

Bovine herpesvirus 1 requires glycoprotein H for infectivity and direct spreading and glycoproteins gH_{W450} and gB for glycoprotein D-independent cell-to-cell spread

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By analogy with glycoprotein H (gH) of herpes simplex virus type 1 (HSV-1) and pseudorabies virus (PRV), gH may also be essential for penetration and cell-to-cell spread of bovine herpesvirus 1 (BHV-1). This was verified with a gH-negative BHV-1 mutant (gH⁻ BHV-1), which replicated normally on gH-expressing cells but was unable to form plaques and infectious progeny on non-complementing cells. The block in entry could be overcome by polyethylene glycol-induced membrane fusion, demonstrating that gH is not essential for egress. Propagation of gH⁻ BHV-1 on cell lines expressing wild-type gH or gH_{W450}, which complements the function of BHV-1 gD for cell-to-cell spread, indicated that gH_{W450} is more efficient than wild-type gH in mediating direct spread of BHV-1. This was supported by the plaque sizes induced by rescued gH⁻ BHV-1 that expressed wild-type gH and gH_{W450}. Infection of cell lines expressing gH of BHV-1, HSV-1 and PRV with gH⁻ BHV-1, HSV-1 and PRV mutants demonstrated that heterologous gH molecules could not complement gH function in penetration or cell-to-cell spread.

The glycoprotein H–glycoprotein L (gH–gL) complex of the alphaherpesviruses herpes simplex virus type 1 (HSV-1) and pseudorabies virus (PRV) has been shown to be essential for entry of the virus into cells and for spread of virus from infected cells to adjacent cells (Gompels & Minson, 1986; Fuller *et al.*, 1989; Forrester *et al.*, 1992; Hutchinson *et al.*, 1992; Peeters *et al.*, 1992; Roop *et al.*, 1993; Klupp *et al.*, 1997). In HSV-1, fusion of the viral envelope with the plasma membrane of the target cells and fusion of the infected cell membrane with that of neighbouring cells also require gB and gD, whereas in PRV gD is dispensable for cell-to-cell spread (reviewed by

Spear, 1993; Mettenleiter, 1994), implying that differences exist in the molecular mechanisms involved in membrane fusion. This view is supported by results demonstrating unidirectional complementation of gB-negative (gB⁻) HSV-1 by gB of PRV (Mettenleiter & Spear, 1994) and of gB⁻ PRV by gB of bovine herpesvirus 1 (BHV-1), which also requires gB and gD for penetration into cells and direct cell-to-cell spread (Fehler *et al.*, 1992; Miethke *et al.*, 1995). However, in a recent report it has been shown that gH_{W450}, encoded by a gene with a single point mutation in codon 450 of the open reading frame (ORF) that results in a glycine-to-tryptophan amino acid exchange, can substitute for the cell-to-cell spread function of BHV-1 gD (Schröder *et al.*, 1997).

In BHV-1, the gH–gL complex is incorporated into virus particles and, by analogy with HSV-1 and PRV, it has been suggested that the complex is required for BHV-1 infectivity (Khattar *et al.*, 1996). To verify this assumption and to compare the activities of wild-type gH and gH_{W450} in BHV-1-induced membrane fusion processes, we isolated a gH⁻, *lacZ*-expressing mutant after co-transfection of 1 µg purified DNA from BHV-1 strain Aus12 (Fehler *et al.*, 1992) and 5 µg plasmid pβgH⁻ on the gH_{W450}-expressing cell line MDBK-gH_{W450} (named MDBK/C20 in Schröder *et al.*, 1997). Progeny virus was titrated on MDBK-gH_{W450} cells and plaques that stained blue under a BlueGal-containing agarose overlay (Fehler *et al.*, 1992) were selected. Virus was further plaque-purified until only blue-staining plaques were detected and no plaque formation was found in normal MDBK-Bu100 cells. Absence of the gH gene in the selected mutant gH⁻BHV-1 was demonstrated by immunoprecipitation with monoclonal antibody BH23 (Baranowsky *et al.*, 1993) and Southern blot hybridizations that confirmed replacement of codons 1 to 825 of the gH ORF by the *lacZ* expression cassette, which in pβgH⁻ is flanked by sequences from the thymidine kinase (TK) gene (nucleotide positions 426 to 713 in Mittal & Field, 1989), located upstream of the gH ORF, and by nucleotide positions 66905 to 67513 of the BHV-1 complete genome (GenBank accession no. AJ004801), encompassing the 27 3'-terminal codons of the gH ORF. The gH⁻ mutant, which is also TK⁻, was subsequently used to infect geneticin-resistant cell clones

