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Analysis of pseudorabies and herpes simplex virus recombinants simultaneously lacking the pUL17 and pUL25 components of the C-capsid specific component

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ABSTRACT

Homologs of the UL17 and UL25 gene products of herpes simplex virus 1 (HSV-1) are conserved throughout the *Herpesviridae* and essential for virus replication. However, their exact function is still unknown. Although both proteins form a complex on DNA-containing C-capsids defects observed in the absence of either protein differ. Absence of pUL17 from HSV-1 or the related alphaherpesvirus pseudorabies virus (PrV) precludes cleavage and packaging of newly replicated viral DNA, whereas in the absence of pUL25 genomic DNA is encapsidated but nuclear egress of capsids to the cytosol is abolished. HSV-1 pUL25 partially complemented the defect in a PrV UL25 deletion mutant indicating overlapping functions. However, reciprocal complementation did not ensue, and the present study demonstrates that UL17-deleted HSV-1 or PrV mutants are also not rescued by heterologous pUL17. To analyze whether simultaneous substitution of both complex partners may allow or increase trans-complementation we generated rabbit kidney cell lines co-expressing either PrV or HSV-1 pUL17 and pUL25, and respective HSV-1 and PrV double deletion mutants. Whereas the defects of both double mutants were *trans*-complemented by cell lines co-expressing the homologous complex partners, productive replication was not restored by heterologous pUL17 and pUL25. Thus, the protein complexes of PrV and HSV-1 either possess distinct functions, or require interactions with other viral proteins which are impaired in a heterologous context.

1. Introduction

Herpesvirus capsids are composed of five different, conserved proteins: pUL19, the major capsid protein which forms the hexons and pentons; the triplex proteins pUL18 and pUL38; the small capsid protein pUL35 located at the tip of hexons; and the portal protein pUL6, which is incorporated as a dodecameric ring at one of the vertices forming the portal through which the viral genome is incorporated (Chang et al., 2007; Newcomb et al., 2001; Steven and Spear, 1997). Products of the UL26.5 and UL26 genes are necessary for formation of the capsid shell constituting the scaffold and the maturational protease. While the scaffold proteins are absent from mature virions, the protease is detectable in purified virus particles (Michael et al., 2006; Sheaffer et al., 2000).

Besides the capsid components and the cleavage-encapsidation apparatus, capsid maturation involves additional proteins. These include homologs of the UL17 and UL25 gene products of HSV-1 which have been identified in all three subfamilies of the *Herpesviridae* (Baer et al., 1984; Chee et al., 1990; McGeoch et al., 1988). pUL17 and pUL25 have been designated either as components of the capsid or the capsid-associated tegument. They are not required for formation of capsids but are essential for maturation into nucleocapsids and/or nuclear egress (reviewed in Baines and Weller, 2005; Mettenleiter et al., 2009). However, their exact function during viral replication is still unclear. In HSV-1 and the related alphaherpesvirus pseudorabies virus (PrV), pUL17 is essential for cleavage and encapsidation of genomic viral DNA (Klupp et al., 2005; Salmon et al., 1998). In contrast, pUL25 has been proposed to play an important role during later stages of the packaging process prior to release of nucleocapsids into the cytoplasm (Klupp et al., 2006; Kuhn et al., 2008; O'Hara et al., 2010; Stow, 2001; Trus et al., 2007). pUL25 may be required for stabilizing the capsid shell since DNA-containing C-capsids were found to have a higher pUL25 content than capsids lacking DNA (Ogasawara et al., 2001; Sheaffer et al., 2001), and absence of pUL25 leads to a decrease in C-capsids (Stow, 2001; Klupp et al., 2006). We showed that PrV and HSV-1 pUL25 is required for primary envelopment of mature C-capsids at the inner nuclear membrane. In the absence of pUL25 nucleocapsids were found in close association with the inner nuclear

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membrane, but budding did not ensue (Klupp et al., 2006; Kuhn et al., 2008).

Recently, an interaction between pUL17 and pUL25 has been proposed to mediate pUL25 binding to capsids. In the absence of pUL17, much lower levels of pUL25 were incorporated into B-capsids, and B-capsids lacking pUL25 contained less pUL17 (Thurlow et al., 2006). In addition, cryoelectron microscopic studies visualized an accessory structural component on C-capsids, designated C-capsid specific component (CCSC), which most probably consists of a pUL17/pUL25 heterodimer and may signal that the C-capsid is ready to exit the nucleus (Trus et al., 2007; Conway et al., 2010).

HSV-1 pUL25 is able to partially complement the defect of a PrV UL25 deletion mutant but not vice versa, indicating overlapping but nonidentical functions of these two conserved herpesvirus proteins (Kuhn et al., 2008). To examine whether trans-complementation occurs after coexpression of pUL17 and pUL25 derived from the same virus, we generated rabbit kidney cell lines co-expressing pUL17/pUL25 of HSV-1 or PrV, and PrV and HSV-1 UL17/UL25 double deletion mutants. The resulting mutants were characterized and subsequently analyzed for trans-complementation by the heterologous pUL17 and/or pUL25.

