Homologous and heterologous interference requires bovine herpesvirus-1 glycoprotein D at the cell surface during virus entry

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Expression of glycoprotein D (gD) of alphaherpesviruses protects cells from superinfection by homologous and heterologous viruses by a mechanism termed interference. We recently showed that MDBK cells expressing bovine herpesvirus (BHV)-1 gD (MDBK\textsuperscript{gD}) resist BHV-1, pseudorabies virus (PRV) and herpes simplex virus-1 (HSV-1) but not the more closely related BHV-5 infection as determined by the number of plaques produced. However, the plaque size is reduced in all four viral infections suggesting a block in cell-to-cell transmission. Here, we show that MDBK cells expressing truncated BHV-1 gD, designated MDBK\textsuperscript{t-gD}, secreted soluble gD and were fully susceptible to infection by all the four viruses when the cells were washed prior to infection. When MDBK cells or MDBK\textsuperscript{t-gD} cells were treated with medium containing truncated gD prior to infection, they partially resisted BHV-1, PRV and HSV-1 but not BHV-5. Interestingly, both BHV-1 and BHV-5 formed normal-sized plaques in MDBK\textsuperscript{t-gD} cells suggesting that the viruses were able to spread efficiently. Thus BHV-1 gD is required at the cell surface at the time of infection in order to block BHV-1, HSV-1 and PRV infections, consistent with a common coreceptor for the three gDs.

Introduction

Alphaherpesvirus entry is complex and involves an initial attachment mediated by viral glycoprotein/s (gB or gC or both) binding to cellular glycosaminoglycans (Spear \textit{et al.}, 1992) followed by penetration of the virus by pH-independent fusion of the viral and cellular membranes mediated by at least five viral envelope glycoproteins, gB, gD, gH, gL and gK (reviewed by Spear 1993\textit{a, b}). Glycoprotein D (gD) is essential for penetration (Ligas & Johnson, 1988; Rauh & Mettenleiter, 1991; Fehler \textit{et al.}, 1992) and cells expressing gD are resistant to infection by the homologous virus, a phenomenon termed ‘interference’ (Campadelli-Fiume \textit{et al.}, 1988; Johnson & Spear, 1989; Chase \textit{et al.}, 1990; Chase & Letchworth, 1994; Tikoo \textit{et al.}, 1990; Petrovskis \textit{et al.}, 1988). Interference against heterologous viruses, termed ‘cross-interference’, usually suggests a common receptor pathway for entry (Weiss, 1993). Cross-interference between herpes simplex virus (HSV)-1 and pseudorabies virus (PRV) (Petrovskis \textit{et al.}, 1988) and between bovine herpesvirus (BHV)-1 and HSV-1 (Chase \textit{et al.}, 1990, 1993; Tikoo \textit{et al.}, 1990) supports the common receptor hypothesis. The observation that the truncated (soluble) gDs of HSV-1 and BHV-1 bind a limited number of cell membrane sites (\(\approx 4 \times 10^5–1 \times 10^6\) per cell) with comparable affinities (\(K_d \approx 10^{-7}\) M) and partially block homologous infections (Johnson \textit{et al.}, 1990; Li \textit{et al.}, 1995) is consistent with the hypothesis but it is not known if HSV-1 and BHV-1 gDs bind the same site(s) on MDBK cells. Recent identification of several human cellular coreceptors for gD (HveA, B, C, D and HIgR), only some of which serve as common receptors for specific alphaherpesviruses, provides further support to the common receptor hypothesis (Montgomery \textit{et al.}, 1996; Whitbeck \textit{et al.}, 1997; Geraghty \textit{et al.}, 1998; Warner \textit{et al.}, 1998; Krummenacher \textit{et al.}, 1998; Cocchi \textit{et al.}, 1998) but it is not known if their animal homologues exist and serve as receptors for HSV. However, certain observations are difficult to explain using the common receptor hypothesis. Firstly, bovine cells expressing BHV-1 gD are resistant to BHV-1, HSV-1 and PRV but cells expressing PRV gD are fully permissive to BHV-1 infection (Chase \textit{et al.}, 1993). Second, several other candidate receptors...
for HSV-1 gD identified earlier (Brunetti et al., 1994; Huang & Campadelli-Fiume, 1996) may be different from the putative BHV-1 gD receptor identified on MDBK cells (Thaker et al., 1994). However, the exact role of these proteins in infection remains unclear. Thirdly, viruses selected for their ability to grow on gD-expressing cells may carry wild-type gDs (Dean et al., 1995; G. K. Dasika & G. J. Letchworth, unpublished results). Additionally, mutations in gD of some interference-resistant viruses may not be sufficient for the unrestricted phenotype (Brandimarti et al., 1994), suggesting that other viral proteins may also participate in gD-mediated interference. Lastly, a point mutation in HSV-1 gD (L25 to P25) enabled mutant HSV-1 to infect cells expressing wild-type HSV-1 gD, but the mutant protein expressed in cells was unable to interfere with wild-type or mutant HSV-1 infection (Campadelli-Fiume et al., 1990). This led the authors to propose an alternative mechanism involving unfavourable interaction between cellular gD and viral gD (or another viral protein) resulting in interference (Campadelli-Fiume et al., 1990; Dean et al., 1994). Recent evidence suggests that an L25 to P25 mutation in gD precludes the use of HveA but the mutant virus utilizes HveC, a coreceptor for HSV-2, BHV-1 and PRV (Geraghty et al., 1998).

