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Short Communication

# The UL49 gene product of BoHV-1: a major factor in efficient cell-to-cell spread

Donata Kalthoff,[1] Harald Granzow,[2] Sascha Trapp[3] and Martin Beer[1]

[1]Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Suedufer 10, 17493 Greifswald, Germany

[2]Institute of Infectology, Friedrich-Loeffler-Institut, Suedufer 10, 17493 Greifswald, Germany

[3]Institute for Medical Microbiology, Infectious and Epidemic Diseases, Faculty of Veterinary Medicine, Ludwig Maximilians University Munich, Germany

Correspondence
Martin Beer
martin.beer@fli.bund.de

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The role of the UL49 gene product, VP22, of bovine herpesvirus type 1 (BoHV-1) in virus replication was characterized with respect to a putative functional interaction of VP22 with the viral glycoprotein E (gE) during BoHV-1 cell-to-cell spread. Deletion of the open reading frames of UL49 and/or gE from an infectious BoHV-1 bacterial artificial chromosome clone did not severely impair the production of viral progeny in single-step growth experiments. However, plaque sizes induced by a VP22-negative BoHV-1 were reduced by 52 %, whilst for the gE/VP22-negative double-deletion mutant a reduction of 83 % could be observed in comparison with parental and revertant viruses, which was consistent with a marked reduction in multi-step growth experiments at early time points. These results suggest that gE and VP22 are important for BoHV-1 cell-to-cell spread, and that both are likely to act independently of each other in a critical pathway for virus cell-to-cell spread.

Bovine herpesvirus type 1 (BoHV-1), a member of the subfamily *Alphaherpesvirinae* (Fauquet *et al.*, 2005), is a major cause of respiratory and genital tract disease in cattle (Gibbs & Rweyemamu, 1977). Like other alphaherpesviruses, such as herpes simplex virus type 1 (HSV-1), pseudorabies virus (PrV) and varicella-zoster virus (VZV), BoHV-1 infects both epithelial and neuronal tissues (Arvin, 1996; Corey & Spear, 1986; Field & Hill, 1975; Tikoo *et al.*, 1995). Within these specialized compartments, virus can disseminate efficiently by means of direct cell-to-cell spread, which protects infectious virions from the adverse effects of neutralizing antibodies (Johnson & Huber, 2002). Virus cell-to-cell spread and entry of extracellular virus particles frequently share mechanistic details, e.g. the utilization of similar membrane fusion machinery, but cell-to-cell spread also involves other intra- and extracellular biophysical events determining virus delivery to cell junctions, as well as the use of receptors found exclusively at cell junctions (Johnson & Huber, 2002).

As in other alphaherpesviruses, the BoHV-1 complex of glycoprotein E (gE) and gI is not involved in the entry of extracellular particles (Rebordosa *et al.*, 1996; Yoshitake *et al.*, 1997) and hence viral gE deletion mutants display unimpaired penetration kinetics and virus yields in cell culture (Rebordosa *et al.*, 1996). However, deletion of BoHV-1 gE is generally associated with a marked reduction in plaque size *in vitro* (Rebordosa *et al.*, 1996; Trapp et *al.*, 2003), and gE deletion mutants have been shown to be attenuated in their respective bovine host (van Engelenburg *et al.*, 1994). Interestingly, for cell-associated alphaherpesviruses, such as VZV and Marek's disease virus (MDV), formation of the gE/gI complex is critical for virus replication *in vitro* (Mallory *et al.*, 1997; Schumacher *et al.*, 2001). With respect to virion morphogenesis, Fuchs *et al.* (2002) demonstrated for PrV that both gE and gM can interact physically with the C-terminal part of VP22, the tegument protein encoded by the UL49 open reading frame (ORF). Moreover, PrVΔgE virions have been shown to incorporate only approximately 50 % of the VP22 tegument protein compared with wild-type virions (Michael *et al.*, 2006). For HSV-1, physical interactions of VP22 with gD and with gE have been demonstrated (Chi *et al.*, 2005; Farnsworth *et al.*, 2007). However, physical or functional interactions of BoHV-1 gE or gD with the VP22 tegument protein have not yet been described.