2. Materials and methods

2.1. Cells and viruses

All virus mutants were derived from PrV strain Kaplan (PrV-Ka; Kaplan and Vatter, 1959) or HSV-1 strain KOS (HSV1-KOS; kindly provided by P. Spear, Northwestern University, Chicago, IL). Viruses were grown on rabbit kidney (RK13) or African green monkey kidney (Vero) cells in minimum essential medium supplemented with 10% or 5% fetal calf serum, respectively. Generation of PrV mutants lacking pUL17 or pUL25, and HSV-1 mutants devoid of pUL25 as well as complementing cell lines have been described (Klupp et al., 2005, 2006; Kuhn et al., 2008).

2.2. Generation of an HSV-1 pUL17 specific antiserum

For generation of a monospecific antiserum against HSV-1 pUL17, the complete open reading frame (ORF) was amplified with primers HUL17For (5′-CACAGAATTCACC**ATG**AACGCGCACTTGGC-3′; nt 33500-33481; UL17 start codon in bold; GenBank Acc. No. X14112; McGeoch et al., 1988) and HUL17Rev (5′-CACAGTCGAC**CTA**GCGAGACCGGCCGTTCC-3′; reverse stop codon in bold; nt 31186-32405; GenBank Acc. No. X14112) and HSV-1 KOS viral DNA. The 2.1-kb PCR product was digested with EcoRI and SalI for which restriction sites were added with the primer sequences (underlined), and cloned into prokaryotic expression vector pGEX-4T-1 (GE Healthcare) or eukaryotic expression vector pcDNA3 (Invitrogen) to yield pGEX-HUL17 or pcDNA-HUL17, respectively. Since the complete UL17 ORF was not efficiently expressed in *Escherichia coli*, pGEX-HUL17 was digested with KpnI, which cleaves at nt position 32449 (GenBank Acc. No. X14112) and SalI, whose cleavage site is located in the vector sequences, to remove 3′-coding sequences. After transformation of pGEX-HUL17-N into *E. coli* an approximately 66 kDa protein was overexpressed which was purified and used for immunization of a rabbit (Klupp et al., 2006).

2.3. Construction of PrV and HSV-1 pUL17/pUL25 co-expressing cell lines

For generation of PrV pUL17/pUL25 co-expressing cell lines, the UL17 and UL25 ORFs were cloned into the eukaryotic double expression vector p3ie under control of either the human or murine cytomegalovirus immediate early promoter/enhancer complex (Klupp et al., 2007). To this end, pcDNA-UL17(PrV) (Klupp et al., 2005) was cleaved with BamHI and EcoRV, pGEX-UL25(PrV) (Klupp et al., 2006) was digested with EcoRI, and the corresponding inserts were consecutively cloned into vector p3ie. For generation of a HSV-1 UL17/UL25 co-expressing cell line, pcDNA-UL17(HSV-1) was digested with BamHI and XbaI, and pGEX-UL25(HSV-1) (Kuhn et al., 2008) was cleaved with EcoRI. The released inserts were then consecutively cloned into vector p3ie. The resulting plasmids p3ie-UL17/UL25(PrV) and p3ie-UL17/UL25(HSV-1) were transfected into RK13 cells using Superfect transfection reagent (Qiagen). An HSV-1 UL17 expressing cell line was derived after transfection of RK13 cells with pcDNA-UL17(HSV) (see above). Transfected cell clones were selected in medium containing 500 μg of Geneticin (Invitrogen) per ml and tested for pUL17 and pUL25 expression by immunofluorescence and Western blot using monospecific antisera.

2.4. Generation of PrV and HSV-1 UL17/UL25 double deletion mutants

For construction of a PrV-UL17/UL25 deletion mutant, the gB-deleted BAC clone pPrV-ΔUL17F (Klupp et al., 2005) was used. For additional deletion of the UL25 coding region, the 1.6-kb EcoRI/XbaI insert of pcDNA-UL25 (Klupp et al., 2006) was cloned into appropriately cleaved pUC19 (New England Biolabs) after inactivation of the resident PstI site, resulting in plasmid pUC19ΔP-UL25. This plasmid was then digested with PstI and ApaI, thereby removing 1398 bp of UL25 coding sequences (Fig. 1B), which were replaced by a kanamycin-resistance gene amplified by PCR from pACYC177 (New England Biolabs) using primers KAN950For (5′-TCCGGATCCCGATTTATTCAACAAAGCCACG-3′) and KAN950Rev (5′-TTCGAATTCGCCAGTGTTACAACCAATTAA-3′). The complete insert of this plasmid was amplified by PCR using pUC-specific primers (New England Biolabs), and the resulting PCR product was used for mutagenesis of pPrV-ΔUL17F in *E. coli*. After isolation of kanamycin-resistant clones, the gB gene was finally restored by cotransfection of pPrV-ΔUL17F/UL25K DNA and plasmid pUC-B1BclI (Kopp et al., 2003) into RK13-UL17/UL25(PrV) cells. Several single plaque isolates of the transfection progeny were screened by Southern blot analysis for the correct deletion (data not shown), and one of the positive isolates was randomly chosen for further characterization. Correct deletion of UL25-specific sequences was verified by sequencing of the PCR product generated with primers UL25For and UL25Rev (Klupp et al., 2006) on PrV-ΔUL17/UL25 DNA.