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Table 1. Cells expressing gH of BHV-1, HSV-1 or PRV do not complement gH functions in heterologous gH⁻ mutants

Wild-type BHV-1 and the gH⁻ mutants gH⁻BHV-1 grown on MDBK-wtgH cells, HSV-1/SCgHZ (gH⁻HSV-1) grown on F6 cells and PRV/gH⁻βgal⁺ (gH⁻PRV) grown on SW78 cells were titrated on the indicated cell lines. After development of plaques in the wild-type BHV-1-infected cultures, cells were fixed and stained for lacZ expression. Formation or absence of plaques is indicated by + and -.

Cell line	Genotype	Plaque formation by			
		gH ⁻ BHV-1	gH ⁻ HSV-1	gH ⁻ PRV	Wild-type BHV-1
Vero	Wild-type	-	-	-	+
MDBK-Bu100	Wild-type	-	-	-	+
SW78	PRV gH ⁺	-	-	+	+
F6	HSV-1 gH ⁺	-	+	-	+
MDBK-wtgH	BHV-1 gH ⁺	+	-	-	+
MDBK-gH _{W450}	BHV-1 gH _{W450} ⁺	+	-	-	+

obtained after co-transfection of purified DNA from plasmids pwtBB8.5 and pAG60 (Colbere-Garapin *et al.*, 1981) into MDBK-Bu100 cells. Plasmid pwtBB8.5 contains the entire gH gene of wild-type BHV-1, including the gH promoter (putative TATA box located at positions 1314 to 1318; Mittal & Field, 1989) (J. Enssle & G. Keil, unpublished), and was constructed as described for pBB8.5, which encodes gH_{W450} (Schröder *et al.*, 1997). Duplicate cultures of cell clones that promoted gH⁻BHV-1-induced plaque formation were tested for release of gH⁻BHV-1 virions capable of inducing plaque formation on MDBK-gH_{W450} cells. One of the positive cultures was named MDBK-wtgH and used for further experiments. Attempts to demonstrate expression of gH and gH_{W450} by indirect immunofluorescence, immunoblotting and radioimmuno-precipitation with MA b BH23, after infection of the respective cell lines with gH⁻BHV-1 to transactivate the gH promoters in the transgenic cells, were not successful, suggesting that the respective cell lines expressed only limited amounts of gH and gH_{W450}.

To test whether gH is indeed essential for cell penetration by BHV-1, MDBK-Bu100 cells were infected with phenotypically wild-type gH-complemented gH⁻BHV-1 and the culture supernatant, collected 48 h post-infection (p.i.), was titrated on MDBK-wtgH cells in the presence or absence of polyethylene glycol (PEG) as described previously (Fehler *et al.*, 1992). Cultures were overlaid with semi-solid medium and plaques were counted 3 days later after staining for β-galactosidase (β-gal) expression (Fehler *et al.*, 1992). In three independent experiments, no plaques were detected in the absence of PEG, whereas PEG treatment led to plaque formation at 8.0×10^4 , 5.7×10^4 and 7.0×10^4 p.f.u./ml. In non-complementing MDBK-Bu100 cultures, only single blue-stained cells were detected after infection in the presence of PEG. Thus, gH⁻BHV-1 virions were released from infected

non-complementing cells and, after having entered the cells by PEG-mediated membrane fusion, were able to replicate in MDBK-wtgH cells, which proves that gH is required for infectious entry of BHV-1 into cells and suggests that gH is not essential for egress. The failure to induce plaques on non-complementing cells further demonstrated that gH is essential for direct spreading.

