Phenotypic similarities and differences between UL37-deleted pseudorabies virus and herpes simplex virus type 1

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In the absence of the tegument protein pUL37, virion formation of pseudorabies virus (PrV) and herpes simplex virus type 1 (HSV-1) is severely impaired. Non-enveloped nucleocapsids accumulate in clusters in the cytoplasm, whereas only a few enveloped particles can be detected. Although a contribution of pUL37 to nuclear egress of HSV-1 has been suggested, the nuclear stages of morphogenesis are not impaired in PrV-ΔUL37-infected cells. Moreover, HSV-1 pUL37 has been described as essential for replication, whereas PrV is able to replicate productively without pUL37, although to lower titres than wild-type virus. Thus, there may be functional differences between the respective pUL37 proteins. This study compared the phenotypes of UL37-deleted PrV and HSV-1 in parallel assays, using a novel pUL37 deletion mutant of HSV-1 strain KOS, HSV-1ΔUL37[86–1035]. Aggregates of seemingly ‘naked’ nucleocapsids were present in the cytoplasm of African green monkey (Vero) or rabbit kidney (RK13) cells infected with HSV-1ΔUL37[86–1035] or PrV-ΔUL37. Nuclear retention of nucleocapsids was not observed in either virus. However, in contrast to PrV-ΔUL37, HSV-1ΔUL37[86–1035] was unable to replicate productively in, and to form plaques on, either Vero or RK13 cells. Trans-complementation of respective deletion mutants with the heterologous pUL37 did not ensue. These data demonstrate that the conserved pUL37 in HSV-1 and PrV have similar but distinct functions.

INTRODUCTION

Herpesvirions consist of four morphological substructures. The DNA-containing core is enclosed in an icosahedral capsid, which, in turn, is surrounded by a proteinaceous tegument and a cell-derived lipid envelope containing viral glycoproteins. Morphogenesis of herpesviruses includes intranuclear assembly of nucleocapsids, which then traverse the nuclear membrane by budding and fusion events (reviewed by Mettenleiter, 2002). Nucleocapsids acquire their final tegument and envelope by budding into trans-Golgi-derived vesicles in the cytosol. Whereas capsid assembly is well understood, the molecular basis for the acquisition of tegument and envelope is largely unclear. Tegumentation follows an intricate network of protein–protein interactions, which occur in different cellular compartments (reviewed by Mettenleiter, 2006; Mettenleiter et al., 2006). Whereas several tegument proteins are conserved throughout the family Herpesviridae, such as the primary tegument protein pUL31 and the inner tegument components pUL36 and pUL37, others are found only in single subfamilies, such as the alphaherpesvirus-specific pUL46, pUL47, pUL48 and pUL49.

The primary tegument protein pUL31 is required for primary envelopment of intranuclear nucleocapsids by complexing with pUL34 at the inner nuclear membrane (Fuchs et al., 2002a; Lake & Hutt-Fletcher, 2004; Reynolds et al., 2002; Sanchez & Spector, 2002). Both proteins are lost from the nascent virus particles when nucleocapsids are released into the cytoplasm after fusion between primary envelope and outer nuclear membrane. Although these newly translocated nucleocapsids appear naked by conventional electron microscopy, immunolabelling studies of pseudorabies virus (PrV) particles have revealed that they carry the tegument proteins pUL36 and pUL37 as well as pUS3 (Fuchs et al., 2002c). pUS3 has also been found in primary virions (Granzow et al., 2004). pUL36, the largest virally encoded protein, presumably forms the inner layer of tegument by interacting either directly (Chen et al., 1999; McNabb & Courtney, 1992; Vittone et al., 2005;
Zhou et al., 1999) or indirectly via pUL25 (Coller et al., 2007) with the nucleocapsid. pUL36 has also been shown to associate with pUL37, which may therefore build up a second layer of tegument (Fuchs et al., 2004; Klupp et al., 2002). Other tegument proteins such as pUL11 and pUL49 assemble at the secondary envelopment site by association with glycoproteins (Farnsworth et al., 2007; Fuchs et al., 2002b). In the absence of pUL37 in PrV (Klupp et al., 2001) and herpes simplex virus type 1 (HSV-1) (Desai et al., 2001), secondary envelopment is impaired and aggregates of nucleocapsids are formed in the cytosol. These data suggest that pUL37 is added early during tegumentation and is required for efficient addition of other tegument proteins (reviewed by Mettenleiter, 2002; Mettenleiter et al., 2006).