For generation of HSV1-ΔUL17, plasmid pcDNA-HUL17 (see above) was digested with BmgBI and BstEII (Fig. 1D) to remove codons 15–522 which were replaced after Klenow polymerase treatment by a 1258-bp blunt-ended BstBI fragment of pKD13 (Datsenko and Wanner, 2000). The resulting plasmid pcDNA-HUL17KF was amplified with vector-specific T7 and SP6 primers, and the product was used for mutagenesis of BAC pHSV1-ΔgJ in *E. coli* as described (Leege et al., 2009a) giving rise to pHSV1-ΔUL17F. For construction of pcDNA-HΔUL25K, the kanamycin-resistance gene was amplified by PCR from pACYC177 and inserted into plasmid pcDNA-HUL25 after digestion with SbfI and AleI and blunt-ending by Klenow polymerase (Fig. 1B). The complete insert of the resulting plasmid pcDNA-HUL25K was amplified by PCR using Pfx polymerase (Invitrogen) and vector-specific T7 and SP6 primers. The PCR product was used for mutagenesis of pHSV1-ΔUL17 in *E. coli*. Finally, the mini-F plasmid vector sequences and the adjacent EGFP expression cassette were removed from the genome after cotransfection of RK13-UL17 or RK13-UL17/25 cells with pHSV1-ΔUL17F or pHSV1-ΔUL17F/UL25K DNA and plasmid pUC-BamJET, and HSV1-ΔUL17 or HSV1-ΔUL17/UL25 were

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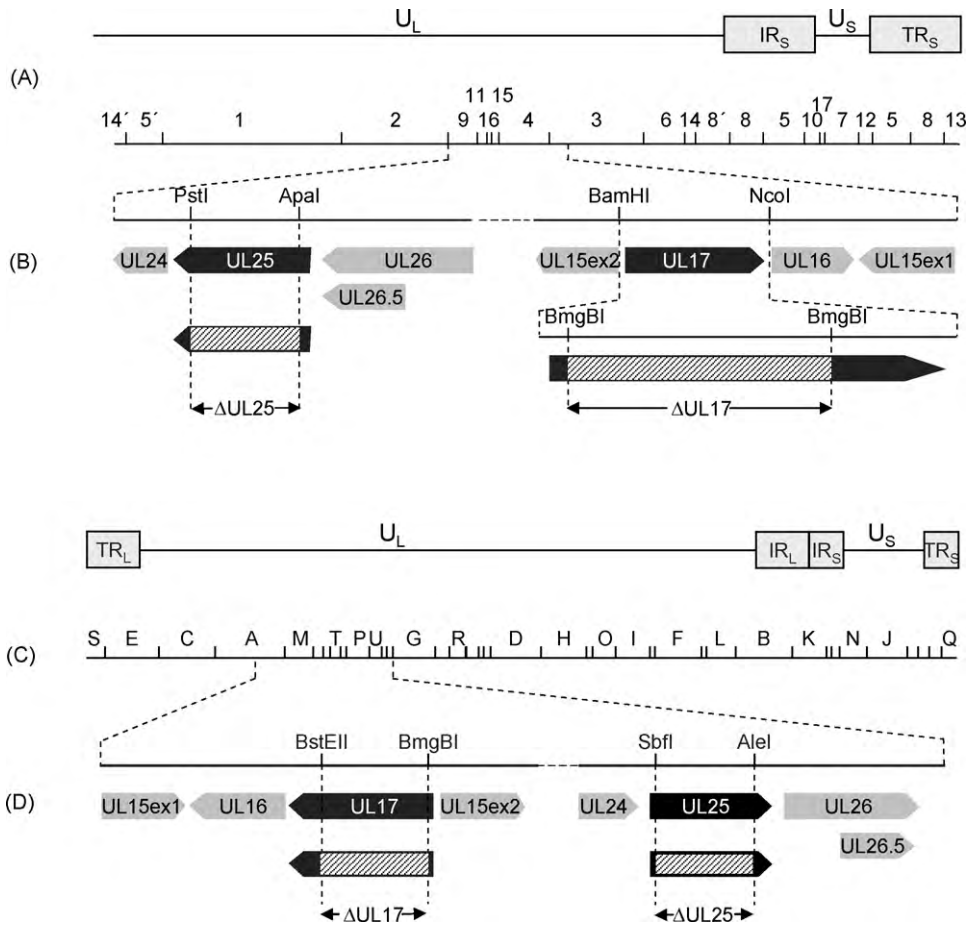

Fig. 1. Construction of UL17/UL25 double deletion mutants. (A) Map of the PrV genome with unique long (U_L), unique short (U_S), and inverted repeat (IR_S, TR_S) sequences. BamHI restriction sites are indicated, and fragments are numbered according to their size. (B) Enlargement of the UL25 and UL17 gene regions. Open reading frames are shown as pointed rectangles. The UL17 and UL16 genes are located within the intron of the spliced UL15 gene. Relevant restriction sites for construction of PrV-ΔUL17/UL25 are indicated, as is the extent of the introduced deletions (hatched). (C) Map of the HSV-1 genome with the unique long (U_L), unique short (U_S), and inverted repeat (TR_L, IR_L, TR_S, and IR_S) sequences. BamHI restriction sites are indicated, and larger fragments are named alphabetically according to their size. (D) Enlargement of the HSV-1 UL17 and UL25 gene regions. The open reading frames are shown as pointed rectangles. Relevant restriction sites for construction of HSV1-ΔUL17/UL25 are indicated, as is the extent of the introduced deletions (hatched).

isolated from non-fluorescent progeny virus plaques. Correct deletion of coding sequences was verified by Southern Blot analyses and sequencing of PCR products spanning the deletions (data not shown).

