Cellular Expression of Bovine Herpesvirus 1 gD Inhibits Cell-to-Cell Spread of Two Closely Related Viruses without Blocking Their Primary Infection

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Alphaherpesviral glycoprotein D (gD) is a critical component of the cell membrane penetration system. Cells that express gD of herpes simplex virus type 1 (HSV1), pseudorabies virus (PRV), or bovine herpesvirus type 11 (BHV11) resist infection by the homologous virus due to interference with viral entry at the level of penetration. BHV11 gD interferes with the distantly related viruses HSV1 and PRV despite only a 30–40% sequence similarity and the complete absence of antigenic cross-reactivity among the three gDs. The six cysteines that form three intrachain disulfide bonds in HSV1 are also present in PRV and BHV11 gD, suggesting structural similarities among the gD homologs. Functional similarities were postulated to be responsible for cross-interference. To test this hypothesis, we constructed a BHV11 gD-expressing cell line (MDBKgD) and assessed its resistance to the homologous BHV11 and two closely related viruses, BHV12 and BHV5. The gDs of these viruses share 98.3% and 86% amino acid identity with BHV11 gD and bound monoclonal antibodies directed against all five neutralizing epitopes mapped on BHV11 gD. MDBKgD cells were resistant to BHV11 but fully susceptible to BHV12 and BHV5 infection as measured by plaque numbers and single cycle growth kinetics. However, all three viruses, but not vesicular stomatitis virus, made smaller plaques on MDBKgD cells than on control cells. These data suggest that gD-mediated interference is expressed both at the level of initial infection and at the level of cell-to-cell spread and that these two levels can be distinguished by using closely related viruses.

INTRODUCTION

Bovine herpesvirus-1 (BHV1), an alphaherpesvirus, infects cattle worldwide and causes ocular, respiratory, genital, and occasionally neurological disease (Gibbs and Rweyemamu, 1977; Ludwig, 1983; Yates, 1982). Restriction enzyme analysis of genomic DNA from BHV1 isolates identified three distinct patterns that roughly correlated with their clinical manifestations. Thus they were classified into respiratory (BHV1.1), genital (BHV1.2), and encephalitic or neuroviral (BHV1.3) subtypes (Engels et al., 1986). The neuroviral subtype has since been reclassified as BHV5 (Roizman et al., 1992).

Glycoprotein D (gD) is essential for alphaherpesviral penetration into cells (reviewed by Spear, 1993a). In addition, cells expressing gD resist infection by the homologous virus, a phenomenon termed "interference." Interference occurs at the level of penetration into cells and not during viral binding or postpenetration steps (Campadelli-Fiume et al., 1988a; Johnson and Spear, 1989). Expression of BHV1.1 gD in bovine cells causes interference not only with BHV1.1 but also with herpes simplex virus 1 (HSV1) and pseudorabies virus (PRV) (Chase et al., 1990). Similarly, PRV gD expression interferes with HSV1 and PRV (Petrovskis et al., 1988; Chase et al., 1993). Consistent with the functional overlap, the three gDs share 30–40% amino acid sequence similarity and the six cysteines that form disulfide bonds at least in HSV1 gD (Long et al., 1992; Tikoo et al., 1990). Although the precise molecular mechanism is unclear, interference is postulated to be a result of competition for a limited number of gD receptors on the cell surface. In support of this, soluble HSV1 gD and UV-inactivated HSV1 bind a limited number of cell membrane sites and block HSV1 infection, whereas HSV1 lacking gD is unable to do so (Johnson et al., 1990; Johnson and Ligas, 1988; Ligas and Johnson, 1988). Consistent with the cross-interference between HSV1 and PRV (Chase et al., 1993; Petrovskis et al., 1988), the two viruses share a binding site on Vero cells, and gD is the most likely ligand (Lee and Fuller, 1993). Recently, HVEM, a novel member of the tumor necrosis factor receptor superfamily, was shown to directly bind HSV gD and mediate viral entry (Whitbeck et al., 1997).

Several observations complicate this simple explanation for interference. First, PRV gD, unlike HSV1gD (Brunetti et al., 1995; Nicola et al., 1996), is dispensable for spread of the virus to neighboring cells once a cell is infected (Hassens et al., 1995; Peeters et al., 1992) and cannot functionally complement HSV1 gD (Muggeridge...
et al., 1990). Second, interference may not always be reciprocal because PRV gD does not interfere with BHV1.1 infection (Chase et al., 1993). Third, single amino acid substitutions in viral HSV1gD permit circumvention of gD-mediated interference (Brandimarti et al., 1994; Campadelli-Fiume et al., 1990; Dean et al., 1994). At least truncated gD from two of the mutant strains (rid1 and ANG) did not bind truncated HVEM, suggesting the use of alternative receptors for entry (Whitbeck et al., 1997).