In addition to its role in virion formation in the cytosol, a function in the nuclear stages of morphogenesis has been postulated for HSV-1 pUL37 based on observations that most viral particles of pUL37-deleted HSV-1 are associated with the nuclear fraction of infected cells, whereas wild-type particles are predominantly found in the cytoplasmic fraction (Desai et al., 2001). However, ultrastructural analysis of PrV-ΔUL37-infected cells has not revealed an effect on nuclear egress of viral particles (Klupp et al., 2001). Moreover, pUL37 has been described as essential for the productive replication of HSV-1 (Desai et al., 2001), whereas pUL37-deleted PrV is still able to replicate productively, although approximately 100-fold less efficiently than wild-type PrV (Klupp et al., 2001). In contrast, PrV and HSV-1 pUL36 execute truly essential functions (Desai, 2000; Fuchs et al., 2004). In PrV, this essential role of pUL36 has been shown to be independent of its interaction with pUL37 (Fuchs et al., 2004).

So far, very few studies have been performed to compare directly the respective deletion mutants in homologous proteins of different herpesviruses in standardized assays. Thus, differing results could be due to differences in virus species or strain, host cells, experimental set-up or read-out system. We recently started to assess whether conservation of protein sequence also translates into conservation of protein function by analysing in parallel, using standardized virus–cell systems, respective deletion mutants of the related alphaherpesviruses HSV-1 and PrV by direct comparison of their phenotypes and by assessing heterologous trans-complementation (Kuhn et al., 2008; Leee et al., 2009). To unravel similarities and differences in pUL37 of HSV-1 and PrV, we compared in parallel the phenotypes of pUL37-deleted PrV (Klupp et al., 2001) and a newly constructed pUL37-deleted HSV-1 mutant based on a bacterial artificial chromosome (BAC) containing the genome of HSV-1 strain KOS (Leege et al., 2009). We also assessed whether cells expressing the PrV and HSV-1 UL37 proteins were able to complement the defect of the other virus mutant in trans.

METHODS

Viruses and cells. HSV-1AUL37[86–1035] was generated by mutagenesis of HSV-1 strain KOS cloned as a BAC (Leege et al., 2009). Wild-type PrV strain Kaplan (Ka; Kaplan & Vatter, 1959) and isogenic PrV-AUL37 (Klupp et al., 2001) have been described previously. Viruses were propagated in rabbit (RK13) or African green monkey (Vero) kidney cells, or in trans-complementing RK13-UL37(PrV) cells (Klupp et al. 2001) or Vero-UL37(HSV-1) cells (see below), respectively. Cells were grown in minimum essential medium containing 5 or 10% fetal calf serum (Invitrogen) and, for plaque assays, 6 g methyl cellulose l–1 (Sigma) was added.

Construction of plasmids, cell lines and virus mutants. The UL37 ORF of HSV-1 strain KOS was amplified from genomic DNA by PCR using Pfu DNA polymerase (Invitrogen), with primers HUL37-F (5′-CAGAAATTCGCGTCTAGGACCCG-3′) containing reverse-strand nt 84073–84090 of the HSV-1 genome sequence (GenBank accession no. X14112; McGeoch et al., 1988) and HUL37-R (5′-CACTGACGCGCCCGACCATAC-3′, nt 80652–80670). Artificial EcoRI and XbaI sites (underlined) facilitated cloning of the PCR product into the appropriately digested eukaryotic expression vector pcDNA3 (Invitrogen). The obtained plasmid, pcDNA-UL37 (Fig. 1c), permitting constitutive expression of the UL37 gene under the control of the human cytomegalovirus immediate-early promoter/enhancer, was used for transfection (FuGene HD reagent; Roche) of Vero cells. After propagation in medium containing 500 µg genetin ml–1 (Invitrogen), Vero UL37(HSV-1) cells were isolated from a single resistant clone.

The EcoRI/XbaI-treated PCR product of HSV-1 UL37 was also inserted into prokaryotic expression vector pGEX-4T-1 (GE Healthcare), which had been digested with EcoRI and SalI. From Escherichia coli transformed with the resulting plasmid, pGEX-UL37 (Fig. 1d), a pUL37 fusion protein with glutathione S-transferase was isolated and used for rabbit immunization to obtain a monospecific antisera as described previously (Fuchs et al., 2002a).