Previously, a BoHV-1ΔUL49 deletion mutant was shown to produce decreased extracellular virus titres and to be avirulent in its bovine host (Liang *et al.*, 1995, 1997), but no further interactions were described. Furthermore, both BoHV-1 VP22 and HSV-1 VP22 exhibit intercellular trafficking, indicating that the protein traverses infected cells without additional viral components (Harms *et al.*, 2000).

To investigate the function of BoHV-1 VP22 in virus cell-to-cell spread, we generated different BoHV-1 mutants with deletion of UL49 or gE, or both, using Red$\alpha/\beta$-based

mutagenesis of a BoHV-1 bacterial artificial chromosome (BAC) clone in *Escherichia coli* (Trapp *et al.*, 2003), as well as conventional homologous recombination in eukaryotic cells. The mutant viruses were syngeneic and based on the BoHV-1 subtype 2 strain Schönböken (Engelhardt & Keil, 1996; Matheka & Straub, 1972).

BoHV-1 Schönböken was propagated in Madin–Darby bovine kidney cells (MDBK; ATCC CCL-22) grown in Dulbecco's modified essential medium supplemented with 10 % fetal calf serum (Engelhardt & Keil, 1996). The BoHV-1ΔgEΔUL49 deletion mutant was generated by Red$\alpha/\beta$-mediated mutagenesis in *E. coli* DH10B cells, as described previously (Trapp *et al.*, 2003). Based on the parental BAC clone, pBoHV-1ΔgE, the UL49 ORF was replaced with a kanamycin resistance gene amplified from plasmid pACYC177 (MBI Fermentas) by PCR using appropriate primers. Revertant viruses with restored gE and UL49 ORFs were generated by co-transfecting the cloned DNAs with the appropriate cloned PCR fragments from strain Schönböken (Fig. 1).

BoHV-1ΔgEΔUL49 could be isolated after transfection of the infectious BAC DNA into non-complementing cells, indicating that both gene products, even in combination, are dispensable for virus replication in cell culture. Samples from infected and uninfected MDBK cells, as well as purified virions, were prepared as described previously (Dietz *et al.*, 2000; Hampl *et al.*, 1984). As expected, in Western blot analyses of infected cell lysates (data not shown) or purified virions (Fig. 2a), VP22-specific reactivity was absent from BoHV-1ΔgEΔUL49- and BoHV-1ΔUL49-infected cells and purified virions. In virion preparations derived from BoHV-1- and BoHV-1ΔgE-infected cells, the 32 kDa VP22 protein readily could be detected by a specific peptide antiserum (polyclonal rabbit anti-UL49 serum; D. Kalthoff and others, unpublished data), and both BoHV-1 and BoHV-1ΔUL49 revealed specific reactivity with a gE-specific monoclonal antibody (mAb 2-1; a kind gift from W. Fuchs, Friedrich-Loeffler-Institut, Germany). Envelope glycoprotein gD (72 kDa) was present in all virus preparations and infected-cell lysates in similar amounts (detected using gD-specific mAb 21/3/3; a kind gift from G. M. Keil, Friedrich-Loeffler-Institut, Germany). In addition, the UL49.5 gene product could be detected in all preparations, indicating that transcription of UL49.5, in the direct vicinity of UL49, remained unaffected in all mutants (data not shown).

