than PrV and HSV-1 and belong to the same genus Varicellovirus within the subfamily Alphaherpesvirinae (Roizman et al., 1992). In contrast, HSV-1 is grouped into a separate genus Simplexvirus. The gB glycoproteins of PrV and BHV-1 exhibit 63% amino acid identity (Robbins et al., 1987; Whitbeck et al., 1988) and both are post-translationally cleaved into disulphide-linked subunits (Hampl et al., 1984; Lukács et al., 1985; Van Drunen Littel-van den Hurk & Babiuk, 1986). These glycoproteins, therefore, are more alike than PrV and HSV-1 gB. We have shown previously that both defects associated with lack of gB in PrV, i.e. lack of penetration and absence of direct cell-to-cell spread, could be complemented in trans by BHV-1 gB-expressing cells (Rauh et al., 1991). Furthermore, a PrV gB- but BHV-1 gB-expressing PrV mutant proved to be fully infectious, indicating that BHV-1 gB was able to provide all functions necessary for productive replication of gB-PrV (Kopp & Mettenleiter, 1992). Since PrV and BHV-1 gB are more similar than PrV and HSV-1 gB, we continued our studies by isolating a gB- BHV-1 mutant after insertional mutagenesis of the BHV-1 gB gene by introduction of a β-galactosidase expression cassette. Analysis of the mutant showed that, as demonstrated for the gB proteins of PrV and HSV-1, BHV-1 gB is essential for penetration of free BHV-1 virions, as well as for direct cell-to-cell spread of BHV-1. Surprisingly, neither the defect in penetration nor the lack of direct cell-to-cell spread of gB- BHV-1 could be complemented by PrV gB. Infection of cell lines expressing chimeric gB proteins showed that this unidirectional complementation was determined by the provenance of the carboxy-terminal half of the molecule identifying this part of the protein as being of primary importance for the observed phenotype.

Methods

Viruses and cells. PrV strain Ka (Kaplan & Vatter, 1959) and BHV-1 strain Schönböken (kindly provided by O. C. Straub, Tübingen, Germany) were grown on Madin-Darby bovine kidney (MDBK) cells. The gB- PrV mutant 4112 carrying a gG (previously called gX) -fl-galactosidase expression cassette in the partially deleted PrV gB-gene was propagated on cell line MT-3 which constitutively expresses PrV gB (Rauh et al., 1991). For isolation of the gB- BHV-1 mutant the constitutively BHV-1 gB-expressing cell line G1 was used (Rauh et al., 1991).

Plasmids. To obtain a gB- BHV-1 mutant, a SaI–BamHI expression cassette encompassing the lacZ gene from E. coli under control of the PrV gG promoter (Mettenleiter & Rauh, 1990) was inserted into the unique XhoI site in the middle of the BHV-1 gB ORF after fill-in of 5' overhangs by Klenow polymerase (Sambrook et al., 1989). This resulted in interruption of the BHV-1 gB ORF in plasmid pdlacZ (Fig. 1). Transcriptional orientation of the lacZ transgene was parallel to the BHV-1 gB gene. Construction of plasmids pMTB-P and pMTB-B for expression of chimeric gB proteins is detailed below.

DNA isolation, transfection and blue plaque screening. Virion DNA was isolated by phenol extraction from virions purified by centrifugation through a 30% sucrose cushion as described (Rauh & Mettenleiter, 1991). For isolation of gB BHV-1, virion DNA and CsiCl-gradient purified pdlacZ DNA were coprecipitated with the calcium phosphate technique (Graham & van der Eb, 1973) and transfected into G1 cells. Transfection progeny was titrated on G1 cells and analysed for blue plaque phenotypes under a Bluo-Gal (BRL) agarose overlay (Mettenleiter & Rauh, 1990). For staining of infected cells, monolayers were fixed for 10 min in 2% formaldehyde-0.2% glutaraldehyde-0.02% Nonidet P-40-0.1% sodium deoxycholate in PBS, washed three times with PBS; and incubated with substrate solution containing 1 mg/ml of X-Gal, 16 mM-potassium ferricyanide, 16 mM-potassium ferrocyanide and 2 mM-magnesium chloride at 37°C until a blue colour developed. Cell lines were established after cotransfection of MDBK cells with recombinant gB plasmid and plasmid pSV2neo (Southern & Berg, 1982) conferring resistance to Geneticin (Sigma).

Southern blot hybridization and radioimmunoprecipitation. For Southern blotting, virion DNA was cleaved with restriction enzymes and the resulting fragments were separated in 0.8% agarose gels. After transfer to nylon membranes, hybridization with β-galactoside expression cassette which were lost during cloning are indicated in parentheses. Arrows denote the transcriptional direction of the gB and β-gal genes.