For deletion of the UL37 gene, pcDNA-UL37 was digested with BstEI and NofI (Fig. 1b, c), and a kanamycin-resistance gene flanked by FpI recombinase recognition target (FRT) sites was inserted. The modified UL37 gene was then amplified and used for mutagenesis of BAC clone pHSV-1Agl as described previously (Leege et al., 2009). After removal of the resistance gene and the BAC vector from the virus genome, HSV-1AUL37[86–1035] retained a substitution of UL37 codons 86–1035 by a single FRT site (36 bp) as the only mutation. This was confirmed by Southern blotting and sequence analysis (data not shown).

Western blotting, one-step growth curves and plaque-size analysis. Western blot analyses of infected cells were performed essentially as described previously (Fuchs et al., 2002a). For detection of the UL37 gene, pcDNA-UL37 was digested with BstEI and NotI (Fig. 1b, c), and a kanamycin-resistance gene flanked by FpI recombinase recognition target (FRT) sites was inserted. The modified UL37 gene was then amplified and used for mutagenesis of BAC clone pHSV-1Agl as described previously (Leege et al., 2009). After removal of the resistance gene and the BAC vector from the virus genome, HSV-1AUL37[86–1035] retained a substitution of UL37 codons 86–1035 by a single FRT site (36 bp) as the only mutation. This was confirmed by Southern blotting and sequence analysis (data not shown).

Analysis of the in vitro growth properties of PrV and HSV-1 followed established procedures (Leege et al., 2009). For determination of one-step replication kinetics, cells were harvested together with the supernatants at the indicated times after synchronized infection at an m.o.i. of 5, and virus progeny was analysed by plaque assays on Vero-UL37(HSV-1) or RK13-UL37(PrV) cells, respectively. Plaques on complementing and non-complementing cells were visualized 2 days after infection with PrV or 3 days after infection with HSV-1 by indirect immunofluorescence of an HSV-1 pUL48-specific antisera or a PrV gC-specific monoclonal antibody (Nixdorf et al., 2000), respectively, and measured microscopically.
**RESULTS**

**Protein analysis**

The novel mutant HSV-1ΔUL37[86–1035] carries a deletion of codons 86–1035 of the UL37 open reading frame (ORF). Codons 1–85 and 1036–1124 are separated out of frame by a 36 bp FRT site that contained translational stop codons prohibiting expression of the 3'-terminus. The 5'- and 3'-terminal codons of the UL37 ORF were retained to avoid interference with the promoters of UL38 and UL36, respectively.

Western blotting using a monospecific rabbit antiserum against HSV-1 pUL37 detected the 120 kDa protein in lysates of Vero cells infected by wild-type HSV-1 strain KOS (Fig. 2a), which was absent in Vero cells infected by HSV-1ΔUL37[86–1035] (Fig. 2a). No signal indicating expression of residual codons 1–85 of UL37 was observed, whereas the tegument protein pUL48 was detected as the loading control (Fig. 2b). However, pUL37 was expressed in Vero-UL37(HSV-1) cells infected by HSV-1ΔUL37[86–1035] (Fig. 2a), and comparable amounts of the protein were also found after infection with PrV-ΔUL37 (Fig. 2c). Correspondingly, similar amounts of PrV pUL37 were present in RK13-UL37(PrV) cells infected with either deletion mutant (Fig. 2d).

**One-step replication curves and plaque formation of HSV-1ΔUL37[86–1035] in different host cells**

In one-step growth analyses after infection at an m.o.i. of 5, maximum titres of HSV-1ΔUL37[86–1035] on Vero-UL37(HSV-1) cells were only slightly lower than those of wild-type HSV-1 KOS, indicating efficient trans-complementation of the deletion mutant by the UL37 protein expressed in the host cells (Fig. 3). In contrast, no productive replication of HSV-1ΔUL37[86–1035] was observed on non-complementing Vero or RK13 cells. The residual low level of infectivity derived from non-complementing cells could not be maintained after subsequent infection of non-complementing cells, indi-
cating that it was probably derived from incorporation of the remaining input pUL37 delivered to the cell by the trans-complemented virions. In parallel with these results, plaque formation of HSV-1D UL37[86–1035] was completely inhibited on RK13 as well as on Vero cells, whereas on Vero-UL37(HSV-1) cells, HSV-1D UL37[86–1035] produced almost wild-type-sized plaques (Fig. 4a).