The alphaherpesvirus BHV-1 infects cattle worldwide and causes a variety of clinical syndromes (Yates, 1982; Ludwig, 1983). On the basis of apparently different clinical manifestations, BHV-1 isolates were classified into respiratory (BHV-1.1), genital (BHV-1.2) and encephalitic (BHV-1.3) subtypes (Engels et al., 1986). The neurovirulent virus (BHV-1.3) was reclassified as a separate type, BHV-5 (Roizman et al., 1986), based on the distinct restriction digest profiles of the genomes although BHV-1.1 and BHV-5 DNAs are 85% hybridizable (Engels et al., 1986). The BHV-5 gD protein shares 86% amino acid identity with BHV-1 gD (Tikoo et al., 1990; Abdelmagid et al., 1995).

We recently showed that full-length BHV-1 gD (referred to as BHV-1 gD here) interfered with infection by the distantly related viruses HSV-1 and PRV, but not with the closely related BHV-5, as determined by the number of plaques produced (Dasika & Letchworth, 1999). However, full-length BHV-1 gD expressed in MDBK cells inhibited cell-to-cell transmission of both BHV-1 and BHV-5 (Dasika & Letchworth, 1999). Since the exact mechanism of gD-mediated interference has not yet been fully elucidated and both truncated (soluble) (Johnson et al., 1990; Li et al., 1995; Nicola et al., 1996) and full-length gDs (Campadelli-Fiume et al., 1988; Chase et al., 1990; Dasika & Letchworth, 1999; Fehler et al., 1992; Johnson & Spear, 1989; Petrovsksii et al., 1988; Tikoo et al., 1990) have been shown separately to interfere with infection by the homologous virus, we hypothesized that if truncated gD interferes with virus infection by a process analogous to full-length gD, truncated BHV-1 gD should block BHV-1, HSV-1 and PRV but not BHV-5 infections. Our results showed the hypothesis to be correct.

Methods

- **Cells and viruses.** All the cell lines and viruses used in this study have been described previously (Dasika & Letchworth, 1999) except MDBKt- Δ5 and MDBKt-control (described below). Growth medium for MDBKt- Δ5, MDBKt- and MDBKt-control cells was supplemented with G418 (200 µg/ml, Gibco-BRL) and replaced with serum-free medium (Gibco-BRL) at the times indicated prior to collection of the supernatant. MDBKt- cells express full-length BHV-1 gD (Dasika & Letchworth, 1999) and MDBKt- cells express truncated BHV-1 gD.