Fig. 1. Construction of gB- BHV-1 mutant 2115. (a) HindIII restriction fragment map of the BHV-1 genome (Mayfield et al., 1983). (b) Detail of fragment A encompassing the BHV-1 gB gene; the unique XhoI site used for insertion is indicated. (c) Insertion of the gG-β-galactosidase expression cassette into the gB gene. The SaI and BamHI sites flanking the β-gal expression cassette which were lost during cloning are indicated in parentheses. Arrows denote the transcriptional direction of the β-gal genes.
One-way complementation of PrV and BHV-1 gB precipitation (Lukács et al., 1985). MAbs 5/14 and 2/22 (anti-PrV gB; Lukács et al., 1985; Schreurs, 1988; kindly provided by H.-J. Rziha, Tübingen, Germany), 42/18/7 and 72/14/6 (anti-BHV-1 gB; Fehler et al., 1992) and 21/3/3 (anti-BHV-1 gD; Fehler et al., 1992) were used for precipitation. Precipitates were separated in SDS-polyacrylamide gels and fluorographic images were obtained on Kodak XAR-5 film.

PEG-induced penetration. Complementing BHV-1 gB-expressing G1 cells in six-well plates were inoculated with the respective viruses for 1 h at 37 °C. Thereafter, the inoculum was removed and the cells were treated with PEG as described (Rauh et al., 1991; Sarmiento et al., 1979).

Immunoelectron microscopy. Confluent monolayers were infected at an m.o.i. of 5 with the respective viruses, and supernatants were harvested after complete degeneration of the cells. Virions were collected after centrifugation through 5 ml of a 30% sucrose cushion, and purified through a discontinuous 50, 40, and 30% sucrose gradient. Immunolabelling and negative staining were performed as reported previously (Rauh et al., 1991).

Results

Isolation of a gB- BHV-1 mutant

Virus progeny resulting from cotransfections of purified BHV-1 DNA and plasmid pDlacZ into BHV-1 gB-expressing G1 cells was harvested and titrated under a Bluo-Gal agarose overlay on G1 cells. Blue staining plaques were purified until a homogeneous blue plaque phenotype was obtained. One plaque isolate, designated 2115, was randomly selected for further analysis. To ascertain correct integration of the mutated BHV-1 gene replacing the wild-type gene, virus DNA from wild-type BHV-1 (Fig. 2, lanes 1) and mutant 2115 (Fig. 2, lanes 2) was cleaved with HindIII; fragments were then separated in a 0.8% agarose gel and stained with ethidium bromide (Fig. 2a). Compared to wild-type DNA, DNA from mutant 2115 exhibited a larger HindIII fragment A, whereas the other fragments comigrated with wild-type BHV-1 fragments. Since the BHV-1 gB gene is localized in HindIII fragment A (Misra et al., 1988; Whitbeck et al., 1988), this increase in size could be explained by insertion of the 3.6kb β-gal expression cassette. Hybridization with a BHV-1 gB-specific probe (Fig. 2b) highlights the size difference in HindIII fragments A of wild-type and mutant 2115 DNA. Only the larger HindIII fragment A of mutant 2115 DNA hybridized with a β-gal-specific probe (Fig. 2c). These data indicate correct replacement of the wild-type BHV-1 gB gene with the mutated gene in mutant 2115.

Mutant 2115 lacks gB

To test for the absence of gB in mutant 2115, BHV-1 gB-expressing G1 cells (Fig. 3, lanes 1–3) or normal MDBK cells (Fig. 3, lanes 7–9) were infected at an m.o.i. of 5 with mutant 2115 propagated on G1 cells, and proteins were labelled with [35S]methionine. After complete CPE was observed, virions were purified from the supernatant and virus proteins were precipitated with anti-BHV-1 gB MAb 42/18/7 (Fig. 3, lanes 1 and 7), anti-PrV gB MAb 5/14 (Fig. 3, lanes 2 and 8) and anti-BHV-1 gD MAb 21/3/3 (Fig. 3, lanes 3 and 9). Whereas BHV-1 gD was precipitated in similar amounts from both preparations, indicating the presence of comparable amounts of virions, BHV-1 gB was only precipitated when mutant 2115 was grown on BHV-1 gB-expressing G1 cells (Fig. 3).
gB-expressing cells and normal MDBK cells.*

2115 virions after replication on noncomplementing infected by phenotypically complemented mutant 2115. BHV-1 gB was also undetectable after immunoprecipitation of whole cell extracts of MDBK cells. BHV-1 gB also recognizes an epitope in the amino-terminal half of mutant 2115, this indicates the absence of gB in mutant 2115, a part of the BHV-1 gB gene resident in G1 cells with a concomitant elimination of the gB defect in the virus genome by the wild-type BHV-1 were inoculated at an m.o.i. of 5 onto either BHV-1 gB-expressing G1 cells [2115 (G1), wt (G1)], normal MDBK cells [2115 (M), Swt (M)] or PrV gB-expressing MT-3 cells [2115 (MT-3)]. After complete CPE was observed, supernatants were harvested and titrated on complementing G1 cells either with or without PEG treatment.