2.5. Western blotting

Trans-complementing cells and either uninfected RK13 or RK13 cells infected at a multiplicity of infection (MOI) of 5 with PrV-Ka, HSV1-KOS or the different trans-complemented single and double deletion mutants were harvested after overnight incubation by scraping into the medium. Cells were collected by centrifugation for 2 min at 16,000 × *g*, washed twice with phosphate-buffered saline (PBS) and resuspended in 100 μl of PBS and the same volume of sample buffer. Proteins were separated in SDS-10% polyacrylamide gels, blotted onto nitrocellulose and incubated with antisera against pUL17 (Klupp et al., 2005; this study), pUL25 (Klupp et al., 2006; Kuhn et al., 2008), pUL37 (Klupp et al., 2001; Leege et al., 2009b), and gB (Kopp et al., 2003). After incubation with peroxidase-conjugated secondary antibodies (Dianova), bound antibody was detected by enhanced chemiluminescence (Super Signal, Pierce) and recorded on X-ray film.

2.6. One-step growth analysis and plaque assays

For analysis of one-step growth kinetics, RK13, RK13-UL17/UL25(PrV), or RK13-UL17/UL25(HSV-1) cells were infected with PrV-Ka, PrV-ΔUL17, PrV-ΔUL25, and PrV-ΔUL17/UL25, or HSV1-KOS, HSV1-ΔUL17, HSV1-ΔUL25, and HSV1-ΔUL17/UL25 with an MOI of 3 on ice for 1 h. Thereafter, the inoculum was removed and prewarmed medium was added. The cells were further incubated for 1 h at 37 °C. Remaining extracellular virus was then inactivated by low pH treatment (Mettenleiter, 1989). Immediately (0 h) and after 4, 8, 12, 24, 36, 48, or 72 h, the cells were scraped into the medium and lysed by freezing (−70 °C) and thawing (37 °C). All samples were centrifuged for 2 min at 16,000 × *g*, and titers of progeny virus in the supernatant were determined on PrV or HSV-1 pUL17/UL25 expressing cells. Mean values of three independent experiments were calculated and plotted with the corresponding standard deviation.

For plaque assays, cells in six-well tissue culture dishes were infected with 100 PFU per well of PrV-Ka, PrV-ΔUL17/UL25 for RK13-UL17/UL25(PrV), HSV1-KOS, and HSV1-ΔUL17/UL25 for RK13-UL17/UL25(HSV-1), or with 1000 PFU of PrV-ΔUL17/UL25 or HSV1-ΔUL17/UL25 for RK13. Two (for PrV) or four days (for HSV-1) after infection, cells were fixed with 5% formalin and stained with crystal violet. Twenty plaques each were measured microscopically, and the average plaque diameters were determined. Values were calculated in comparison to those of PrV-Ka or HSV1-KOS, which were set at 100%. Average percentages and standard deviations were calculated from three independent experiments.

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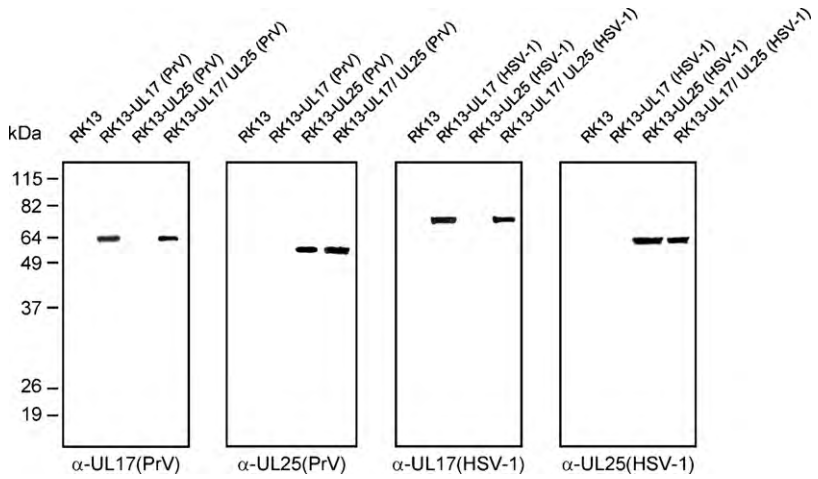

Fig. 2. Characterization of transgenic cell lines. Lysates of cells stably expressing the corresponding proteins were separated on SDS-10% polyacrylamide gels and incubated with monospecific antisera against PrV or HSV-1 pUL17 and pUL25. The approximately 64-kDa pUL17 and 57-kDa pUL25 of PrV, as well as the 75-kDa pUL17 and 63-kDa pUL25 of HSV-1 are detectable in the corresponding lanes. The locations of molecular mass markers are indicated on the left.