- **Construction of expression plasmids for transfection.** The plasmids p-gD, and p-control, were constructed as follows (Fig. 1). To construct p-gD, an opal codon was inserted into the BHV-1 gD open reading frame (ORF) (Tikoo et al., 1990) at position 1077 by PCR amplification with a mutagenic 3’ oligonucleotide primer 1161R’gD (5’ GCGGAGCTCGAGTTTGGCGGCCGGCGTGA 3’) and a wild-type 5’ primer (72FgD) including the start codon (5’ CGAGCCGGCGCAACATGCAAGG 3’). Amplification was done in a programmable tempcycler (model 50 Tempcycler, Coy Laboratory Prod. Inc.) with an initial denaturation at 95°C for 5 min, followed by 30 cycles through 95°C for 1 min, 60°C for 1 min, 72°C for 1:5 min and terminated by a final extension step at 72°C for 10 min. The PCR product was purified with the GeneClean II kit (Bio 101), after isolation of the 1098 bp band from low melting temperature gel electrophoresis (Sea Plaque GTG agarose, FMC Bioproducts) and cloned into PCRII (Invitrogen) to create the plasmid p-TAgD. The truncated gD gene with EcoRI ends was then transferred to pcDNA3 (Invitrogen) to create the plasmid p-gD, such that gD had a CMV promoter upstream and BGH poly(A) signals downstream from the gene. To construct the plasmid p-control, the plasmid p-TAgD was digested with SalI and religated thereby releasing a 786 bp fragment from the gD ORF. Truncated gD with a SalI deletion was then transferred to pcDNA3 in the opposite orientation.

- **Construction of cell lines.** The plasmids p-gD and p-control, (Fig. 1) were transfected into subconfluent MDBK cells using Lipofectin (GIBCO-BRL) and selected for G418 resistance as described previously (Dasika & Letchworth, 1999). G418-resistant clones were tested for truncated gD (t-gD) expression in the supernatants using immunodot blots and Western blots using polyclonal (a kind gift from X. Zhu) and monoclonal antibodies (Marshall et al., 1986) against BHV-1 gD. Affinity purified BHV-1 gD (Zhu & Letchworth, 1996) was used as the positive control in immunodot blots. One representative clonal line that expressed t-gD was designated MDBKt and a negative control line (MDBKt-control) that was transfected with the plasmid p-control, and selected for G418 resistance was used in these studies.

- **SDS–PAGE and Western blot analysis.** Immunodot blots were performed as an initial screen to pick t-gD-expressing and control non-expressing clones. None of the clones that resulted from p-control transfection expressed t-gD. G418-resistant clones were grown to confluency in 24-well plates (Costar); the cells were then washed with MEM and the medium replaced with 1 ml serum-free MEM. An aliquot (200 µl) from the supernatant was made to bind to nitrocellulose through negative pressure and screened for the presence of gD using polyclonal or monoclonal anti-BHV-1 gD MAb (3402) as described for Western blots (Dasika & Letchworth, 1999).

- **Estimation of truncated BHV-1 gD concentration.** In order to estimate the concentration of gD in the supernatant, serum-free supernatant (10 ml) from 75 cm² flasks was collected after 24 h and 25 µl of serial twofold dilutions was separated by SDS–PAGE on an 8 % gel.
Truncated BHV-1 gD-mediated interference

Fig. 1. Construction of expression plasmids used for transfection. To construct the truncated gD expression plasmid, DNA encoding the ectodomain domain of BHV-1 gD was PCR amplified using primers 72FgD and 1161RgD. The amplified product was purified and ligated into PCRII plasmid (Invitrogen) to generate PCR.gDt. The t-gD ORF contained in EcoRI fragment was then transferred into pcDNA3 plasmid to yield pcDNA3-gDt. To construct the control plasmid, PCR.gDt was digested with SalI and religated releasing a 786 bp SalI fragment from the t-gD ORF. Truncated gD ORF with an internal SalI deletion was then transferred to pcDNA3 in the opposite orientation to create the control plasmid designated pcDNA3-control.

and stained with Coomassie blue (see Fig. 3). Undiluted control supernatant (25 µl) was also run on a similar gel alongside. Serial dilutions of BSA run on a similar gel and stained with Coomassie blue (data not shown) was used as a standard for visual comparison and estimation of the concentration of t-gD.