† After infection of G1 cells by mutant 2115 grown on normal MDBK cells, no plaques were observed at the lowest dilution of 10^{-1} tested.

‡ Ratio of titres with and without PEG treatment is indicated.

**BHV-1 gB is essential for penetration of BHV-1**

To analyse whether gB is essential for replication of BHV-1, BHV-1 gB-expressing G1 and normal MDBK cells were infected with phenotypically complemented mutant 2115 at an m.o.i. of 5. After complete CPE had developed, supernatants were harvested and titrated. Results are shown in Table 1. As expected, wild-type BHV-1 exhibited similar titres on G1 and MDBK cells. In contrast, mutant 2115 after propagation on complementing G1 cells was able to efficiently form plaques on G1 cells, whereas only very few plaques were detected on MDBK cells. These single plaques on MDBK cells were due to rescue of the gB defect in the virus genome by the wild-type gB gene resident in G1 cells with a concomitant elimination of the lacZ transgene. Plaques were not observed after inoculation of either G1 or MDBK cells with virus progeny resulting from infection of normal MDBK cells by phenotypically complemented mutant 2115. In addition, after X-Gal staining no blue cells were observed. These data show that gB is essential for productive replication of BHV-1. They also demonstrate that mutant 2115 virions released from noncomplementing cells are noninfectious and that the defect in infection by gB^- BHV-1 virions resides prior to expression of the lacZ transgene, presumably during entry.

To test for this possibility, wild-type BHV-1 and mutant 2115 were propagated on BHV-1 gB-expressing G1 or normal MDBK cells, and supernatants were assayed on G1 cells in the presence or absence of PEG. PEG has been shown to induce membrane fusion which can overcome a defect in penetration associated with the lack of an essential glycoprotein (Sarmiento et al., 1979).

Table 2 shows the results of a representative experiment. Wild-type BHV-1 propagated on G1 or MDBK cells exhibited a similar titre of between 1.0 x 10^7 and

**Table 2. PEG-induced plaque formation of gB^- BHV-1***

<table>
<thead>
<tr>
<th>PEG treatment</th>
<th>wt (G1)</th>
<th>wt (M)</th>
<th>2115 (G1)</th>
<th>2115 (M)</th>
<th>2115 (MT-3)</th>
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<tr>
<td>+</td>
<td>1.4 x 10^7</td>
<td>1.0 x 10^7</td>
<td>2.5 x 10^6</td>
<td>8.2 x 10^5</td>
<td>1.7 x 10^6</td>
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<tr>
<td>-</td>
<td>1.6 x 10^7</td>
<td>1.4 x 10^7</td>
<td>3.2 x 10^6</td>
<td>&lt; 10^7</td>
<td>38</td>
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* Phenotypically complemented gB^- BHV-1 mutant 2115 or wild-type BHV-1 were inoculated at an m.o.i. of 5 onto either BHV-1 gB-expressing G1 cells [2115 (G1), wt (G1)], normal MDBK cells [2115 (M), Swt (M)] or PrV gB-expressing MT-3 cells [2115 (MT-3)]. After complete CPE was observed, supernatants were harvested and titrated on complementing G1 cells either with or without PEG treatment.

† After infection of G1 cells by mutant 2115 grown on normal MDBK cells, no plaques were observed at the lowest dilution of 10^{-1} tested.

‡ Ratio of titres with and without PEG treatment is indicated.