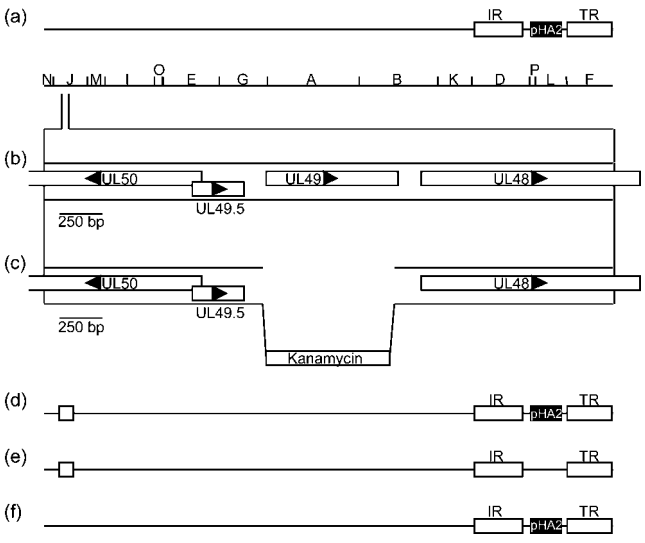


**Fig. 1.** Schematic illustration of the construction of BoHV-1ΔgEΔUL49 virus from pBHV-1ΔgE. (a) BoHV-1 genome organization in the infectious BAC clone (pBHV-1ΔgE) and *Hin*dIII restriction map. (b, c) Detailed organization of the target gene UL49 and the neighbouring ORFs (b), together with a depiction of kanamycin resistance gene integration into the UL49 ORF (c). (d–f) Schematic design of the mutants BoHV-1ΔgEΔUL49 (d), BoHV-1ΔUL49gErev (e) and BoHV-1ΔgEUL49rev (f). pHA2 defines the inserted mini F-plasmid cassette encoding chloramphenicol resistance and enhanced green fluorescent protein (Trapp *et al.*, 2003).

Growth properties of the generated mutant viruses were compared with those of the parental BoHV-1 strain. Single-step and multi-step growth kinetics from two independent experiments were assessed using MDBK cells inoculated with the parental and mutant viruses at an m.o.i. of 1 or 0.01, respectively, for 1 h. Virus particles that had not entered the cells after 1 h were inactivated by low-pH treatment using a citrate buffer (Highlander *et al.*, 1987). At the indicated times post-infection (p.i.), intra- and extracellular virus titres were determined (Fig. 2b–e). Single-step growth kinetics revealed a maximum 37-fold reduction in extracellular virus titres of the gE/UL49 double-deletion mutant with the greatest difference at 24 h p.i. (Fig. 2b) and a 24-fold reduction in intracellular virus titres at 48 h p.i. (Fig. 2c). Thus, gE and VP22 are apparently not crucial for secondary envelopment and/or egress of BoHV-1, as even a double deletion of gE and UL49 did not result in a drastic reduction in the number of viral progeny in the supernatant of the single-step growth experiment. In contrast, multi-step growth kinetics demonstrated markedly reduced extracellular (up to 6500-fold at 24 h p.i.) as well as intracellular (up to 75-fold at 12 h) virus titres for BoHV-1ΔUL49gErev compared with wild-type BoHV-1, which were adjusted to a minimal reduction at 48 or 72 h p.i. (Fig. 2d and e). Furthermore, in comparison with BoHV-1ΔgE, a greater than 230-fold reduction at 24 h p.i. was observed (Fig. 2d).

In order to examine the effect of the gE/UL49 double deletion on virus cell-to-cell spread, MDBK cells were seeded in six-well plates and 200 p.f.u. of the viral mutants was used to infect $10^6$ cells per well. At 2 days p.i. under a methylcellulose overlay (Neubauer *et al.*, 1997), the diameters of at least 100 plaques were measured for each virus and mean diameters ($\pm$SD) were calculated. Values for the parental strain Schönböken were set at 100 % and