Blocking assay with truncated BHV-1 gD. The blocking assay was similar to that developed by Johnson et al. (1990) with minor modifications. Briefly, MDBK or MDBK-t-gD cells grown overnight in 24-well plates (≈ 1 x 10⁵ cells per well) were rinsed twice with MEM and treated with 500 µl of supernatant containing truncated gD (≈ 200 µg) or control supernatant for 1 h at 4 °C. Approximately 100 p.f.u. of each virus was added in duplicates on cells (MDBK or MDBK-t-gD) and incubated for an additional hour at 4 °C. Supernatant containing the unbound proteins and virus was removed and the cells were overlaid with MEM containing agarose (medium EEO, Sigma) as described previously (Dasika & Letchworth, 1999). Three days after infection, the
cells were fixed, stained and the plaques were counted. The number of plaques in control treated cells was normalized to 100 and means and standard deviations were calculated.

**Effect of truncated gD on plaque size.** Approximately 100 p.f.u. of each virus (BHV-1, BHV-5, PRV and HSV-1) was used to infect confluent MDBK\(^{t-gD}\) or MDBK (control) monolayers in a 24-well plate in duplicates after rinsing the cells with MEM twice. After incubating at 37 °C for 1 h the cells were washed with MEM and overlaid with MEM containing 0-5 % agarose (Fisher Scientific) supplemented with 5 % FBS, and then placed in an incubator at 37 °C with 5 % CO\(_2\). Monolayers were fixed with 10 % formaldehyde 3 days after infection and stained with crystal violet. The diameters of 37–40 random isolated plaques from each virus-infected monolayer were measured under a microscope (Olympus magnification) using an ocular micrometer, and the mean and standard deviations were calculated.

**Results**

**MDBK\(^{t-gD}\) cells secreted truncated BHV-1 gD**

Immunodot blot assay revealed BHV-1 gD expression in several of the clones transfected with plasmid p-gD\(_t\), but not in supernatant from MDBK\(^{t-control}\) cells (data not shown). The level of t-gD expression varied among different clones and a positive clone (clone 1.2) that expressed high levels was chosen and analysed further. Western blot analysis of the supernatant from MDBK\(^{t-gD}\) demonstrated the presence of t-gD that migrated faster (at \(\approx 64\) kDa) than full-length BHV-1 gD from MDBK\(^{t-gD}\) cells (Dasika & Letchworth, 1999) used as control (Fig. 2). At least one of the two additional bands, at \(\approx 140\) kDa, is probably a homodimer of gD since it reacted with MAb 3402. As expected, the control supernatant did not contain truncated BHV-1 gD.

The concentration of t-gD (mol. mass \(\approx 64\) kDa) in supernatant from MDBK\(^{t-gD}\) was estimated to be \(\approx 100–120\) µg/ml (Fig. 3, see Methods). The \(\approx 64\) kDa band was absent in control supernatant although two other protein bands at \(\approx 100\) kDa and \(\approx 120\) kDa were present. The concentration of t-gD in the same supernatant could be increased to about 450–500 µg/ml by collecting the supernatant after 3–4 days. If the supernatant was not replaced with fresh medium, the cells showed cytotoxic effects after 5–6 days as evidenced by vacuolation, rounding off and detaching from the surface similar to MDBK\(^{t-gD}\) cells upon induction (Dasika & Letchworth, 1999). Such an effect was not seen in MDBK\(^{t-control}\) cells and replacing the medium from MDBK\(^{t-gD}\) cells restored the cells to normal, suggesting that accumulation of gD caused the toxic effects.

**Truncated BHV-1 gD blocked BHV-1, HSV-1 and PRV but not BHV-5 infection**

Supernatant containing about 200 µg of gD was sufficient to partially block infection with BHV-1 (Fig. 4a) suggesting that t-gD was functional in a blocking assay. A similar effect was not seen with 200 µg of BSA and truncated gD did not inhibit vesicular stomatitis virus (VSV) infection (not shown). Additionally, supernatant containing truncated BHV-1 gD specifically inhibited BHV-1, HSV-1 and PRV plaques (\(\approx 60\%\)). There was no apparent reduction in BHV-5 plaque numbers, consistent with the results obtained with MDBK cells expressing full-length BHV-1 gD (Dasika & Letchworth, 1999).