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Fig. 3. Radioimmunoprecipitation of mutant 2115 virions grown on different host cells. BHV-1 gB-expressing G1 (lanes 1-3), PrV gB-expressing MT-3 (lanes 4-6) or normal MDBK cells (lanes 7-9) were infected at an m.o.i. of 5 with phenotypically complemented gB^- BHV-1 mutant 2115 and labelled from 2 h.p.i. until 18 h.p.i. with [35S]methionine. After virus purification proteins were precipitated with anti-BHV-1 gB MAb 42/18/7 (lanes 1, 4 and 7), anti-PrV gB MAb 5/14 (lanes 2, 5 and 8) and anti-BHV-1 gD MAb 21/3/3 (lanes 3, 6 and 9). Precipitates were separated in a nonreducing SDS-8% polyacrylamide gel.
One-way complementation of PrV and BHV-1 gB

Fig. 4. Plaque formation of phenotypically complemented gB− BHV-1 and gB− PrV on G1, MT-3 and normal MDBK cells. PrV gB-expressing MT-3 cells, BHV-1 gB-expressing G1 cells and normal MDBK cells were infected under plaque assay conditions with gB− PrV and gB− BHV-1 after phenotypic complementation by propagation on MT-3 or G1 cells, respectively. Two days after infection plaques were stained with X-Gal. To more clearly show lack of complementation of plaque formation of gB− BHV-1 on MT-3 and MDBK cells as well as of gB− PrV on MDBK cells, 100-fold higher concentration of virus was used for inoculation. Bar, 300 μm.

BHV-1 gB is essential for plaque formation of BHV-1

Penetration of free infectious virions and plaque formation by direct cell-to-cell spread are related but distinct processes during herpesvirus infection (Mettenleiter, 1994; Spear, 1993). Since gB− BHV-1 mutant 2115 exhibited a defect in penetration we were interested in analysing its ability to form plaques. To this end, BHV-1 gB-expressing G1 and normal MDBK cells were infected under plaque assay conditions with phenotypically complemented mutant 2115. Two days after infection cells were fixed and stained with X-Gal. As demonstrated in Fig. 4, plaques only developed on BHV-1 gB-expressing G1 cells, whereas after infection of normal MDBK cells only single blue-staining cells appeared. This indicated that phenotypically BHV-1 gB complemented mutant 2115 was able to infect primary target cells and express β-galactosidase. However, virus spread leading to plaque formation did not result in noncomplementing cells, demonstrating that gB is essential for direct cell-to-cell spread of BHV-1.

PrV gB fails to restore plaque formation by gB− BHV-1

Our studies demonstrated that BHV-1 gB exerts similar functions during the viral replicative cycle as does PrV gB: both are essential for infectious entry of free virions.
as well as direct cell-to-cell spread. Since BHV-1 gB has already been shown to complement gB- PrV (Kopp & Mettenleiter, 1992; Rauh et al., 1991), we then analysed whether reciprocal complementation could be observed, i.e. whether PrV gB was able to complement the defect in gB- BHV-1. To assay for complementation of the cell-to-cell spread defect of gB- BHV-1, PrV gB-expressing MT-3 cells were infected under plaque assay conditions with gB- PrV or gB- BHV-1 which had been phenotypically complemented by propagation on PrV gB or BHV-1 gB-expressing cells, respectively. The results after X-Gal staining are shown in Fig. 4. Whereas MT-3 cells readily supported plaque formation of gB- PrV, they behaved like noncomplementing MDBK cells after infection with gB- BHV-1 mutant 2115, i.e. only single infected blue-staining cells appeared. In contrast, both G1 and MT-3 cells were able to complement plaque formation of gB- PrV. Therefore, although both cell lines are derived from the same parental cell, and both constitutively express the respective glycoprotein in comparable amounts (Rauh et al., 1991), complementation of gB- BHV-1 plaque formation by PrV gB did not occur.

PrV gB fails to restore infectivity of gB- BHV-1 virions

To test for complementation of infectivity of gB- BHV-1 virions, MT-3 cells were infected with phenotypically BHV-1 gB-complemented mutant 2115 at an m.o.i. of 5. After complete CPE had developed, supernatants were titrated on G1 cells with or without PEG treatment (Table 2; see also Fig. 10). Without PEG treatment 38 p.f.u./ml were obtained, which increased after PEG treatment approx. 4500-fold to a titer of 1.7 x 10^5 p.f.u./ml. Mutant 2115 virions lacking any gB after replication in MDBK cells did not form plaques at the lowest dilution of 10^-1 tested. Similar results were obtained in two independent experiments. These data show that MT-3 cells essentially do not complement infectivity of free gB- BHV-1 virions.