Lack of trans-complementation of PrV and HSV-1 UL37 deletion mutants by heterologous pUL37

To analyse whether the pUL37 proteins of HSV-1 and PrV were able to trans-complement the UL37 defect in the respective non-homologous virus, PrV-ΔUL37 (Klupp et al., 2001) was assayed on Vero-UL37(HSV-1) cells and HSV-1ΔUL37[86–1035] on RK13-UL37(PrV) cells. Productive replication of HSV-1ΔUL37[86–1035] did not ensue after infection of RK13-UL37(PrV) cells (Fig. 3) and PrV-ΔUL37 replicated on Vero-UL37(HSV-1) cells to titres similar to those on non-complementing Vero cells (Fig. 3). Plaque sizes of PrV-ΔUL37 were also reduced to a similar extent on Vero cells expressing HSV-1 pUL37 as on native Vero or RK13 cells (Fig. 4b), demonstrating a lack of complementation by HSV-1 pUL37. In the reverse experiment, HSV-1ΔUL37[86–1035] was unable to replicate productively or to form plaques on RK13-UL37(PrV) cells (Figs 3 and 4a). Wild-type HSV-1 KOS and PrV-Ka (Kaplan & Vatter, 1959) were able to form plaques efficiently on all cells tested (Fig. 4a, b).

Ultrastructural phenotype of pUL37 mutants

For ultrastructural analyses, RK13 (Figs 5a–c and 6a–c) and Vero cells (Figs 5d–f and 6d–f) were infected with phenotypically complemented HSV-1ΔUL37[86–1035] (Fig. 5) or PrV-ΔUL37 (Fig. 6) at an m.o.i. of 1 for 16 h, and processed for transmission electron microscopy as described previously (Klüpp et al., 2001). In both cell lines infected with either UL37 deletion mutant, clusters of non-enveloped nucleocapsids were detected in the cytoplasm (Figs 5c, f and 6c, f), and enveloped virions were found only rarely. However, the cytoplasmic nucleocapsids were not associated with electron-dense tegument material, as in cells infected for example with PrV unable to express glycoproteins E/I and M (Brack et al., 1999) or with PrV and HSV-1 lacking pUL11 and gM (Kopp et al., 2004; Leege et al., 2009). Nucleocapsids at the inner nuclear membrane or primary virions in the perinuclear cleft were present in HSV-1D UL37[86–1035]-infected cells (Fig. 5b, e) as well as in PrV-ΔUL37-infected cells (Fig. 6b, e), indicating that pUL37 is not required for nuclear egress.

Analysis of nuclear egress of HSV-1 and PrV pUL37 mutants

To analyse in more detail whether the absence of pUL37 had any effect on nuclear egress, intranuclear and intracytoplasmic DNA-containing C capsids were counted in representative thin sections of 20 Vero cells infected with wild-type or UL37-negative PrV or HSV-1. The results are shown in Table 1. The intracellular distribution of C capsids in Vero cells infected by pUL37-deleted HSV-1 or PrV was nearly identical, and the overall numbers of intranuclear C capsids were similar to those found in cells infected with the respective wild-type viruses. No defect in nuclear egress, i.e. intranuclear accumulation of C capsids, was observed in either of the mutants. The smaller numbers of intracytoplasmic C capsids in both wild-type
viruses reflected efficient secondary envelopment and release of viral particles, which was impaired in the pUL37 deletion mutants. Thus, in these parallel assays, UL37-deleted HSV-1 and PrV displayed similar ultrastructural phenotypes, indicating that deletion of pUL37 leads to similar defects in either virus.

**DISCUSSION**

Herpesvirus tegument proteins play important roles in the formation of progeny virions. Whereas several of them, including the inner tegument proteins pUL36 and pUL37, are conserved throughout the family *Herpesviridae*, others are found only in specific subfamilies, such as the outer tegument proteins pUL46, pUL47, pUL48 and pUL49 of the alphaherpesviruses. To assay whether conservation of protein sequence also means conservation of protein function, we started to analyse respective deletion mutants of the alphaherpesviruses HSV-1 and PrV in standardized virus–cell systems in parallel to gain insight into similarities and differences in the function of homologous proteins in these two viruses.