2.7. Preparation of viral DNA and Southern blotting

RK13 cells were infected with PrV-Ka, PrV-ΔUL17, PrV-ΔUL25, or PrV-ΔUL17/UL25, or HSV1-KOS, HSV1-ΔUL17, HSV1-ΔUL25, or HSV1-ΔUL17/UL25 at an MOI of 1, and infected cell DNA prepared 16 h post-infection was digested with BamHI, separated on 0.8% agarose gels, blotted onto nylon membranes, and probed with radioactively labeled genome-end specific BamHI-fragment 14′ for PrV or with BamHI-Fragment K for HSV-1 (see Fig. 1 for location). Bound radioactivity was recorded with a phosphoimager (FLA-3000; Raytest).

2.8. Electron microscopy

For ultrastructural analyses, RK13 cells were infected with an MOI of 1 with PrV-Ka, HSV1-KOS, and the corresponding single and double deletion mutants which had been propagated on cell lines expressing homologous pUL17 and pUL25. After 14 h (PrV) or 20 h (HSV-1) cells were fixed and embedded as described previously (Granzow et al., 1997). Ultrathin sections were counterstained with uranyl acetate and lead salts, and examined with a model Tecnai 12 electron microscope (Philips).

3. Results

3.1. Isolation of PrV- and HSV1-ΔUL17/UL25 mutants and trans-complementing cells

Cryoelectron microscopic analysis indicated that HSV-1 pUL17 and pUL25 form a complex on mature C-capsids (Trus et al., 2007; Conway et al., 2010) with pUL17 being required for association of pUL25 to capsids (Thurlow et al., 2006). Since HSV-1 pUL25 can, at least partially, substitute for PrV pUL25 (Kuhn et al., 2008) we examined whether trans-complementation could be improved by the coexpression of pUL17 and pUL25 derived from the same virus. Thus, we generated PrV and HSV-1 pUL17/pUL25-expressing rabbit kidney (RK13) cell lines, as well as PrV and HSV-1 UL17/UL25 double deletion mutants. To this end, RK13 cells were transfected with double expression vectors p3ie-UL17/UL25(HSV-1) or p3ie-UL17/UL25(PrV), and selected in medium containing Geneticin. Cell lines co-expressing pUL17/UL25 of HSV-1 or PrV were identified by indirect immunofluorescence and Western blot analysis with the α-UL17 and α-UL25 sera. For comparison cell lines expressing either protein were included. All cell lines expressed or coexpressed the respective viral protein(s) at a molecular mass as expected (Fig. 2).

PrV-ΔUL17/UL25 was constructed in a bacterial artificial chromosome (BAC) clone of PrV-Ka and carried a deletion of codons 23–444 of the 597 codon UL17 open reading frame, and codons 29–495 of the 534 codon UL25 gene (Fig. 1B). Infectious progeny virus could only be isolated on RK13-UL17/UL25(PrV) cells. Sequencing (data not shown) and Western blot analysis verified absence of PrV pUL17 and pUL25 (Fig. 3). Whereas in cells infected by wild-type PrV-Ka the monospecific α-UL17 and α-UL25 antisera detected the 64-kDa pUL17 and 57-kDa pUL25, they were absent from cell lysates infected by PrV-ΔUL17/UL25 (Fig. 3, upper panels). In congruence, cells infected by HSV1-ΔUL17, HSV1-ΔUL25 and HSV1-ΔUL17/UL25 also lacked the respective proteins (Fig. 3, lower panels). As loading controls detection of envelope glycoprotein B, which in PrV, in contrast to HSV-1, is cleaved by a cellular protease into two subunits (Lukàcs et al., 1985), and inner tegument protein pUL37, is shown.

3.2. In vitro replication of PrV- and HSV1-ΔUL17/UL25 mutants

To investigate the defect of the single and double deletions on virus replication in cell culture, one-step growth analyses (Fig. 4) and plaque assays (Fig. 5) were performed. PrV-Ka, PrV-ΔUL17, and PrV-ΔUL25 replicated with comparable kinetics on RK13-UL17/UL25(PrV) cells (Fig. 4A), indicating full complementation of the respective defect by the co-expressing cells. In contrast, PrV-ΔUL17/UL25 replicated on these cells to approximately 10-fold reduced titers (Fig. 4A) and approximately 30% reduced plaque sizes (Fig. 5). On RK13 cells only few infectious particles were produced (Fig. 4A) for all deletion mutants tested underlining the essential function of these proteins. RK13-UL17/UL25(HSV-1) cells complemented replication of the single and double HSV-1 deletion mutants to titers comparable to wild-type virus demonstrating full complementation by the generated cell line (Fig. 4B). This is also supported by plaque assays. Plaques on RK13-UL17/UL25(HSV-1) cells reached diameters similar to wild-type strain KOS (Fig. 5), while on non-complementing RK13 cells only single infected cells were detectable and very little infectious progeny was observed (Fig. 4B). In summary, both co-expressing cell lines showed homologous complementation which was a prerequisite for the heterologous assay.