PrV gB is incorporated into gB- BHV-1 virions

Since incorporation of the glycoprotein into the virion envelope is a prerequisite for its function during penetration, we tested for the presence of PrV gB in gB- BHV-1 virions after infection of PrV gB-expressing MT-3 cells by phenotypically complemented mutant 2115. Lysates of purified virions were precipitated by anti-BHV-1 gB MAb 42/18/7 (Fig. 3, lane 4), anti-PrV gB MAb 5/14 (Fig. 3, lane 5) or anti-BHV-1 gD MAb 21/3/3 (Fig. 3, lane 6). It is evident that PrV gB was precipitated from mutant 2115 virions after propagation on MT-3 cells, whereas BHV-1 gB was absent. Precipitation of BHV-1 gD served as a positive control.
Construction of cell lines expressing chimeric gB molecules

The observed unidirectional transcomplementation pattern between otherwise highly homologous glycoproteins prompted us to attempt to delineate a region on the respective gB protein responsible for this phenotype. To this end, cell lines expressing chimeric gB molecules consisting of one part PrV gB and one part BHV-1 gB were constructed. An XhoI site conserved between PrV and BHV-1 gB which is located approx. 50 nt upstream of the sequences encoding the proteolytic cleavage site in BHV-1 gB and approx. 60 nt upstream in PrV gB was used for gene fusion. Therefore, the fusions essentially led to exchange of the proteolytically processed subunits. As shown in Fig. 6(a) for the construction of plasmid pMTP-B the PrV gB gene from plasmid pMTgII was subcloned after BamHI–SphI cleavage. Then, the amino-
Fig. 8. Radioimmunoprecipitation of chimeric gB proteins. G1 (lanes 1, 5, 9 and 13), MT-3 (lanes 2, 6, 10 and 14), MCB-1 (lanes 3, 7, 11 and 15) and MCB-2 cells (lanes 4, 8, 12 and 16) were infected with phenotypically complemented gB- PrV at an m.o.i. of 5 and proteins were labelled with [35S]methionine. At 18 h p.i. when complete CPE was visible, virus was purified from the supernatants, lysed, and precipitated with anti-PrV gB MAbs 5/14 (lanes 1-4) and 2/22 (lanes 5-8) or anti-BHV-1 gB MAbs 42/18/7 (lanes 9-12) and 72/14/6 (lanes 13-16). Precipitates were separated in a SDS-8% polyacrylamide gel under nonreducing conditions (a) or in a SDS-10% polyacrylamide gel under reducing conditions (b). Positions of uncleaved precursor gBa, as well as cleavage products gBb and gBc are indicated in (b). Positions of molecular mass markers are also indicated.

terminal part of the PrV gB gene was excised by complete BamHI and incomplete XhoI cleavage and inserted downstream from the mouse metallothionein promoter into plasmid pMTgI replacing the corresponding BamHI-XhoI fragment of BHV-1 gB. To construct plasmid pMTB-P (Fig. 6 b) the BamHI-XhoI fragment of pMTgI encompassing the carboxy-terminal part of BHV-1 gB was replaced by the corresponding fragment from pMTgII containing the carboxy terminus of PrV gB by fusing a 3.6 kb XhoI-BamHI fragment of pMTgII containing the carboxy terminus of PrV gB to a 6.5 kb BamHI-XhoI fragment of pMTgI encompassing the metallothionein promoter and the amino-terminus of BHV-1 gB. Correct fusion was verified by both plasmids by sequencing (data not shown). Both plasmids were used in cotransfections with the selectable vector pSV2neo (Southern & Berg, 1982) and Geneticin-resistant cell clones were picked and assayed for expression of functional gB by their ability to complement plaque formation of a gB- PrV mutant. Two cell lines, MCB-1 containing pMTP-B and MCB-2 containing pMTB-P, were analysed in detail. The structural properties of the respective gB proteins are summarized in Fig. 7.

To analyse gB expression in recombinant cell lines, BHV-1 gB-expressing G1 cells (Fig. 8, lanes 1, 5, 9 and 13), PrV gB-expressing MT-3 cells (Fig. 8, lanes 2, 6, 10 and 14), MCB-1 cells expressing P-B gB (Fig. 8, lanes 3, 7, 11 and 15) and MCB-2 cells expressing B-P gB (Fig. 8, lanes 4, 8, 12 and 16) were infected with phenotypically complemented gB-PrV at an m.o.i. of 5, and labelled for 16 h with [35S]methionine. Thereafter, virions were purified from supernatants, lysed, and precipitated with anti-PrV gB MAb 5/14 (Fig. 8, lanes 1-4), anti-PrV gB.
One-way complementation of PrV and BHV-1 gB