Since gH is one of the most highly conserved herpesvirus glycoproteins, we investigated whether heterologous gH molecules were able to complement the replication defect of gH⁻ BHV-1, HSV-1 and PRV. In contrast to HSV-1 and PRV, which induce plaques with comparable efficiency on MDBK-Bu100 cells and Vero cells, the plating efficiency of BHV-1 on Vero cells is about 1000-fold lower than on MDBK-Bu100 cells. To ascertain that a comparable number of infected cells could be monitored for plaque formation, approximately 10^7 p.f.u. of each of the following viruses were titrated on the various cell lines listed: wild-type gH-complemented gH⁻BHV-1, wild-type BHV-1 (as a control), gH⁻HSV-1 grown on the HSV-1 gH-expressing cell line F6 (derived from Vero cells; virus and cells kindly provided by A. C. Minson, Department of Virology, University of Cambridge, UK) and gH⁻PRV propagated on the PRV gH-expressing cell line SW78 (derived from Vero cells; virus and cells kindly provided by B. G. Klupp, Federal Research Centre for Virus Diseases of Animals, Insel Riems, Germany) were titrated on normal Vero cells, MDBK-Bu100 cells, SW78 cells, F6 cells, MDBK-wtgH cells and MDBK-gH_{W450} cells. After the appearance of plaques in the cultures infected with wild-type BHV-1, cells infected with the mutant viruses were fixed and stained for β-gal expression. Blue-staining plaques were only observed in cultures expressing homologous gH molecules (Table 1), whereas, at the appropriate dilutions, only single cells expressed β-gal in the normal cell cultures or in heterologous