Indeed, at least four different coreceptors for alphaherpesviral gDs have recently been discovered (Geraghty et al., 1998; Montgomery et al., 1996; Warner et al., 1998). Because BHV1.1 gD alone is sufficient to interfere with infection by two distantly related heterologous viruses (PRV and HSV1), we wanted to test whether it would interfere with two more closely related viruses, BHV1.2 and BHV5. The BHV1.2 and BHV5 predicted gD polypeptides share 98.3% and 86% amino acid identity with BHV1.1 gD, respectively (Abdelmagid et al., 1995; Leung-Tack et al., 1994; Tikoo et al., 1990). All three gD homologs are of an identical length (417 amino acids) and share sequence similarities with PRV and HSV1 gDs (Fig. 1). In this study, we showed that BHV1.1, 1.2, and 5 share all five monoclonal antibody (mAb) epitopes previously mapped to be neutralizing epitopes on BHV1.1 gD (Marshall et al., 1988). We constructed a stable Madin-Darby bovine kidney (MDBK) cell line that expressed full-length BHV1.1gD under the control of MT promoter (Fig. 3C). The level of gD expression could be increased by supplementing the medium with 1–4 \( \mu \text{M} \) cadmium chloride as demonstrated by immunoprecipitation and indirect immunofluorescence assay (Fig. 3D). Recombinant gD was localized to plasma membrane and around the nucleus in uninduced cells (Fig. 3C). On induction with 1 \( \mu \text{M} \) cad-

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**RESULTS**

Establishment of a stable line expressing full-length BHV1.1gD

A stable cell line expressing BHV1.1 gD and an appropriate control were constructed using MDBK cells as the parental line. The plasmids p-gD and p-control (Fig. 2) were transfected into MDBK cells using liposomes (Rose et al., 1991), and plasmids p-gD and p-control were amplified using the MULALIGN program (G. Gonnet, unpublished data) on the computational biochemistry research group (CBRG, ETH, Zurich, Switzerland) server. The entire mature polypeptide of BHV1.1 gD is shown, and all amino acids except the six cysteines that are identical in the other gDs have been replaced with a period. Amino acids that align identically in all the five proteins have been represented in bold with an asterisk at the bottom. Only cysteines and amino acids that are different in the other four proteins have been shown. Gaps introduced by the algorithm are marked by a solid line, and areas in which there is no similarity with BHV1gD are marked by a dashed line. The N-linked glycosylation sites (+ + +) in BHV1gD and the transmembrane domain have been marked. The BHV5 gD sequence differs from BHV1gD in the mAb 3402 epitope (92–106) by four amino acids and lacks the first N-linked glycosylation site (Abdelmagid et al., 1995). A putative interference domain including the often mutated L25 and Q27 (in bold) in the amino-terminus of BHV1 gD is also shown.

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**INTERFERENCE DOMAIN**

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**TRANSMEMBRANE DOMAIN**

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**REFERENCES**

Establishment of a stable line expressing full-length BHV1.1gD

A stable cell line expressing BHV1.1 gD and an appropriate control were constructed using MDBK cells as the parental line. The plasmids p-gD and p-control (Fig. 2) were transfected into MDBK cells using liposomes (Rose et al., 1991), and G418-resistant clones were isolated. Clones carrying p-gD were screened for gD expression using ELISA. Fourteen clones expressed BHV1.1gD-MEDIATED ALPHAHERPESVIRAL INTERFERENCE

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**FIG. 1.** Multiple sequence alignment of gD homologs compared with BHV1 gD. BHV1.1 gD (Tikoo et al., 1990), BHV1.2 gD (Leung-Tack et al., 1994), BHV5 gD (Abdelmagid et al., 1995), PRV gD (Petrovskis et al., 1986), and HSV1 (KOS) gD (Watson et al., 1982) mature polypeptides were aligned using the MULALIGN program (G. Gonnet, unpublished data) on the computational biochemistry research group (CBRG, ETH, Zurich, Switzerland) server. The entire mature polypeptide of BHV1.1 gD is shown, and all amino acids except the six cysteines that are identical in the other gDs have been replaced with a period. Amino acids that align identically in all the five proteins have been represented in bold with an asterisk at the bottom. Only cysteines and amino acids that are different in the other four proteins have been shown. Gaps introduced by the algorithm are marked by a solid line, and areas in which there is no similarity with BHV1gD are marked by a dashed line. The N-linked glycosylation sites (+ + +) in BHV1gD and the transmembrane domain have been marked. The BHV5 gD sequence differs from BHV1gD in the mAb 3402 epitope (92–106) by four amino acids and lacks the first N-linked glycosylation site (Abdelmagid et al., 1995). A putative interference domain including the often mutated L25 and Q27 (in bold) in the amino-terminus of BHV1 gD is also shown.
mium chloride, the level of cytoplasmic expression was increased (Fig. 3D). The level of background fluorescence was minimal when an isotype matched anti-BHV1.1 gB mAb (mAb 5106) was used (Fig. 3B).