To test for heterologous complementation, RK13-UL17/UL25(HSV-1) and RK13-UL17/UL25(PrV) cells were

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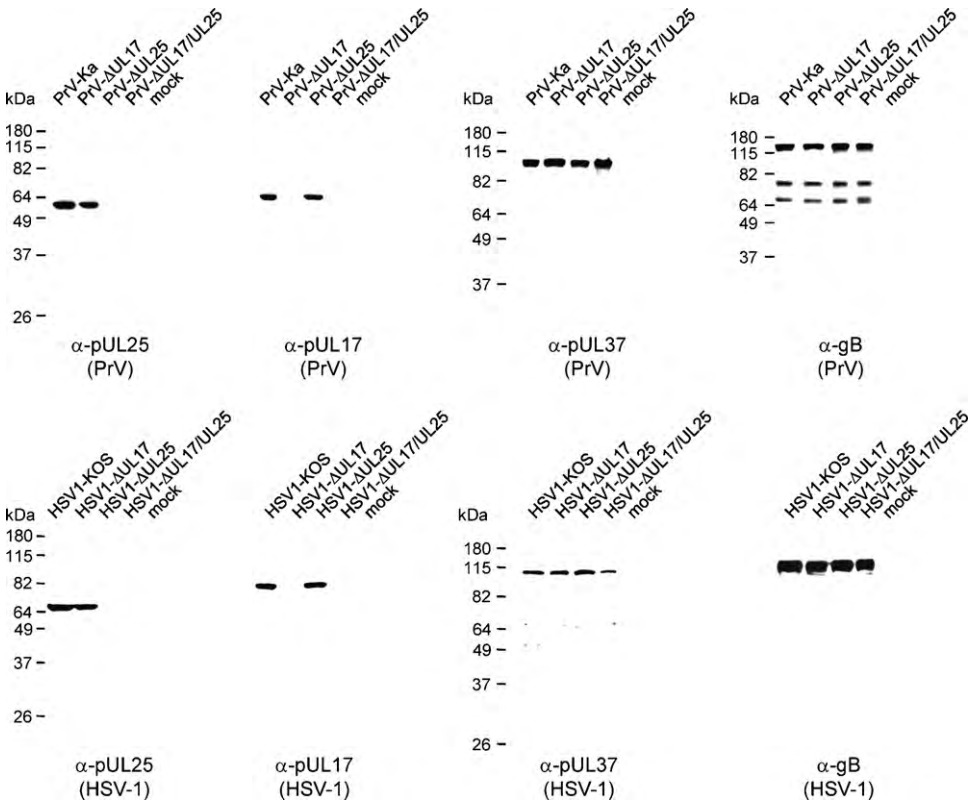

Fig. 3. Protein expression by PrV and HSV-1 deletion mutants. RK13 cells were infected with PrV and respective mutant viruses (upper row) or HSV-1 and respective mutant viruses (bottom row). Infected cell lysates were separated on sodium dodecyl sulfate-10% polyacrylamide gels and incubated with monospecific antisera against pUL25, pUL17, pUL37, and gB. Unlike HSV-1 gB the PrV gB is cleaved during maturation by a cellular protease into two subunits, resulting in three different gB specific bands (Lukàcs et al., 1985). The location of molecular mass marker proteins is indicated on the left.

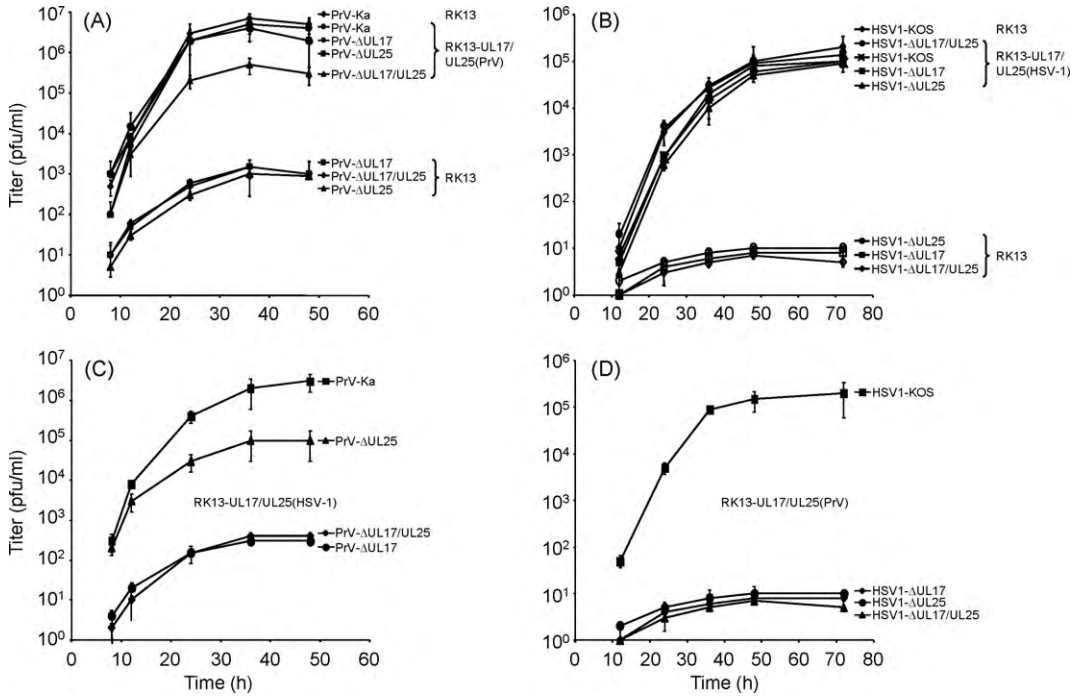

Fig. 4. One-step growth kinetics of PrV and HSV-1 deletion mutants. RK13 and transgenic cells were infected at an MOI of 3 with PrV-Ka, PrV-ΔUL17, PrV-ΔUL25 or PrV-ΔUL17/UL25 (A and C), or with HSV1-KOS, HSV1-ΔUL17, HSV1-ΔUL25 or HSV1-ΔUL17/UL25 (B and D), harvested at the indicated times after infection, and titrated on either RK13-UL17/UL25(HSV-1) (for HSV-1 mutants) or RK13-UL17/UL25(PrV) cells (for PrV mutants). Average titers (pfu/ml) and standard deviations from three independent experiments are shown.