Our studies showed that the inner tegument protein pUL37 executes similar functions in the virion morphogenesis of PrV and HSV-1 in the cytosol, but has no apparent role in nuclear egress of either virus. The ultrastructural phenotypes observed in the two tested cell lines, RK13 and Vero, were similar for pUL37 deletion mutants of both viruses with similar levels of accumulation of nucleocapsids in the cytoplasm, apparently resulting from impairment of a...
Fig. 4. Plaque formation of UL37-deleted HSV-1 and PrV. Native and pUL37-expressing Vero and RK13 cells were infected under plaque assay conditions with wild-type and UL37-deleted HSV-1 (a) or PrV (b) as indicated. After 2 (PrV) or 3 (HSV-1) days, the diameters of 30 plaques per assay were determined in three independent experiments. Mean relative sizes are shown as percentage ± SD of the corresponding wild-type sizes. Images of representative plaques are shown in the panels below the graphs.

Fig. 5. Ultrastructural analysis of HSV-1ΔUL37-infected cells. RK13 (a–c) and Vero (d–f) cells were infected with phenotypically complemented HSV-1ΔUL3786–1035 at an m.o.i. of 1 and analysed 16 h after infection. Overviews of the infected cells are shown in (a) and (d), whilst (b) and (e) show stages of nuclear egress and (c) and (f) show intracytoplasmic aggregates of nucleocapsids. Bars, 4 μm (a); 2 μm (d); 300 nm (c, e, f); 200 nm (b).
crucial step in tegumentation and virion formation. These data confirm previous results (Desai et al., 2001; Klupp et al., 2001). As a marked difference between the two deletion mutants, PrV-ΔUL37 was capable of productive replication and direct cell-to-cell spread in RK13 and Vero cells, whereas HSV-1ΔUL37[86–1035] was not. Thus, whereas pUL37 was dispensable for productive replication of PrV, it was essential for HSV-1 in both cell types tested (Vero and RK13). In contrast, the interacting inner tegument protein pUL36 has been found to be essential for the replication of either virus (Desai, 2000; Fuchs et al., 2004).

It has recently been shown that pUL37 of HSV-1 is needed for the formation of virions, which parallels our results, but that it is not required during the initial stages of infection (Roberts et al., 2009). PrV pUL37 has also been demonstrated to be non-essential for entry, although the transport of incoming capsids via the cellular microtubule system to the nuclear pore is delayed in the absence of pUL37 (Krautwald et al., 2009). Taken together, these results imply that pUL37 plays a more important role for virion formation of HSV-1 than of PrV, despite the similarity in ultrastructural phenotypes as observed in the present study. It is conceivable that certain maturation steps of HSV-1 and PrV are generally different, or that PrV is able to utilize an alternative, UL37-independent pathway that is not accessible to HSV-1. More likely, pUL37 of HSV-1 might contribute to a conserved function necessary for virion formation that is supplied by other protein(s) in PrV.

In our comparative ultrastructural analyses, pUL37-deleted PrV and HSV-1 presented similar phenotypes in Vero and RK13 cells, and, in contrast to previous findings for pUL37-deleted HSV-1 (Desai et al., 2001), an impairment of nuclear egress was not observed with HSV-1ΔUL37[86–1035].

**Table 1.** Distribution of DNA-containing non-enveloped nucleocapsids 16 h after infection of Vero cells

<table>
<thead>
<tr>
<th>Virus</th>
<th>Intranuclear</th>
<th>Intracytoplasmic</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-1</td>
<td>202 (42 %)</td>
<td>284 (58 %)</td>
<td>486</td>
</tr>
<tr>
<td>HSV-1ΔUL37</td>
<td>222 (24 %)</td>
<td>689 (76 %)</td>
<td>911</td>
</tr>
<tr>
<td>PrV</td>
<td>262 (62 %)</td>
<td>160 (38 %)</td>
<td>422</td>
</tr>
<tr>
<td>PrV-ΔUL37</td>
<td>370 (23 %)</td>
<td>1243 (77 %)</td>
<td>1613</td>
</tr>
</tbody>
</table>

**Fig. 6.** Ultrastructural analysis of PrV-ΔUL37-infected cells. RK13 (a–c) or Vero (d–f) cells were infected with phenotypically complemented PrV-ΔUL37 at an m.o.i. of 1 and analysed 16 h after infection. Overviews of the infected cells are shown in (a) and (d), whilst (b) and (e) show stages of nuclear egress and (c) and (f) show intracytoplasmic aggregates of nucleocapsids. Bars, 5 μm (a); 4 μm (d); 500 nm (b, c, e, f).