To determine whether gD synthesized by MDBK gD cells is full-length BHV1.1 gD, MDBKgD, MDBK control, and MDBK cells infected with BHV1.1 were grown in the presence of 1 μM cadmium chloride for 6 h to induce gD expression, metabolically labeled with [35S]methionine and cysteine, and immunoprecipitated with anti-BHV1.1 gD mAb 1106. The 77-kDa full-length gD and the 64-kDa immature protein were recognized in both MDBK gD and BHV1.1-infected MDBK cells but not in MDBK control cells (Fig. 4). MDBK gD cells expressed gD for at least 25 passages.

MDBKgD cells resist BHV1.1, HSV1, and PRV infections

To determine whether MDBK cells were susceptible to HSV1 infection, serial 10-fold dilutions of HSV1 (KOS) were plaqued in duplicate on monolayers of MDBK and Vero cells, and essentially equal numbers of plaques were observed (data not shown). To confirm that MDBKgD cells had the interference phenotype previously reported for BHV1.1 gD-expressing cells (Chase et al., 1990; Tikoo et al., 1990), we plaqued dilutions of BHV1.1, HSV1, and PRV on MDBKgD and MDBK control cells and compared plaque numbers after 3–4 days. Uninduced MDBKgD cells had 99% fewer BHV1.1 plaques, 82% fewer HSV1 plaques, and 92% fewer PRV plaques than MDBK control cells (Fig. 5), consistent with previous studies (Chase et al., 1990; Tikoo et al., 1990). This inhibition was specific for the herpesviruses and was not exerted on an unrelated virus (VSV). Similar results were obtained with other clonal lines (data not shown).

BHV1.1, BHV1.2, and BHV5 are antigenically similar

Because the three bovine herpesviral gDs share high degree of sequence identity (Fig. 1), we used a panel of anti-BHV1.1 gD mAbs that neutralize BHV1.1 efficiently in the absence of complement (Marshall et al., 1988) to test the functional conservation of these epitopes among the two closely related viruses and HSV1 in a standard neutralization assay (Table 1). mAbs 4603 (epitope I), 5006 (epitope I), and 1102 (epitope IV) neutralized all the three viruses, while mAbs 4906 (epitope III) and 3402 (epitope V) neutralized BHV1.1 and BHV1.2, and mAbs 0722 (epitope I) and 1106 (epitope II) were able to neutralize only BHV1.1. None of the antibodies neutralized HSV1.

To test whether type specificity in neutralization correlated with a loss of the specific epitopes in the related viruses, we immunoprecipitated each metabolically labeled infected lysate with equal amounts of each anti-BHV1.1 gD antibody or control antibodies N9 (anti-VSV G) and 5106 (anti-BHV1.1 gB) (Fig. 6). Interestingly, all anti-gD mAbs recognized gD from all the three viruses, suggesting that all the epitopes were conserved and that antibody binding is not sufficient for neutralization activity. Specificity of anti-gD antibodies is indicated by the fact that the control antibodies, 5106 and N9, did not precipitate a protein of identical molecular weight as gD. gD of BHV5 migrates slightly faster (≃74 kDa) than BHV1.1 and BHV1.2 gD (≃77 kDa).
Our results were inconsistent with those of others (Abdelmagid et al., 1995) who reported an absence of mAb 3402 epitope (92–106) in BHV5 gD (Fig. 1) based on the inability of the antibody to recognize BHV5 gD on a Western blot. Therefore, we compared the ability of mAbs 3402, 0722, 5006, and 4906 to recognize BHV1.1 and BHV5 gDs in a similar assay. Consistent with the observation of Abdelmagid et al. (1995), mAb 3402 did not recognize denatured BHV5 gD immobilized on a nitrocellulose membrane but bound BHV1.1 gD. In addition, mAb 4906 (epitope III) was unable to recognize either gD, suggesting that epitope III may be conformational. mAbs 5006 (epitope I) and 0722 (epitope I) recognized both the gDs, and the intensity of the reaction roughly correlated with their neutralization ability (data not shown).

**BHV1.1 gD-expressing cells are susceptible to BHV1.2 and BHV5**

Because MDBK<sup>gD</sup> cells resisted infection with BHV1.1 and distantly related herpesviruses like HSV1 and PRV despite a lack of cross-neutralization epitopes in gD, we predicted that closely related viruses also would be susceptible to BHV1.1 gD-mediated interference. Surprisingly, BHV1.2 and BHV5 made approximately equal number of plaques in MDBK<sup>gD</sup> and MDBK<sup>control</sup> cells (Fig. 7). Furthermore, [<sup>35</sup>S]methionine labeling of BHV1.1- and
BHV5-infected MDBK<sup>gD</sup> (as well as two additional gD-expressing clonal lines) and MDBK<sup>control</sup> monolayers revealed efficient host–protein shutoff and synthesis of viral polypeptides in gD-expressing cells by both viruses (data not shown).