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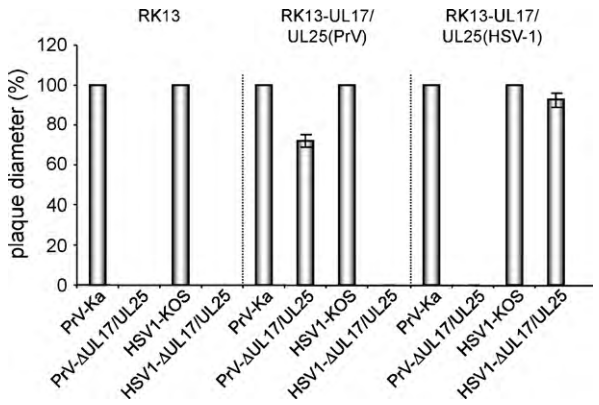

Fig. 5. Plaque formation of pUL17/25 double deletion mutants. Plaques on RK13, RK13-UL17/UL25(PrV), or RK13-UL17/UL25(HSV-1) cells infected with PrV-Ka and PrV-ΔUL17/UL25 or HSV1-KOS and HSV1-ΔUL17/UL25 were microscopically measured at two (for PrV) or four days (for HSV-1) post-infection. Relative plaque sizes were calculated and compared to those of PrV-Ka or HSV-1 KOS, which were set 100%. Average values and standard deviations from three independent experiments are shown.

infected either with PrV-Ka, PrV-ΔUL17, PrV-ΔUL25 and PrV-ΔUL17/UL25, or with HSV-1 KOS, HSV1-ΔUL17, HSV1-ΔUL25 and HSV1-ΔUL17/UL25. As shown earlier, HSV-1 pUL25 partially complemented the defect of the PrV pUL25 deletion mutant (Kuhn et al., 2008), and a corresponding level of complementation was also observed on RK13-UL17/UL25(HSV-1) cells (Fig. 4C). No complementation of PrV-ΔUL17 by HSV-1 pUL17 was detectable and no complementation was found for PrV-ΔUL17/UL25 on RK13-UL17/UL25(HSV-1) cells. In the reverse experiment, also no complementation was observed for the corresponding HSV-1 mutants on PrV pUL17/UL25 expressing cells (Fig. 4D).

3.3. Comparison of ultrastructural phenotypes of PrV- and HSV1-ΔUL17 and -ΔUL25 mutants

To further investigate the phenotypes of the double deletion mutants, RK13 cells were infected either with parental strains PrV-Ka, HSV1-KOS (Fig. 6A and B), the single UL17 (Fig. 6C and D), the single UL25 (Fig. 7A and B), and the double deletion mutants (Fig. 7C and D) at an MOI of 1, and examined at 14 h (for PrV) or 20 h (for HSV-1) after infection. The different times of incubation are due to the differences in the replication cycle between HSV-1 and PrV.

For HSV-1 and PrV, even in the absence of pUL25, C-capsids were formed in the nuclei of infected cells, but primary envelopment of these capsids did not ensue (Fig. 7A and B; Klupp et al., 2006; Kuhn et al., 2008). In contrast, in electron microscopic analyses of HSV1- and PrV-ΔUL17 infected cells, only B-capsids are detectable (Fig. 6C and D) and cleavage of concatemeric DNA into unit-length genomes did not take place (Fig. 8) (Klupp et al., 2005; Salmon et al., 1998). Although the electron density of B-capsids differs between PrV and HSV-1, in comparison with C-capsids in wild-type virus infected cells (Fig. 6A and B), it is evident that in the simultaneous absence of pUL17 and pUL25, B-capsids accumulated in the nuclei of infected cells, whereas no mature C-capsids were observed (Fig. 7C and D). This indicates that in the concomitant absence of pUL17 and pUL25 DNA packaging is blocked before DNA is inserted into the capsid and before the scaffold protein is expelled. This is supported by Southern blot analyses of DNA isolated from cells infected by the different mutant viruses. As shown in Fig. 8, the presence of terminal fragments Q and S of HSV-1 DNA as well as terminal fragment 14′ in PrV DNA is visible in cells infected by the respective wild-type