MAb 2/22 (Fig. 8, lanes 5–8), anti-BHV-1 gB MAb 42/18/7 (Fig. 8, lanes 9–12) and anti-BHV-1 gB MAb 72/14/6 (Fig. 8, lanes 13–16). Precipitates were separated under nonreducing (Fig. 8a) and reducing conditions (Fig. 8b). For reactivity of antibodies see Fig. 7. It is evident that both anti-BHV-1 antibodies precipitated BHV-1 gB from G1 cells (Fig. 8, lanes 9 and 13), and failed to precipitate PrV gB from MT-3 cells (Fig. 8, lanes 10 and 14). In contrast, both anti-PrV antibodies precipitated PrV gB (Fig. 8, lanes 2 and 6), but not BHV-1 gB (Fig. 8, lanes 1 and 5). MAb 2/22 which recognizes an epitope in the amino-terminal subunit of PrV gB (Schreurs, 1988) precipitated B–P gB expressed by cell line MCB-1 (Fig. 8, lane 7), as did MAb 72/14/6 recognizing an epitope in the carboxy-terminal subunit of BHV-1 gB (Fig. 8, lane 15). In contrast, MAb 5/14 reacting with the carboxy-terminal subunit of PrV gB (Fig. 8, lane 4) as well as MAb 42/18/7 reacting with the amino-terminal subunit of BHV-1 gB (Fig. 8, lane 12) precipitated B–P gB expressed by MCB-2 cells. Reciprocal assays did not lead to precipitation of a specific protein. Similar results were obtained when respective cell lines were labelled in the absence of virus infection and precipitated accordingly (data not shown). These data show that cell lines MCB-1 and MCB-2 expressed chimeric gB molecules. It is noteworthy that the B–P gB complex precipitated by MAbs 5/14 (Fig. 8, lane 4) and 42/18/7 (Fig. 8, lane 12) exhibited a different pattern to the other gB proteins. Whereas in all other cases only one gB-specific band was observed, B–P gB migrated as two distinct proteins of approx. 120 and 150 kDa, as well as a high molecular mass species (> 200 kDa). Upon reduction, however, the presence of subunits with the expected molecular masses was observed (Fig. 8b, lanes 4 and 12). The reason for this behaviour is unclear.

Complementation of plaque formation of gB− PrV and BHV-1 by chimeric gB proteins

To test for complementation of the cell-to-cell spread defect in gB− PrV and BHV-1, MCB-1 and MCB-2 cells were infected under plaque assay conditions with gB− PrV and gB− BHV-1 phenotypically complemented by the respective gB. Two days after infection cells were fixed and stained with X-Gal. As shown in Fig. 9, both MCB-1 and MCB-2 cells complemented plaque formation of gB− PrV. In contrast, only in MCB-1 cells was gB− BHV-1 able to form plaques whereas MCB-2 cells exhibited the same phenotype as normal MDBK cells (see Fig. 4). The fact that both cell lines comple-
very low levels, that could significantly be enhanced by PEG enhancement. In contrast, after propagation on normal MDBK cells infectivity was only detectable in gB− BHV-1 propagated on MT-3 or normal MDBK cells after PEG enhancement. MCB-2 cells which did not support plaque formation of gB− BHV-1 also failed to restore infectivity of free virions. Infection occurred only after PEG treatment. After propagation on MCB-1 cells which did complement plaque formation of gB− BHV-1, infectivity was restored to gB− BHV-1 to only a very low level which was significantly enhanced by PEG. In summary, the data show that complementation of infectivity of free infectious virions, although generally exhibiting the same pattern, was considerably less efficient than restoration of plaque formation. However, also in this case, the presence of the carboxy-terminal part of BHV-1 gB was essential for bidirectional complementation of gB− PrV and BHV-1.

**Discussion**

Of all herpesvirus glycoproteins identified so far, only homologues of glycoproteins B, H, L and M of HSV-1 appear to be conserved in sequence and/or function throughout the herpesviruses. The highest homology at the amino acid level is found among the glycoprotein gB homologues, indicating that these proteins play a crucial role in the replicative cycle of the respective virus. Herpesvirus gB glycoproteins are involved in membrane fusion processes during infection including penetration, direct viral cell-to-cell spread and syncytia formation (reviewed in Mettenleiter, 1994; Spear, 1993), and glycoprotein B null mutants of HSV-1 and PrV depend on complementing gB-expressing cells for productive replication (Cai et al., 1988; Peeters et al., 1992; Rauh et al., 1991). Interestingly, Epstein–Barr virus and murine gammaherpesvirus 68 gB was not detected in the virion, indicating different requirements for gB in alpha- and gammaherpesviruses (Gong et al., 1987; Stewart et al., 1994). We describe here the first isolation of a gB− mutant of BHV-1 on a BHV-1 gB-expressing cell line. Insertional mutagenesis using lacZ under control of the PrV gG (previously called gX) promoter has been used in several instances to isolate PrV, HSV-1 or BHV-1 mutants lacking specific gene products (Fehler et al., 1992; Mettenleiter & Rauh, 1990; Mettenleiter & Spear, 1994; Rauh et al., 1991). Insertion of the expression cassette into the XhoI site in the BHV-1 gB gene, as used for isolation of the gB− BHV-1 mutant 2115, theoretically still allows expression of the amino-terminal part of BHV-1 gB up to the insertion site. However, in a number of experiments using different immunological reagents...
no corresponding gene product was ever observed, either in infected cells, or in virions or media (data not shown). Anti-BHV-1 gB MAb 42/18/7 used in this study recognizes an epitope within the amino-terminal part of BHV-1 gB (G. M. Keil, unpublished). Yet it failed to detect any protein in infected cells (not shown) or purified virions (Fig. 3). Therefore, it appears unlikely that a truncated expression product of the interrupted BHV-1 gB gene might interfere with the reported studies.