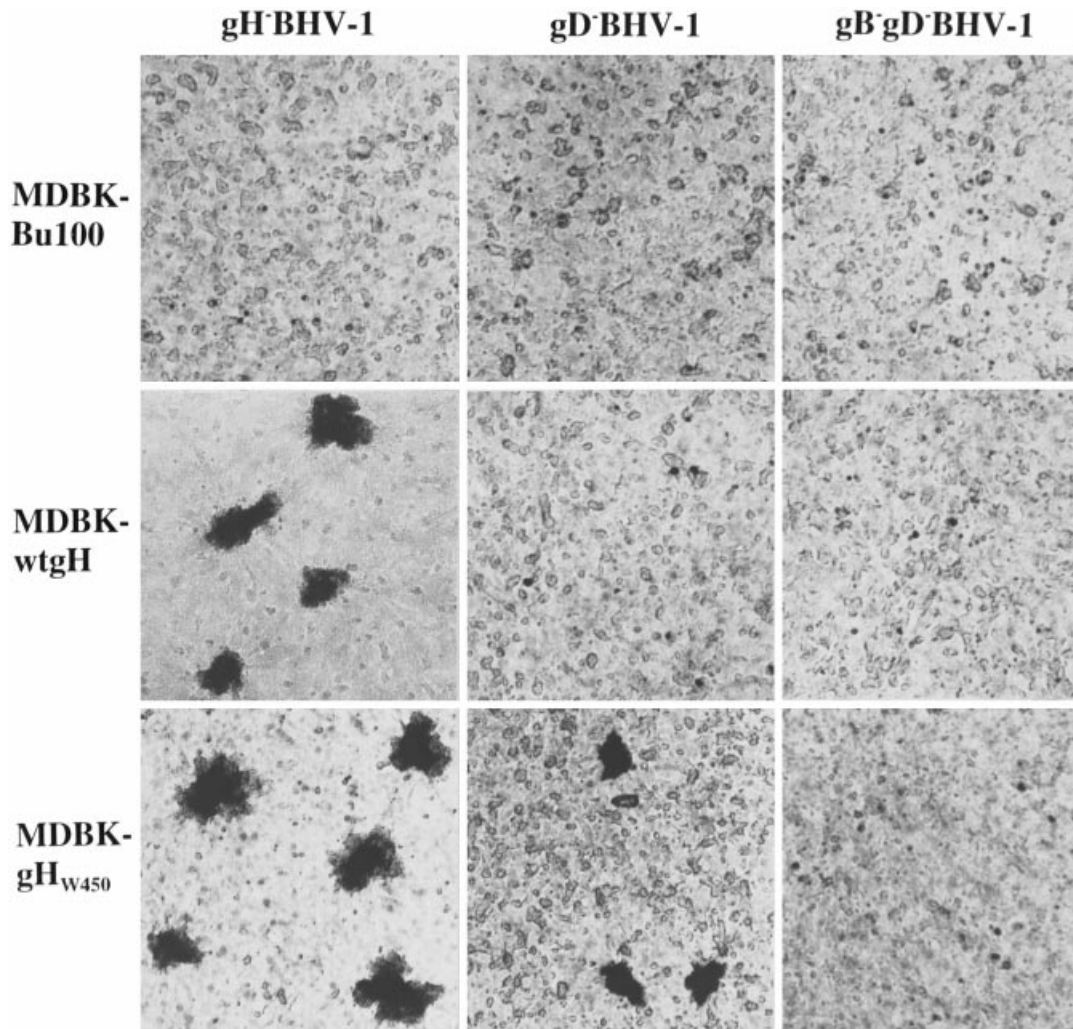


Fig. 1. Trans-complementation of BHV-1 deletion mutants by BHV-1 gH-expressing cells. MDBK-Bu100 cells, MDBK-wtgH cells and MDBK-gH_{W450} cells were infected with diluted, phenotypically gH⁻BHV-1, phenotypically gD⁻-complemented gD⁻BHV-1 and phenotypically gB⁻ and gD⁻-complemented gB⁻gD⁻BHV-1. Cultures were maintained under semi-solid medium for 2 days, fixed and stained for *lacZ* expression.

gH-expressing cells, demonstrating that none of these alpha-herpesvirus gH molecules was able to cross-complement.

To examine the effect of the glycine-to-tryptophan exchange in gH_{W450} on direct cell-to-cell spread, approximately 200 p.f.u. gH⁻BHV-1 grown on MDBK-wtgH cells was used to infect MDBK-Bu100 cells, MDBK-wtgH cells and MDBK-gH_{W450} cells. Cultures were overlaid with semi-solid medium and stained for β -gal expression 3 days later. As shown in Fig. 1, plaques developed on MDBK-wtgH cells and MDBK-gH_{W450} cells, whereas only single blue-staining cells were detected on MDBK-Bu100 cultures. The plaques induced on MDBK-gH_{W450} cells appeared slightly larger than those on MDBK-wtgH cells (Fig. 1). This suggests that gH_{W450} might be more efficient than wild-type gH at mediating direct spreading of BHV-1. To verify this suggestion, the genes encoding wild-type gH and gH_{W450} were integrated into

gH⁻BHV-1 by homologous recombination after co-transfection of gH⁻BHV-1 DNA and plasmids containing a 5.7 kbp *Th111I* fragment (positions 62336 to 68003, numbered according to the complete genome sequence of BHV-1), encompassing the entire TK and gH genes and flanking sequences, of wild-type BHV-1 and BHV-1/80-221gH_{W450} (Schröder *et al.*, 1997). The rescued viruses for which replacement of the *lacZ* cassette by the respective gH genes and reconstitution of the authentic gene order was confirmed by Southern blot hybridizations (not shown) were plaque-purified twice on MDBK-Bu100 cells. Two isolates, named BHV-1/gH_{W450}-I and BHV-1/gH_{W450}-II, and one isolate, named BHV-1/gH_{res}, were titrated on MDBK-Bu100 cells and incubated under semi-solid medium. The parent strain BHV-1/Aus12 was included as a control. Three days after infection, the diameters of 50 plaques were determined under a