**BHV1.1 gD inhibits plaque enlargement**

Herpesviral plaques on MDBK<sup>control</sup> cells appeared 2 days after infection and enlarged greatly by 3 days. In contrast, herpesviral plaques on MDBK<sup>gD</sup> cells appeared at 2 days after infection but grew very little between 2 and 3 days, resulting in tiny plaques. VSV plaques grew steadily despite BHV1.1 gD expression. The sizes of plaques in MDBK<sup>control</sup> and MDBK<sup>gD</sup> cells were measured at 24 h (VSV), 48 h (BHV5, BHV1.2), and 72 h (BHV1.1) after infection because the plaque sizes in control cells were comparable at these times (Fig. 8). BHV1.1 gD expression reduced the size of herpesviral plaques by 60–80% but did not inhibit VSV plaque enlargement.

**BHV1.1 gD does not interfere with growth kinetics of the viruses**

Because BHV1.2 and BHV5 were able to infect gD-expressing cells but were unable to form normal-sized plaques, we wanted to rule out a possible block in replication due to gD expression. Of the two viruses, BHV5 formed smaller plaques on MDBK<sup>gD</sup> cells (Fig. 8). Therefore, we compared the viral growth kinetics of BHV5 infection on MDBK<sup>gD</sup> and MDBK<sup>control</sup> cells. Viral growth was measured at 6-h intervals after infection at an m.o.i. of 10. The growth kinetics and titers of BHV5 on MDBK<sup>gD</sup> cells were comparable to those on control cells (Fig. 9a). A similar experiment with BHV1.1 (Fig. 9b) yielded identical kinetics but lower titers on gD-expressing cells compared with control cells, comparable to the observations of others in different interference systems (Petrovskis et al., 1988). Thus BHV1.1 gD was unable to interfere with BHV5 infection and replication but inhibited the enlargement of plaques.

![FIG. 4. MDBK<sup>gD</sup> cells express BHV1 gD. Confluent monolayers of MDBK<sup>gD</sup>, MDBK<sup>control</sup>, and MDBK cells infected with BHV1 were metabolically labeled with [35S]methionine and cysteine for 6 h, and lysates were prepared as described under Materials and Methods. The lysates were immunoprecipitated with anti-BHV1 gD mAb 1106 and electrophoresed under denaturing conditions. BHV1 gD that was of identical molecular weight (<77 kDa) was precipitated from both BHV1-infected cells and MDBK<sup>gD</sup> cells but not from MDBK<sup>control</sup> cells.](image)

**TABLE 1**

<table>
<thead>
<tr>
<th>BHV1 gD mAb</th>
<th>Epitope Group</th>
<th>Reaction on a Western Blot</th>
<th>Neutralization Titer</th>
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<tr>
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<tr>
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<tr>
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<td>V</td>
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<td>1:800 1:800 1:2</td>
</tr>
</tbody>
</table>

*Note. ND, not done.*

![FIG. 5. MDBK<sup>gD</sup> cells are resistant to homologous and heterologous herpesviral infections. Number of pfus on MDBK<sup>gD</sup> expressed as a percentage of pfu scored on MDBK<sup>control</sup> cells. The cells were exposed to serial dilutions of BHV1, HSV1, PRV, or VSV for 1 h at 37°C. The inocula were removed and replaced with 0.5% agarose overlay containing growth medium. The cells were fixed and stained 72 h postinfection for herpesviruses and 24 h postinfection for VSV. The results represent the mean number of pfu from triplicate determinations on gD-expressing cells given as a percentage of pfu from triplicate determinations on control cells. The standard deviations are also marked.](image)
DISCUSSION

gD on the virion envelope is essential for viral penetration and, when expressed on the cell membrane, interferes with entry of the homologous virus presumably by competing for gD coreceptor or coreceptors. Cross-interference patterns in other viral systems have usually corresponded with host range patterns and allowed the classification viruses according to different receptor groups (Weiss, 1993). However, the following results have revealed unexpected complexity. Cross-interference between HSV1 and PRV (Chase et al., 1993; Petrovskis et al., 1988) is consistent with the notion that HSV1 and PRV gDs share a binding site, at least on Vero cells (Lee and Fuller, 1993). However, lack of reciprocity in gD-mediated interference between BHV1.1 and PRV gD is inconsistent with the proposed mechanism (Chase et al., 1993). All the herpesviruses used in this study can infect cattle and bovine cells in culture, suggesting that a similar classification among these herpesviruses should be possible. The present study was done to test interference between BHV1.1 gD and two very closely related bovine herpesviruses with gD sequences much more closely related than the viruses tested previously (Fig. 1), with the prediction that they would be susceptible to BHV1.1 gD-mediated interference.