Using recombinant cell lines expressing BHV-1 gB it has previously been suggested that BHV-1 gB might have fusogenic properties (Blewett & Misra, 1991; Fitzpatrick et al., 1988), although neither G1 cells nor MT-3 cells exhibited spontaneous cell fusion (Rauh et al., 1991). Analysis of the gB- BHV-1 mutant unambiguously showed a requirement for gB for infectivity of free BHV-1 virions as well as for the propensity of BHV-1 to directly spread from cell to cell. Therefore, in this respect, gB- mutants of HSV-1, PrV and BHV-1 exhibit the same phenotype, at least in vitro. This adds further evidence to the notion that gB homologues play similar roles in replication of the different herpesviruses.

Since gB glycoproteins are highly homologous and exhibit similar functional properties we analysed heterologous transcomplementation between different herpesvirus gB mutants and corresponding gB-expressing cell lines. The results showed that gB- HSV-1 was able to productively replicate in a PrV gB-expressing cell line (Mettenleiter & Spear, 1994). PrV gB complemented the penetration defect of gB- HSV-1 virions and restored plaque formation, i.e. cell-to-cell spread capability of gB- HSV-1. Interestingly, complementation of either virus entry or direct cell-to-cell spread of gB- PrV by HSV-1 gB was not observed. HSV-1 and PrV gB exhibit a sequence homology of only 50% as compared to 63% between BHV-1 and PrV gB (Bzik et al., 1984; Robbins et al., 1987; Whitbeck et al., 1988). In addition, PrV and HSV-1 gB differ in post-translational modification. Whereas PrV gB is cleaved by a cellular protease located in the trans-Golgi at a consensus processing site into subunits that remain linked via disulphide bonds (Whealy et al., 1990; Wölfer et al., 1990), this consensus sequence is absent in HSV-1 gB, and consequently, proteolytic processing does not occur (Claesson-Welsh & Spear, 1986). Although this proteolytic cleavage has been shown to be non-essential for gB function, at least for the in vitro function of BHV-1 gB in either a BHV-1 or PrV background (Kopp et al., 1994), the absence or presence of proteolytic cleavage might nevertheless influence the heterologous transcomplementation capabilities of gB proteins. The lack of complementation of gB- PrV by HSV-1 gB might therefore be due to the more limited sequence homology and/or the differences in post-translational processing.

BHV-1 and PrV gB are 63% identical in amino acid sequence and they resemble each other also in post-translational processing since both are cleaved into two disulphide-linked subunits (Hampl et al., 1984; Lukács et al., 1985; Van Drunen Littel-van den Hurk & Babiuk, 1986). Given this similarity it was surprising that unidirectional complementation was observed between these two glycoproteins. Whereas BHV-1 gB was able to fully complement infectivity of a gB- PrV mutant (Kopp & Mettenleiter, 1992; Rauh et al., 1991) complementation of gB- BHV-1 by PrV gB was not observed. gB- BHV-1 was capable of direct cell-to-cell spread in BHV-1 gB-expressing G1 cells, but was unable to form plaques in PrV gB-expressing MT-3 cells despite a similar level of expression of the respective gB protein in MT-3 and G1 cells (Rauh et al., 1991). PrV gB was also unable to restore infectivity to gB- BHV-1 virions in spite of its presence in the BHV-1 envelope. Although difficult to quantify, radioimmunoprecipitation and electron microscopy indicated a similar amount of PrV gB in gB- BHV-1 virions after propagation on MT-3 cells as compared to the level of BHV-1 gB after propagation on G1 cells (see Figs 3 and 5).

Therefore, although very closely related, BHV-1 and PrV gB exhibit strikingly different properties as regards heterologous transcomplementation. The observation that infectivity of BHV-1 gB- BHV-1 virions cannot be complemented by PrV gB, although it appears to be incorporated into the virus envelope quite efficiently, indicates that the mere presence of the glycoprotein in the envelope is not sufficient for its function. We hypothesize that interaction of the heterologous gB with other virus components, presumably other virus envelope glycoproteins, is essential for its function. In this case, the lack of functional interaction between PrV gB and components of the BHV-1 virion might lead to lack of complementation, whereas BHV-1 gB functions efficiently in a PrV background.