**MDBK\(^{t-gD}\) cells support herpesvirus replication and form normal-sized plaques**

Since MDBK cells that expressed full-length BHV-1 gD were partially resistant to BHV-1 infection and formed tiny plaques on infection with BHV-1 or BHV-5 (Dasika & Letchworth, 1999), we tested if MDBK\(^{t-gD}\) cells were resistant to superinfection and compared the plaque sizes of BHV-1 and BHV-5 on MDBK\(^{t-gD}\) and MDBK cells. We also wished to rule out the possibility that MDBK\(^{t-gD}\) cells were inherently resistant to specific herpesviral infections by some unknown mechanism. When the cells were washed and infected with the herpesviruses used in this study without prior incubation with t-gD-containing supernatant, the number of plaques in MDBK\(^{t-gD}\) cells and MDBK cells was approximately the same,
Fig. 3. Estimation of truncated gD concentration in the supernatant. The concentration of t-gD in the supernatant was estimated by visual comparison of Coomassie blue-stained gels of serial twofold dilutions of serum-free t-gD supernatants and a similarly stained gel containing known amounts of BSA used as a standard (not shown). Undiluted supernatant from MDBKt-control cells electrophoresed and stained similarly is also shown (right).

Fig. 4. Truncated gD blocks specific herpesviral infections and is required at the cell membrane. (a) Inhibition of specific herpesviral infections by truncated gD. Incubation of MDBK cells with truncated gD-containing medium but not control medium inhibited plaque formation by HSV-1, PRV and BHV-1 as evidenced by the reduction in plaque numbers. No inhibitory effect was evident upon BHV-5 infection. The plaque numbers from control medium-treated duplicate cultures were normalized to 100 and the relative number of plaques in t-gD-treated cultures is shown. (b) Prior incubation of MDBKt-gD cells with t-gD medium restores interference. Incubation of MDBKt-gD cells with truncated gD-containing medium but not control medium inhibited plaque formation by HSV-1, PRV and BHV-1 as evidenced by the reduction in plaque numbers. No inhibitory effect was evident upon BHV-5 infection. The plaque numbers from duplicate cultures treated with control medium were normalized to 100 and the relative number of plaques in t-gD treated cultures is shown.

suggesting that MDBKt-gD cells were not resistant to super-infection and that sufficient t-gD was not retained on the cell membrane of MDBKt-gD cells after washing with MEM. Infection of MDBKt-gD cells in the presence of t-gD-containing, but not control, supernatant resulted in a $\approx 40-55\%$ reduction ($P < 0.01$) in plaque numbers of BHV-1, PRV and HSV-1 but not BHV-5 (Fig. 4b).

Since MDBKt-gD cells were continuously producing t-gD, we tested if cell-to-cell transmission of BHV-1 or BHV-5 was affected in these cells by measuring the sizes of plaques of BHV-1 and BHV-5 on MDBKt-gD cells and control MDBK cells. The plaque sizes were virtually identical in both the cell types with either virus (data not shown), suggesting that there was no significant inhibition of cell-to-cell transmission.

Discussion

Since truncated gD from HSV-1 or BHV-1 blocks super-infection by the homologous virus (Ligas & Johnson, 1988; Li et al., 1995), we attempted to confirm this and extend it to heterologous viruses. Cell culture supernatant containing about 200 µg of gD was sufficient to partially block plaque formation by BHV-1 (Fig. 4a). A similar effect was not seen with 200 µg/ml of BSA nor did a similar concentration of
truncated gD inhibit VSV infection (data not shown). Additionally, supernatant containing about 200 µg of t-gD specifically inhibited the formation of BHV-1, HSV-1 and PRV plaques (P < 0.001) (≈ 60%) but had no apparent effect on BHV-5. This is consistent with our results obtained with MDBK cells expressing full-length BHV-1 gD (Dasika & Letchworth, 1999), indirectly suggesting that BHV-5 may utilize alternative gD coreceptors for entry in MDBK cells. The reduced level of inhibition of specific herpesviral infections by truncated gD when compared to that of full-length gD may at least in part be due to a possible monomeric interaction of the former with cellular receptor(s) as opposed to a multimeric interaction of the latter.