A bovine kidney cell line, MDBK, that is also susceptible to infection by BHV1.2 and BHV5. Number of PFUs on MDBK gD expressed as a percentage of PFU scored by the same virus sample on MDBK control cells. Monolayers of MDBK gD and MDBK control cells were exposed to serial dilutions of BHV1, BHV1.2, BHV5, or VSV in duplicates and overlaid with growth medium containing 0.5% agarose. The cells were fixed and stained after 48–72 h (for herpesviruses) or 24 h (for VSV) after infection. The results represent mean number of pfu from duplicate determinations on MDBK gD cells given as a percentage of pfu from duplicate determinations on MDBK control cells from two independent experiments. The standard deviations have been marked.
Recent evidence suggests that despite only a 15–20% amino acid sequence identity among the three virus gDs, all the three viruses can use a common gD coreceptor (HveC) for entry (Geraghty et al., 1998). One potential explanation is that a bovine homolog of HveC (or some other cellular molecule) may serve as a common receptor for entry for these viruses and expression of BHV1.1 gD thus may interfere with the entry of all three viruses.

Interestingly, MDBK cells expressing BHV1.1 gD were fully permissive to both BHV1.2 and BHV5, whereas they resisted >2 logs of BHV1.1 (Fig. 4). BHV gDs are highly conserved (Fig. 1), and gDs of BHV1.2 and BHV5 share 98.3% and 86% amino acid identity with BHV1.1 gD, respectively (Abdelmagid et al., 1995; Leung-Tack et al., 1994; Tikoo et al., 1990). We used a panel of anti-BHV1.1 gD mAbs that were mapped to five antigenic areas (Marshall et al., 1988) and neutralize BHV1.1 efficiently in vitro in the presence or absence of complement (Dubuisson et al., 1992) to determine the conservation of the five epitopes among the three closely related viruses. The neutralization epitope recognized by mAb 3402 in the amino-half of BHV1.1 gD (92–106) was reported absent in BHV5 (Abdelmagid et al., 1995). Our results confirmed that BHV5 was resistant to neutralization by mAb 3402.
HveA, a HSV1 gD receptor (Whitbeck et al., 1993) demonstrated that the mutant gD was unable to bind gD that resulted in the loss of a neutralization epitope coreceptors for entry or that different regions of gD may contribute to BHV1.2 and BHV5 (Fig. 6), consistent with the high sequence conservation among the three homologs (Fig. 1). Because equal amounts of a given antibody were used in each immunoprecipitation and equal PFUs of each virus were used against a serial dilution of each antibody in the neutralization assay, a simple interpretation would be that the epitopes recognized by mAb 3402, 1106, and 0722 are present in all of the three viruses but the antibodies are unable to neutralize the viruses despite binding them or that the epitopes are masked on the virions of BHV1.2 and BHV5. Because all of the five epitopes are recognized by the bovine immune system (Marshall et al., 1988), we speculate that these closely related viruses may have evolved mechanisms of neutralization evasion by certain antibodies even though the epitopes are retained. Resistance to neutralization by some anti-BHV1.1 gD mAbs as well as to BHV1.1 gD-mediated interference is consistent with the hypotheses that BHV1.2 and BHV5 may use different coreceptors for entry or that different regions of gD may be critical for entry. Recent studies with a HSV1 gD interference resistance mutant with a point mutation in gD that resulted in the loss of a neutralization epitope demonstrated that the mutant gD was unable to bind HveA, a HSV1 gD receptor (Whitbeck et al., 1997), but would instead use HveC or HveB, a coreceptor for HSV2 gD and PRV (Warner et al., 1998). In addition, the domain of HSV1 gD that interacts with HveA is different from the domain that interacts with HveC (Krucmenacher et al., 1998).

The present study also revealed a role for cellular BHV1.1 gD in preventing cell-to-cell spread of bovine herpesviruses that may be independent of its role in preventing initial infection. Although BHV1.2 and BHV5 were able to infect BHV1.1 gD-expressing cells, the enlargement of the plaques was inhibited, suggesting a block after initial infection (Fig. 8). Of the two viruses, BHV5 was more sensitive to inhibition of plaque enlargement. Single step growth kinetics of BHV5 in BHV1.1 gD-expressing cells was similar to that in MDBK control cells, suggesting an absence of any block in replication (Fig. 9). Taken together, these data suggested an impairment in cell-to-cell transmission of BHVs as the likely cause for the appearance small plaques on BHV1.1 gD-expressing cells. Another potential explanation would be that BHV5 infecting MDBK 1D cells is transcomplemented by BHV1.1 gD, and thus the virus spreading to neighboring cells is sensitive to BHV1 gD-mediated interference and results in small plaques. This is unlikely the case because the level of BHV5 gD produced in MDBK 1D cells infected with BHV5 will be significantly greater than the low level of BHV1 gD being expressed, especially in view of the host–protein shutoff due to BHV5 infection. In addition, BHV5 grown on MDBK 1D cells (as well as two other gD-expressing clonal lines) was able to efficiently infect MDBK 1D cells (data not shown), suggesting that the virus was not enhanced in its sensitivity to BHV1 gD-mediated interference. Similarly, HSV1 rid mutants grown on HSV1 (KOS) gD-expressing cells remained resistant to gD-mediated interference, presumably because cellular gD is not very efficiently trans-complemented (Dean et al., 1994). Because the plasma membranes of MDBK cells are known to be polarized and herpesviral glycoproteins, including HSV gD, sort preferentially to the basolateral surface (Srinivas et al., 1986), an alternative explanation would be that significantly more BHV1.1 gD was present on the basolateral than on the apical surface and this large amount of gD inhibited the cell-to-cell spread of all three bovine herpesviruses. Because the relative distribution of BHV1.1 gD on the apical and basolateral surfaces is unknown, this must remain speculative.