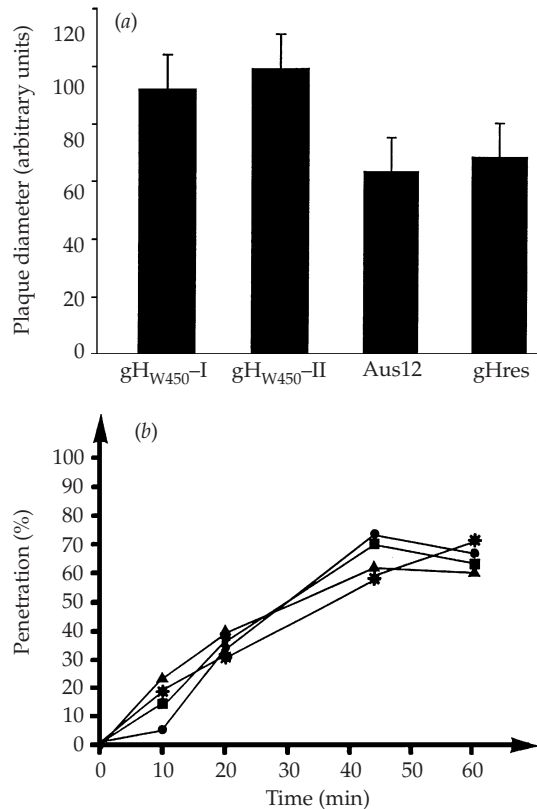


Fig. 2. The mutant gH_{W450} improves direct spreading of BHV-1 but is neutral for entry. (a) MDBK-Bu100 cells were infected with BHV-1 wild-type strain Aus12 and rescued strains gH_{W450}-I, gH_{W450}-II and gHres. At 3 days p.i., the diameters of 50 plaques were determined under a microscope by using a graduated ocular. Average diameters are shown in arbitrary units. Error bars indicate SD. (b) Stocks of BHV-1/gH_{W450}-I (●), BHV-1/gH_{W450}-II (■), BHV-1/gHres (▲) and BHV-1/Aus12 (*) were diluted to yield approximately 200 p.f.u. per cell culture dish. Penetration kinetics were determined as described previously (Fehler *et al.*, 1992).

microscope. The result is shown in Fig. 2(a) and demonstrated that plaques generated by rescued gH_{W450}-expressing gH⁻BHV-1 were clearly larger than plaques induced by the corresponding wild-type gH-expressing revertant or BHV-1/Aus12. In contrast, penetration of the rescued virus into target cells was independent of the gH expressed and comparable to the entry kinetics of wild-type BHV-1, suggesting that the mutation in gH_{W450} is neutral with regard to fusion between the membranes of extracellular virions and target cells. However, it should be noted that, although radioimmunoprecipitations with the gH-specific MAb BH23 (Baranowski *et al.*, 1993), and gC-specific MAb 118/2/4 (Schröder *et al.*, 1997) as an internal control, revealed comparable expression of gH in cells infected with the rescued virus and wild-type BHV-1, the possibility that small differences in the amount of gH expressed by the recombinant viruses may contribute to the effects observed cannot be excluded.

As mentioned before, the glycine-to-tryptophan exchange in gH_{W450} complements the function of gD for direct cell-to-

cell spread, a capacity demonstrated by plaque formation after infection of MDBK-gH_{W450} cells with phenotypically complemented virions of the gD⁻ mutant BHV-1/80-221 (Schröder *et al.*, 1997), which cannot spread in MDBK-Bu100 cells or MDBK-wtG cells (Fig. 1). To clarify whether gH_{W450} had acquired intrinsic membrane fusion activity, MDBK-Bu100 cells, MDBK-wtG cells and MDBK-gH_{W450} cells were infected with about 200 p.f.u. phenotypically gB⁻ and gD⁻ complemented gB⁻gD⁻ double mutant (gB⁻gD⁻BHV-1, kindly provided by F. Fehler, Federal Research Centre for Virus Diseases of Animals). As shown in Fig. 1, gB⁻gD⁻BHV-1 was unable to induce plaques on MDBK-gH_{W450} cells. Thus, gH_{W450} is unable to mediate direct spreading of BHV-1 from infected to neighbouring cells in the absence of gB, which also suggests that gH and gD might be involved in different steps during fusion of infected cell membranes.

In summary, our results demonstrate that gH is essential for infectious entry and cell-to-cell spread of BHV-1 and that no cross-complementation occurs between the gH molecules of BHV-1, HSV-1 and PRV. Furthermore, they provide evidence that different domains within gH might be involved in penetration and direct spreading, an assumption supported by Browne *et al.* (1996), who demonstrated that mutations in the cytoplasmic tail of HSV-1 gH affected both plaque formation and penetration of HSV-1 recombinants, whereas in our study gH_{W450} improved only cell-to-cell spread.

Note added in proof. After submission of this article, Meyer *et al.* (*Journal of General Virology* 79, 1983–1987, 1998) published their finding that gH is essential for penetration and propagation of BHV-1 in cell culture.

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