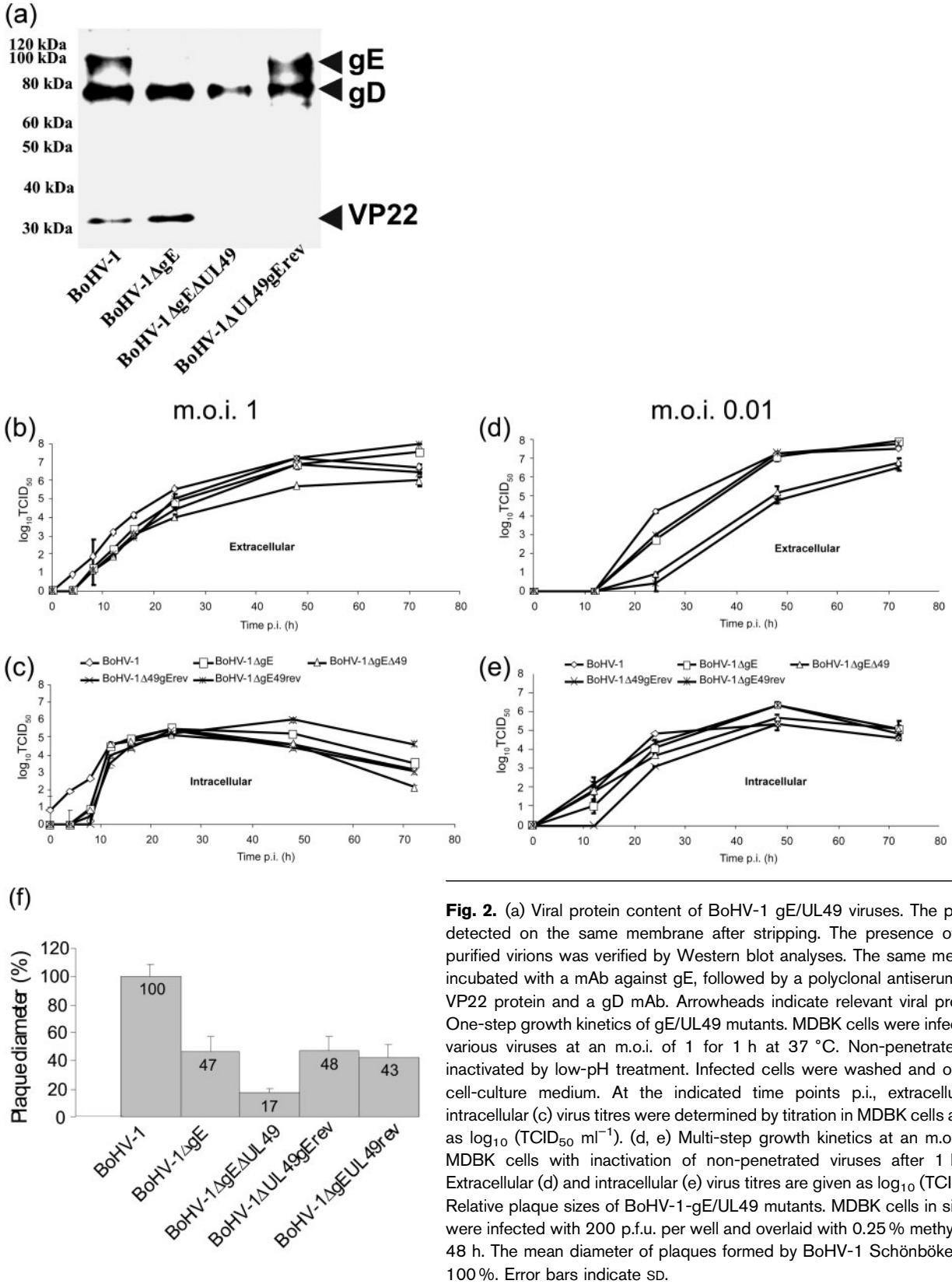


**Fig. 2.** (a) Viral protein content of BoHV-1 gE/UL49 viruses. The proteins were detected on the same membrane after stripping. The presence of proteins in purified virions was verified by Western blot analyses. The same membrane was incubated with a mAb against gE, followed by a polyclonal antiserum against the VP22 protein and a gD mAb. Arrowheads indicate relevant viral proteins. (b, c) One-step growth kinetics of gE/UL49 mutants. MDBK cells were infected with the various viruses at an m.o.i. of 1 for 1 h at 37 °C. Non-penetrated virus was inactivated by low-pH treatment. Infected cells were washed and overlaid using cell-culture medium. At the indicated time points p.i., extracellular (b) and intracellular (c) virus titres were determined by titration in MDBK cells and are given as $\log_{10}$ ($TCID_{50}$ $ml^{-1}$). (d, e) Multi-step growth kinetics at an m.o.i. of 0.01 in MDBK cells with inactivation of non-penetrated viruses after 1 h at 37 °C. Extracellular (d) and intracellular (e) virus titres are given as $\log_{10}$ ($TCID_{50}$ $ml^{-1}$). (f) Relative plaque sizes of BoHV-1-gE/UL49 mutants. MDBK cells in six-well plates were infected with 200 p.f.u. per well and overlaid with 0.25 % methylcellulose for 48 h. The mean diameter of plaques formed by BoHV-1 Schönböken was set at 100 %. Error bars indicate SD.

the plaque diameters observed for the mutant viruses were expressed relative to this value (Fig. 2f). Deletion of gE resulted in a 53 % reduction in plaque diameter, whereas the single deletion of UL49 resulted in a reduction of 52 %. Simultaneous deletion of gE and UL49, however, resulted in virus plaques exhibiting a reduction of 83 % in diameter. Statistical analysis using the Scheffé test (alpha level set at 0.05) revealed no significant differences in plaque