gD of HSV1 has been implicated in cell fusion and cell-to-cell transmission (Brunetti et al., 1995) in contrast to the role of PRV gD (Peeters et al., 1992; Rauh and Mettenleiter, 1991). HveA (HVEM), a HSV1 gD receptor, can mediate cell-to-cell spread of HSV1 (Roller and Rauh, 1998) and virus-induced cell fusion (Terry-Allison et al., 1998). gD of HSV1 or BHV1.1 expressed alone was sufficient for induction of spontaneous fusion and syncytium formation according to some (Campadelli-Fiune et al., 1988; Tikoo et al., 1990) and not according to some others (Chase et al., 1990). This may be due to the level of gD being expressed on the plasma membrane, the cell type used, or the stoichiometry of gD and its cellular receptor or receptors. mAbs to HSV1 gD have been shown to reduce the frequency of cell fusion observed in HSV1 gD-expressing cells (Campadelli-Fiune et al., 1988b) and HSV1-infected cells (Noble et al., 1983) in a manner that is independent of their ability to neutralize exogenous HSV1 infectivity (Minson et al., 1986). Our cells exhibited polykaryocyte formation only on induction of high-level gD expression, suggesting a direct role for BHV1.1 gD in cell fusion. However, it is difficult to rule out the possibility of nuclear division without cell division evident only on high-level expression of BHV1.1 gD. Because BHV1.1 gD at levels sufficient to interfere with BHV1.1, HSV1, and PRV was apparently unable to interfere with different subtypes of BHV1 but could inhibit plaque enlargement, we suggest that the process of spread of the virus may in some ways be different from the initial exogenous infection. Indeed, only a subset of the proteins implicated in cell-to-cell transmission are required for entry (Davis-Poynter et al., 1994; Spear, 1993b), and the apical and basolateral membrane com-
positions in polarized cells are different in part due to preferential sorting of proteins and impedance in protein mobility by desmosomes.

The most favored hypothesis for gD-mediated interference is the utilization of a limited number of gD receptors, and at least four different alphaherpesviral gD receptors have been identified (HveA–D) (Geraghty et al., 1998; Montgomery et al., 1996; Warner et al., 1998). Although gD is the major determinant, other unidentified gene or genes may play a role in resistance to interference (Brandimarti et al., 1994; Dean et al., 1995). Expression of US-11, a nonstructural HSV1 protein that binds RNA, in cells induced resistance to infection, and a mutation in gD enabled HSV1 to overcome US11-mediated restriction (Roller and Roizman, 1994). Although BHV1 does not encode an obvious US11 gene, the existence of a functional homolog cannot be ruled out. Thus it is not surprising that cross-interference between alphaherpesviruses does not correlate with the gD sequence conservation (Fig. 1). It is possible that the ability of BHV1.2 and BHV5 to overcome BHV1.1 gD-mediated interference is a function of respective gDs or some other protein in the virus. Further understanding of viral and cellular proteins that play a role in herpesviral entry and spread is essential to unravel the molecular mechanism of gD-mediated interference, and bovine herpesviruses provide unique tools.

**MATERIALS AND METHODS**

**Cells and viruses**

MDBK cells purchased from American Type Culture Collection (ATCC CCL 22; Rockville, MD) were used because they are equally susceptible to infection and plaque formation with all the viruses used in this study. MDBK and African green monkey kidney cells (Vero, ATCC CCL 81) were passaged in minimal essential medium (MEM) (GIBCO BRL) supplemented with 5% fetal bovine serum (FBS) (Hyclone Labs).

All viruses were grown on MDBK cells at a low multiplicity, titered on MDBK cells, and stored at −70°C until future use. BHV1.1 (Cooper) was purchased from ATCC (VR-864), HSV1 (KOS) was a generous gift of Dr. C. Brandt, and PRV (Sullivan) was kindly provided by Dr. B. C. Easterday. The TX-89 isolate of BHV5 obtained from the brain of a steer that died of encephalitis (D’Offay et al., 1993) was kindly provided by Dr. S. I. Chowdhury. The ST strain of BHV1.2 was generated by transfecting genomic BHV1.2 DNA into MDBK cells. BHV1.2 genomic DNA was a kind gift from Dr. J. C. Audonnet (Leung-Tack et al., 1994). Vesicular stomatitis virus (VSV Indiana) was a gift from Dr. S. D. Vernon. Infectious BHV1.2 was generated by transfecting BHV1.2 genomic DNA into MDBK cells using Lipofectamine (GIBCO BRL).