The interference function required gD to be on the cell surface at the time of infection. Since MDBK<sup>gD</sup> and MDBK<sup>gD<sub>b</sub></sup> were constructed from the same parent cells and since both these cell lines express different forms of the same gD and the former unlike the latter are fully susceptible to infection, we conclude that truncation of gD resulted in loss of the interference function. Since pre-incubation of the susceptible MDBK<sup>gD</sup> cells with gD-containing medium partially restored the ability to resist infection (Fig. 4b), it appears that retention of gD in/on the cells may be a prerequisite for interference. Studies with truncated HSV-1 gD demonstrated that incubation of HSV-1 with t-gD does not reduce the infectivity of the virus (Johnson et al., 1990). Taken together, it appears that gD acts at the cell membrane possibly blocking a receptor at the time of infection to mediate a block. If the blocking function of gD is same as the interference function, these data suggest that the carboxyl 58 amino acids of gD may not be absolutely required (Fig. 4d). Truncated gD was able to block herpesviral infections only to about 40% of the controls in MDBK<sup>gD</sup> cells when compared to 60% in MDBK cells. This is consistent with the speculation that gD receptors in MDBK<sup>gD</sup> cells may be slightly upregulated but the exact reason is unknown. Additionally, MDBK<sup>gD</sup> cells formed normal-size plaques, suggesting that there was no apparent block in cell-to-cell transmission. This is in contrast to our results with MDBK<sup>gD<sub>b</sub></sup> cells, which formed tiny plaques when infected with BHV-1 as well as BHV-5 (Dasika & Letchworth, 1999). Several factors could have contributed to this result. The transmembrane (TM) and/or cytoplasmic (cyt) domain of gD may be required to inhibit cell-to-cell transmission. Since the m.o.i. was < 0.01 and the cells neighbouring a particular infected cell would have continued to produce truncated gD even if host-cell protein synthesis was shut off in infected cells, and since the level of truncated gD was much higher than that of full-length gD in MDBK<sup>gD<sub>b</sub></sup> cells (Fig. 2 and data not shown), it can be speculated that truncation of gD eliminated the block in cell-to-cell transmission. Alternatively, since the level of inhibition of infection mediated by truncated gD is only a fraction of the interference due to full-length gD, then if the inhibition during cell-to-cell spread is proportionately less, our assay may not have been able to detect it. If the TM and cyt domains indeed code for a function associated with cell-to-cell transmission, it can be tested by constructing cells expressing chimeric gD that carry TM and cyt domains of heterologous glycoproteins, e.g. VSV G protein. We speculate that the block in cell-to-cell transmission mediated by full-length BHV-1 gD in MDBK cells is dependent on basolateral sorting of gD similar to HSV-1 gD, and truncation at amino acid 359 may have deleted the sorting signals of BHV-1 gD resulting in apical secretion of truncated gD from MDBK<sup>gD<sub>b</sub></sup> cells. Indeed we discovered a putative basolateral-targeting signal in the carboxyl terminus of gD (Table 1). This would conceivably result in lack of t-gD at the basolateral surface resulting in no block in cell-to-cell transmission.