In an attempt to delineate a region on the gB proteins responsible for the observed unidirectional complementation, two cell lines were established which expressed chimeric gB proteins composed of PrV and BHV-1 gB. Fusion at a conserved XhoI site located closely upstream from the sequences encoding the conserved proteolytic cleavage site essentially led to an exchange of the respective proteolytic subunit. Both chimeric gB proteins efficiently complemented plaque formation of gB- PrV, a propensity they shared with both parental glycoproteins. In contrast, plaque formation of gB- BHV-1 was only restored by authentic BHV-1 gB and the chimeric protein composed of the amino terminus of PrV gB and the carboxy terminus of BHV-1 gB. The reverse chimeric protein did not complement plaque formation. Restoration of infectivity
of free infectious virions generally followed the same pattern, although it proved to be much less efficient than complementation of plaque formation. Obviously there are different and probably more stringent requirements for gB function in entry than in direct cell-to-cell spread. Using monoclonal antibodies different parts of gB have indeed been implicated in the different membrane fusion processes in HSV-1 infection (Navarro et al., 1992). If this is also true for PrV and BHV-1 gB it could help to explain our findings. Taken together, the presence of the BHV-1 gB carboxy-terminal subunit, either in authentic BHV-1 gB or in the P-B chimera expressed by MCB-1 cells, is necessary for complementation of gB BHV-1. This indicates that properties essential for gB function which differ between PrV and BHV-1 gB are located within this part of the gB protein.

It is conceivable that BHV-1 gB has essential functional roles in addition to those of PrV gB. Indirect evidence has been obtained which indicates that BHV-1 gB might play a role in the attachment of BHV-1 virions (Liang et al., 1991), whereas gB does not appear to be involved in PrV attachment (Mettenleiter et al., 1990). In HSV-1, gB has been shown to bind heparan sulphate (Herald et al., 1994), a step which might be crucial for infection of cells in the absence of the major heparan sulphate-binding glycoprotein gC. In contrast, PrV gB does not appear to bind heparan sulphate during infection of cells by PrV even in the absence of PrV gC (Mettenleiter et al., 1990). This indicates functional differences which might also apply for BHV-1 and PrV. However, despite the difference in attachment properties, PrV gB was able to substitute for HSV-1 gB in HSV-1 infection (Mettenleiter & Spear, 1994). At present it is unclear whether BHV-1 gB, in the absence of BHV-1 gC, is also able to bind heparan sulphate or other cellular surface receptors.

Some properties of the chimeric gB proteins are noteworthy. As summarized in Fig. 7, consensus N-linked glycosylation sites of PrV and BHV-1 gB are unevenly distributed between the two subunits exchanged in the chimeras. Four sites reside within the amino-terminal part of BHV-1 gB, whereas the amino-terminal part of PrV gB only specifies three sites. In the carboxy-terminal subunits, PrV gB contains three possible N-glycosylation sites compared to two in BHV-1 gB. This leads to the situation that chimera P-B gB contains five N-glycosylation sites, whereas chimera B-P gB contains seven sites. Whether all these sites are indeed glycosylated and whether the number of possible N-glycosylation sites influences functional properties of the respective protein remains to be elucidated. The aberrant migration in SDS-PAGE of the B-P gB complex is still puzzling. Whereas authentic PrV and BHV-1 gB as well as P-B gB exhibited only one protein band of the expected size, B-P gB migrated as two protein species of approx. 150 and 120 kDa, as well as a higher molecular mass species of > 200 kDa. Upon reduction, however, subunits of the expected size appeared. Therefore, the aberrant migration appears to be connected with disulphide bonding in the gB complex. It is clear, however, that B-P gB is functional since it efficiently complemented plaque formation and, less efficiently, infectivity of gB- PrV.

In summary, our studies show inherent differences in the properties of otherwise highly homologous glycoproteins. Although all the gB homologues analysed so far, including BHV-1 gB as shown in this report, are essential for penetration and direct cell-to-cell spread of the respective virus, they exhibit differences in trans-complementation of gB defects in heterologous viruses. In the case of BHV-1 gB, the ability for bidirectional complementation could be correlated with the presence of the carboxy-terminal subunit of the BHV-1 gB molecule. This indicates that the amino-terminal subunits of BHV-1 and PrV gB, as assayed in our system, are functionally more similar than the carboxy-terminal parts. We are currently in the process of analysing in more detail the functional properties of different regions in herpesvirus gB proteins.

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