**Construction of plasmids for transfection**

To generate the plasmid p-control (Fig. 2a), a G418-resistance cassette carrying both prokaryotic and eukaryotic transcription signals (Matthias et al., 1983) was ligated into the unique HindIII site of plasmid, p341 (Eiden et al., 1985). This cassette allowed us to select for its expression in bacteria using kanamycin (50 μg/ml) and in mammalian cells using G418 (Geneticin; GIBCO BRL). The plasmid p341 contains a metallothionein (MT) promoter and SV40 polyadenylation signal flanking a unique BglII site. To generate the plasmid p-gD (Fig. 2b), the BHV1.1 gD open reading frame contained in an Mael fragment (Tikoo et al., 1990) was transferred from polink gIV (a kind gift from Dr. T. J. Zamb) into the BglII site of p341 after the addition of BamHI linkers such that gD was under the control of the MT promoter. Proper orientation of the gD gene was determined by Smal restriction digestion. The G418 resistance cassette was then cloned into the unique HindIII site.

**Synthesis of liposomes, transfection, and selection of G418-resistant clones**

Liposomes were prepared as described by Rose et al. (1991) with minor modifications. Briefly, 1 ml of the cationic lipid dimethyl dioctadecyl ammonium bromide (DDAB 0.4 mg/ml in chloroform from Sigma) was mixed with 100 μl of the neutral lipid solution dioleoyl-L-α-phosphatidylethanolamine (PtdEtn; 10 mg/ml; Sigma) and evaporated to dryness at room temperature in a SpeedVac (Savant Instruments) overnight. The pellet was resuspended in 1 ml of sterile deionized water (Millipore) and sonicated using a Branson Sonifier (125 W, 117 V) with intermittent cooling on ice until the solution was clear.

The plasmids p-control and p-gD were used to construct the cell lines MDBK<sup>control</sup> and MDBK<sup>gD</sup>, respectively. MDBK cells grown overnight in 6-well plates to 70–80% confluency were used for transfection. Freshly prepared liposomes (60 μg in 1 ml of MEM) were mixed with 20 μg of either plasmid DNA (p-gD or p-control) and incubated at room temperature for 15 min. The monolayers were washed with serum-free MEM and overlaid with the liposome–DNA mixture for 3 h at 37°C. An additional 2 ml of MEM containing 20% FBS was added and incubated for another 24 h. The medium was then replaced with MEM containing 5% FBS and incubated for an additional 24 h for the expression of selectable marker. The medium was then replaced with MEM containing 1 mg/ml active G418 (Geneticin; GIBCO BRL) supplemented with 5% FBS and similarly replaced every 3–4 days. Individual G418-resistant clones were picked 15 days after transfection. After selection, the cells were maintained with 200 μg/ml active G418.
Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was used as an initial screen to detect gD-expressing clones. Briefly, transfected clones or untransfected control cells were plated onto 96-well plates (Costar) at a density of 20,000 cells/well and incubated for 12 h at 37°C. Duplicate wells of untransfected MDBK cells were infected with BHV1.1 at an m.o.i. of 1 and incubated overnight at 37°C to serve as positive controls. The cells were washed twice with PBS and fixed with methanol (kept at −20°C) to make the cells permeable to antibodies. They were then subjected to ELISA using a 1:2000 dilution of mouse ascites fluid containing anti-BHV1.1 gD mAb (mAb 1106) (Marshall et al., 1986). Reactions were detected with Vectastain (ABC) reagents according to the manufacturer’s instructions (Vector Laboratories).

Fluorescent antibody test

To identify clones that express gD homogeneously, three p-gD transfected clones that tested positive by ELISA and nonexpressing p-control transfected cells (MDBKcontrol) were grown on chamber slides (Nunc Labs). Nontransfected MDBK cells were infected with BHV1.1 to serve as positive controls. Replicate cultures were induced with medium containing 1 μM cadmium chloride for 18 h. Cells were then washed with ice-cold PBS containing 1% BSA and 0.02% sodium azide and fixed with methanol (−20°C) on ice for 30 min. The cells were washed again and incubated either with anti-BHV1.1 gD mAb (mAb 1106) or anti-BHV1.1 gB antibody (mAb 5106, isotype matched control) on ice for 45 min. The cells were then washed twice with PBS and incubated with 50 μl of FITC-conjugated goat anti-mouse antibody (1:30; Cappel, West Chester, PA). The cells were washed again with PBS and evaluated by both phase contrast and UV microscopy. Because the cells were subjected to identical treatments in parallel and photographed identically using a CCD camera, the intensities are directly comparable.