sizes for BoHV-1ΔgE versus BoHV-1ΔUL49gErev, BoHV-1ΔgE versus BoHV-1ΔgEUL49rev and BoHV-1ΔUL49gErev versus BoHV-1ΔgEUL49rev, whereas all other measured plaque size differences were clearly statistical significant. In conclusion, the observed plaque size effect strongly suggested that gE and VP22 are both involved in direct cell-to-cell spread.

The morphogenesis of BoHV-1 mutants was also investigated by electron microscopy. MDBK cell cultures were fixed at different times p.i. for 60 min with 2.5 % glutaraldehyde buffered in 0.1 M sodium cacodylate (pH 7.2, 300 mosmol), scraped off the plate, pelleted by low-speed centrifugation and embedded in low-melting-point agarose. Small pieces were post-fixed in 1.0 % aqueous $OsO_4$ and stained with uranyl acetate. After stepwise dehydration in ethanol, the cells were cleared in propylene oxide, embedded in glycid ether 100 and polymerized at 59 °C for 4 days. Ultrathin sections of embedded material, counterstained with uranyl acetate and lead salts, were examined with an electron microscope (Tecnai 12; Philips). The electron microscopic analysis of cells infected by the mutant viruses clearly revealed that all of the main steps of wild-type virion morphogenesis were unaffected following deletion of gE and/or UL49 (Fig. 3).

Both single-step growth kinetics data and electron microscopy suggested that particle egress of UL49 single-deletion and gE/UL49 double-deletion mutants was relatively unaffected. These data contrast in part with a study by Liang *et al.* (1995); however, the UL49-deleted mutants used in that study, which showed a 1000-fold reduced titre at 12 h p.i., were reported also to have a defect in expression of the UL49.5 gene product, which is in clear contrast to our mutants. Furthermore, no data were provided about the m.o.i. used for the reported growth kinetics.