Epithelial cells are polarized with spatially and functionally asymmetric apical and basolateral membranes separated by tight junctions (zonulae occludentes) (reviewed by Le Gall et al., 1995). MDBK cells, like other kidney cells, are polarized and HSV glycoproteins including gD sort to the basolateral surface (Srinivas et al., 1986). Basolateral-sorting signals usually include a critical tyrosine residue (Y-X-X-hydrophobic) (reviewed by Rodriguez-Boulan & Zurzolo, 1993). Computer predictions and structural analysis of peptides have shown that they form a beta turn, suggesting that such a loop structure may interact with other factors responsible for proper translocation (Collawn et al., 1990; Eberle et al., 1991). The tyrosine is critical both for internalization and basolateral transport for several proteins including viral haemagglutinin (HA). HA is an apical protein, but mutation at position 543 from C to Y in the cytoplasmic domain targets it to the basolateral membrane where it is rapidly internalized through coated pits (Brewer & Roth, 1991). VSV G protein uses the classic Y-X-X-aliphatic motif (YTDI) to mediate its basolateral sorting in MDCK cells (Thomas et al., 1993). Interestingly, BHV-1, BHV-5, PRV and HSV-1 gD's have tyrosines in their cytoplasmic tails, which could potentially be a part of the sorting signal (Table 1). Bovine herpesvirus gDs and PRV gD have the classic Y-X-X-L motif, which is very likely a basolateral-sorting signal for gD.

**Table 1. Cytoplasmic basolateral-sorting signals include a critical tyrosine residue**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Basolateral-sorting signal</th>
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<tbody>
<tr>
<td>p75 NGF receptor</td>
<td>FKRTNSLYSLP</td>
</tr>
<tr>
<td>Influenza virus HA (Tyr mutant)</td>
<td>CSNGSLQYRICI</td>
</tr>
<tr>
<td>Lpg 120</td>
<td>RRKRSHAGYQTT</td>
</tr>
<tr>
<td>Fc Receptor</td>
<td>EAENTITYSLLKH</td>
</tr>
<tr>
<td>BHV-1.1, -1.2, -5 gD</td>
<td>FGNVNYSAFP&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>HSV-1 gD</td>
<td>DQPSSHQPLFY&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>PRV gD</td>
<td>KGYRLGGPA&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* Significance unknown.
Truncated BHV-1 gD-mediated interference

Consistent with this hypothesis, upon induction for over-expression, we observed accumulation of BHV-1 gD in the cytoplasm of cells expressing full-length BHV-1 gD (Dasika & Letchworth, 1999), possibly due to overwhelming of the sorting machinery from trans-Golgi to the plasma membrane. However, this needs to be experimentally demonstrated, e.g. by mutating the putative critical tyrosine to alanine. Thus it is possible that t-gD was unable to block cell-to-cell transmission in MDBK\textsuperscript{t-gD} due to apical delivery of the protein in the absence of basolateral-sorting signals (Fig. 5).

More basic information about BHV-1 gD synthesis, processing and sorting is required for proper interpretation of experimental observations and understanding of gD function. We conclude the following. (a) The extracellular domain of BHV-1 gD is sufficient to block infection both against homologous and heterologous viruses. Although the level of inhibition is several-fold lower when compared with interference mediated by full-length gD, the specificity of block mediated by truncated BHV-1 gD is qualitatively similar to the interference function of full-length BHV-1 gD. The amount of BHV-1 gD sufficient to block BHV-1, HSV-1 and PRV infection is unable to block the closely related BHV-5. (b) Cells expressing truncated gD are fully susceptible to infection unless pre-incubated with truncated gD and form normal-sized plaques, suggesting that retention of gD in/on the cells may be required for inhibition of infection and cell-to-cell transmission. Thus gD acts at the level of cell membrane at the time of membrane penetration.

We speculate that MDBK\textsuperscript{t-gD} cells were improperly targeting t-gD to the apical surface and thus there was no block in cell-to-cell transmission despite secretion of high levels of t-gD. Similarly, significantly more BHV-1 gD may be required to block BHV-5 and due to preferential basolateral sorting of gD (if true), MDBK\textsuperscript{t-gD} cells were able to block cell-to-cell transmission of BHV-5 but not the initial infection. Construction of gD-expressing cell lines with a mutation in the putative basolateral targeting signal (Y-S-A-L) should allow us to define if the gD-mediated block in cell-to-cell transmission is dependent on basolateral targeting or if another functional domain in the TM or cyt domain is responsible. Similar to the proposed mechanism of gD-mediated interference, the gD-mediated block in cell-to-cell transmission may involve sequestering or blocking of a cellular factor present in the region of cell-to-cell contact that may or may not be identical to the cellular receptor/s required for initial entry.

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Truncated BHV-1 gD-mediated interference


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