Radioimmunoprecipitation and gel electrophoresis

Radioimmunoprecipitation was used to confirm the production of BHV1.1 gD by MDBKcontrol cells and to show the antigenic relationship between the bovine herpesviruses. Confluent monolayers of stable transfecants carrying p-gD or p-control in 6-well plates were induced with 1 μM cadmium chloride, labeled with 50 μCi/ml of [35S]methionine and cysteine from 6 to 18 h postinfection. The anti-BHV1.1 gD mAbs 0722, 1102, 1106, 5006, 4906, and 3402 used in this experiment were made by Marshall et al. (1986) and mapped to five antigenic areas of BHV1.1 gD (Marshall et al., 1988). All the antibodies neutralize BHV1.1 in the absence of the complement (Dubuisson et al., 1992). mAb 3402 recognizes an epitope on BHV1.1 gD (92–102) and fails to neutralize BHV5 or recognize BHV5 gD on a Western blot (Abdelmagid et al., 1995). A neutralizing monoclonal antibody (N9) directed against VSV G protein (Lefrancois and Douglas, 1982) was used as the negative control. Each lysate (5% v/v of the lysate) was immunoprecipitated with each anti-BHV1.1 gD mAb or one anti-BHV1.1 gB antibody (5106) and one anti-VSV G antibody (N9). The precipitates were resolved on 8% SDS–polyacrylamide gels and subjected to autoradiography as described above.

Neutralization assay

To determine the conservation of neutralizing epitopes, BHV1.1, BHV1.2, BHV5, and HSV1 were tested for neutralization by a panel of anti-BHV1.1 gD mAbs and anti-VSV control antibody (N9) in the absence of the complement in two sets. Approximately 100 pfu of each virus in duplicates was subjected to neutralization by a serial 2-fold dilution of monoclonal anti-gD antibodies starting with 1:100 in 96-well plates (set 1). Serial 2-fold dilutions of each antibody were incubated with the same amount of virus in parallel to more sensitively determine the titer of non-neutralizing and poorly neutralizing antibodies (set 2). They were incubated for 1 h at 37°C, after which ~105 MDBK cells in MEM supplemented with 5% FBS were added to all the plates and incubated for an additional 72 h. The lowest concentration of a given antibody that was able to neutralize each virus was recorded as the neutralization titer of the antibody for each virus.

Western immunoblotting

To further define antigenic relationship between the bovine herpesviruses, Western blots were performed. Briefly, equal amounts of virus-infected cell lysates or mock-infected lysates were boiled and resolved on 8% SDS–PAGE under reducing conditions (Laemmli, 1970), and the proteins were transferred to nitrocellulose membrane (Bio-Rad). The blot was blocked, incubated with each anti-BHV1.1 gD mAb, followed by secondary antibody (rabbit anti-mouse IgG conjugated to alkaline phos-
Plaque assay to measure relative sensitivities to gD-mediated interference

MDBK<sup>gD</sup> and MDBK<sup>control</sup> cells (passage 7–10) were plated onto 24-well plates and grown to confluency overnight. They were washed with MEM and infected with serial 10-fold dilutions of virus (HSV1, BHV1.1, PRV, BHV1.2, BHV5, or VSV) in triplicates and incubated at 37°C for 1 h. The inoculum was then removed and overlaid with MEM supplemented with 5% FBS and 0.5% agarose (Medium EEO; FMC Biochemicals). When plaques were easily visible at 24 h (VSV) or 72 h (all other viruses), the cells were fixed with 10% formalin and stained with crystal violet. Plaques were counted to determine virus titers. Mean and S.D. values were calculated. Mean values for each virus on MDBK<sup>control</sup> cells were normalized to 100% to facilitate comparisons. The ability of BHV1.1 gD to interfere with BHV1.1, BHV1.2, and BHV5 was measured in two separate experiments.

BHV1.1 gD-mediated inhibition of plaque enlargement

Confluent MDBK<sup>gD</sup> and MDBK<sup>control</sup> monolayers in 6-well plates were infected at ~100 pfu/well of BHV1.2, BHV5, or VSV. Because BHV1.1 produced ~1000-fold fewer plaques on MDBK<sup>gD</sup> cells, ~10<sup>6</sup> pfu of BHV1.1 was used to infect MDBK<sup>gD</sup> cells and only 10<sup>5</sup> pfu was used on MDBK<sup>control</sup> cells. The plates were incubated for 1 h with the virus, washed with MEM, and overlaid with MEM containing 0.5% agarose and 5% FBS. Infected cells were fixed after 1 day (VSV), 2 days (BHV1.2 and BHV5), or 3 days (BHV1.1) when plaque sizes were <100 μm units in diameter. The diameter of ≥30 isolated plaques from each infected monolayer were measured using an ocular micrometer. Mean plaque diameters and the standard deviations were calculated.

BH5 growth kinetics on BHV1.1 gD-expressing and control cells

MDBK<sup>gD</sup> and MDBK<sup>control</sup> cells were plated onto 24-well plates and grown overnight in MEM containing 5% FBS. Cells were infected with BHV1.1 or BHV5 at an m.o.i. of 10 and incubated for 1 h at 37°C. The inoculum was removed, the cells were washed with MEM, and 0.5 ml of MEM supplemented with 5% FBS was added to each well. Infected monolayers were incubated at 37°C and frozen at 6-h intervals. Intracellular virus was released by lysing the cells through three freeze–thaw cycles. The amount of virus present in duplicate cultures at each time point for each virus was determined by plaque assay performed in duplicate.

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