Interestingly, in our study, plaque size measurements revealed massively impaired cell-to-cell spread for BoHV-1ΔUL49gErev, as well as for BoHV-1ΔgEΔUL49, which was consistent with the observed multi-step growth kinetics data. The different intracellular virus titres of the

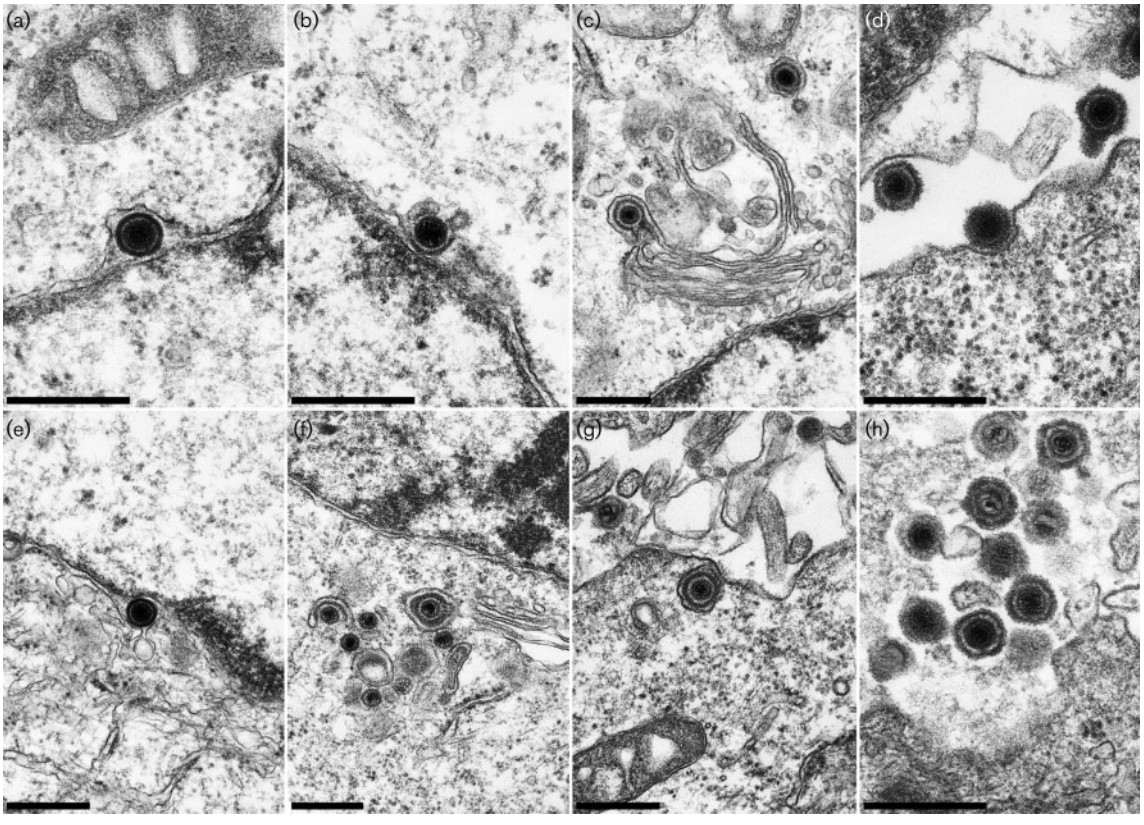


**Fig. 3.** Electron microscopy analyses of BoHV-1ΔUL49gErev (a–d) and BoHV-1ΔgEΔUL49 (e–h). Virion morphogenesis was impaired in both deletion mutants. Selected stages of morphogenesis are depicted for both mutants: (a, e) primary enveloped virion; (b) de-envelopment; (c, f) secondary envelopment; (g) vesicular transport; (d, h) extracellular virions. Bars, 400 nm.

multi-step growth curves at 12 h p.i. with no detectable extracellular infectivity (Fig. 2d and e) can best be explained by a different efficiency of cell-to-cell spread of the tested BoHV-1 mutants. Further differences in the extracellular titres can in addition probably be attributed to the reduced cell-to-cell spread and thus the reduced numbers of infected cells at the different time points. However, an additional minor role of a decrease in virus release, as reported for an HSV-1ΔUL49 mutant (Duffy *et al.*, 2006), cannot be ruled out completely, especially following the observed differences between the BoHV-1ΔgE and BoHV-1ΔUL49ΔgErev mutants in the multi-step growth kinetics (Fig. 2d and e). However, Rijsewijk *et al.* (1999) demonstrated an improved release of BoHV-1ΔgE from infected cells compared with wild-type BoHV-1. Therefore, a possible explanation for the observed differences may be that, in cells infected with BoHV-1ΔgE mutants, more virions could be secreted into the medium.

By analysis of gE and UL49 single- and double-deletion mutants as well as revertant viruses, we have shown here that, in addition to gE, the VP22 tegument protein is a major factor in cell-to-cell spread – at least in epithelial cells. Given that VP22 also interacts with several other BoHV-1 glycoproteins, as has been shown for other alphaherpesviruses (Farnsworth *et al.*, 2007; Fuchs *et al.*, 2002; Michael *et al.*, 2006), it is conceivable that it is expressed to direct capsids to neighbouring cells. The single deletion of UL49 had a comparable effect on BoHV-1 cell-to-cell spread to the gE deletion (52 and 53 % reductions in plaque size), and the effect on cell-to-cell spread was more than additive in the characterized gE/UL49 double-deletion mutant (83 % reduction in plaque size). Therefore, our data not only identified VP22, in addition to gE, as a crucial factor for BoHV-1 cell-to-cell spread, but also indicated that both viral proteins may act independently in the same mechanistic pathway of cell-to-cell spread of BoHV-1. Nevertheless, deletion of UL49 also resulted in a detectable reduction in virus titres in comparison with wild-type BoHV-1, which is, in our opinion, in accordance with the observed plaque size reduction, whilst virion morphogenesis – as demonstrated by electron microscopic analysis – was not affected.

The relevance of VP22 for virion morphogenesis and cell-to-cell spread appears to be highly variable among the different members of the subfamily *Alphaherpesvirinae*. PrV lacking VP22 shows no distinct phenotype (del Rio *et al.*, 2002) and this deletion has only minor effects on virus replication (Fuchs *et al.*, 2002). However, titres of HSV-1 VP22 deletion mutants are decreased 50-fold compared with wild-type virus, and a marked delay in the onset of viral protein synthesis has been described (Elliott *et al.*, 2005). Interestingly, a reduction in virus titres could only be observed in MDBK cells and not in Vero cells, which are known to display only a few cell junctions (Polcicova *et al.*, 2005). These findings indicate that cell lines with more pronounced epithelial characteristics such as MDBK cells allow the altered phenotype of HSV-1ΔUL49 mutants. Duffy *et al.* (2006) characterized a UL49-deleted HSV-1 mutant exhibiting plaque sizes that were reduced by 95 % compared with the parental virus. The authors explained the observed massive reduction by a decrease in virus release, as assessed by multi-step growth kinetics, rather than by a dysfunctional cell-to-cell spread. In addition, for viruses that depend fully on cell-to-cell spread for their growth, such as the cell-associated MDV, VP22 is an essential gene product, as is gE (Dorange *et al.*, 2002). In our opinion, the differences between UL49-deleted mutants of BoHV-1, PRV, MDV and HSV-1 may on the one hand be related to epithelial characteristics of the infected cell type and on the other to a different compensation capacity concerning the UL49-dependent cell-to-cell spread, leading to minor (e.g. PRVΔUL49), medium (e.g. BoHV-1ΔUL49) or severe (e.g. MDVΔUL49) defects.

Taken together, our observations for BoHV-1 strongly support the hypothesis that alphaherpesvirus secondary envelopment, egress and direct cell-to-cell spread are independent of each other, although fusion of membranes containing viral proteins is required for both processes. Finally, and most importantly, we identified gE and VP22 as two equally important factors for BoHV-1 cell-to-cell spread that are likely to act independently from each other in a critical pathway for virus cell-to-cell spread.

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