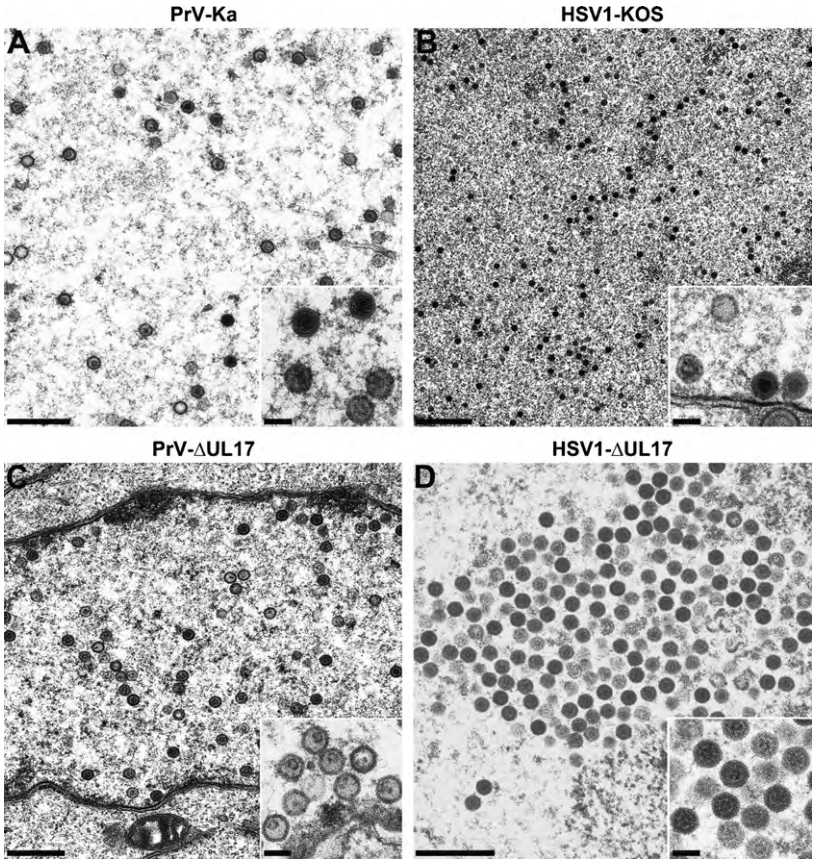

Fig. 6. Ultrastructural analysis of PrV and HSV-1 wild-type and pUL17 deletion mutants. RK13 cells were infected at an MOI of 1 with PrV-Ka (A) or HSV-1 KOS (B), or UL17-deleted PrV (C) or HSV-1 (D), and analyzed 14 h (for PrV) or 20 h (for HSV-1) after infection. Scale bars: (A, C, and D) 500 nm, (B) 1 μm, insets 100 nm.

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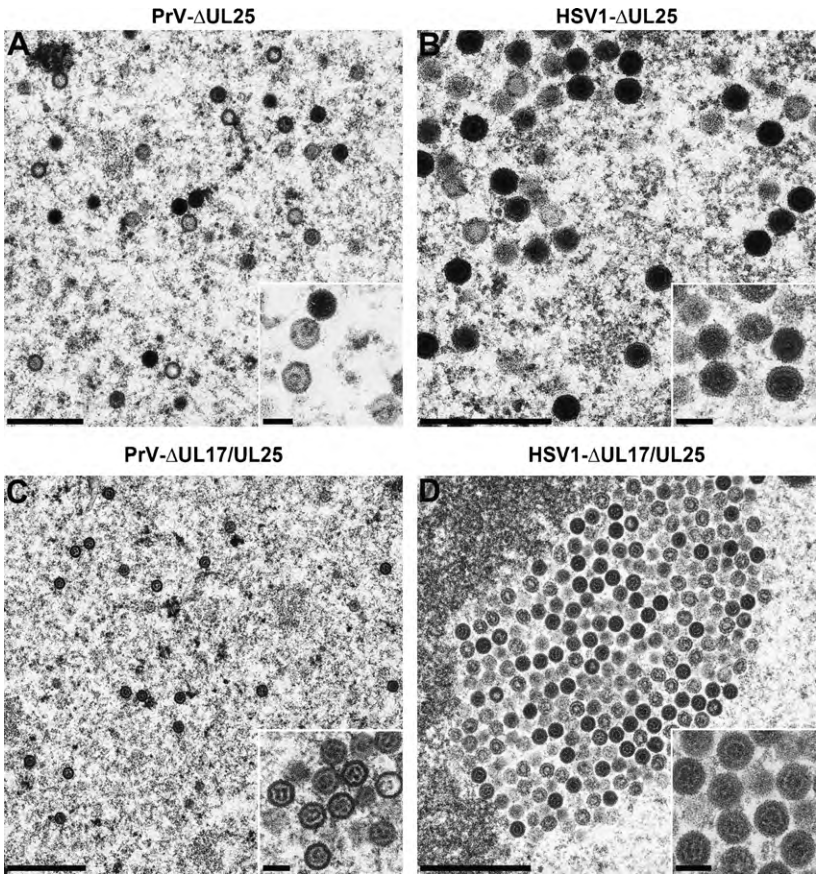

Fig. 7. Ultrastructural analysis of PrV and HSV-1 pUL25 and pUL17/pUL25 deletion mutants. RK13 cells were infected at an MOI of 1 with PrV- (A) or HSV1-ΔUL25 (B), or UL17 and UL25 deleted PrV (C) or HSV-1 (D), and analyzed 14 h (for PrV) or 20 h (for HSV-1) after infection. Scale bars: (A and B) 500 nm, (C and D) 700 nm, insets 100 nm.

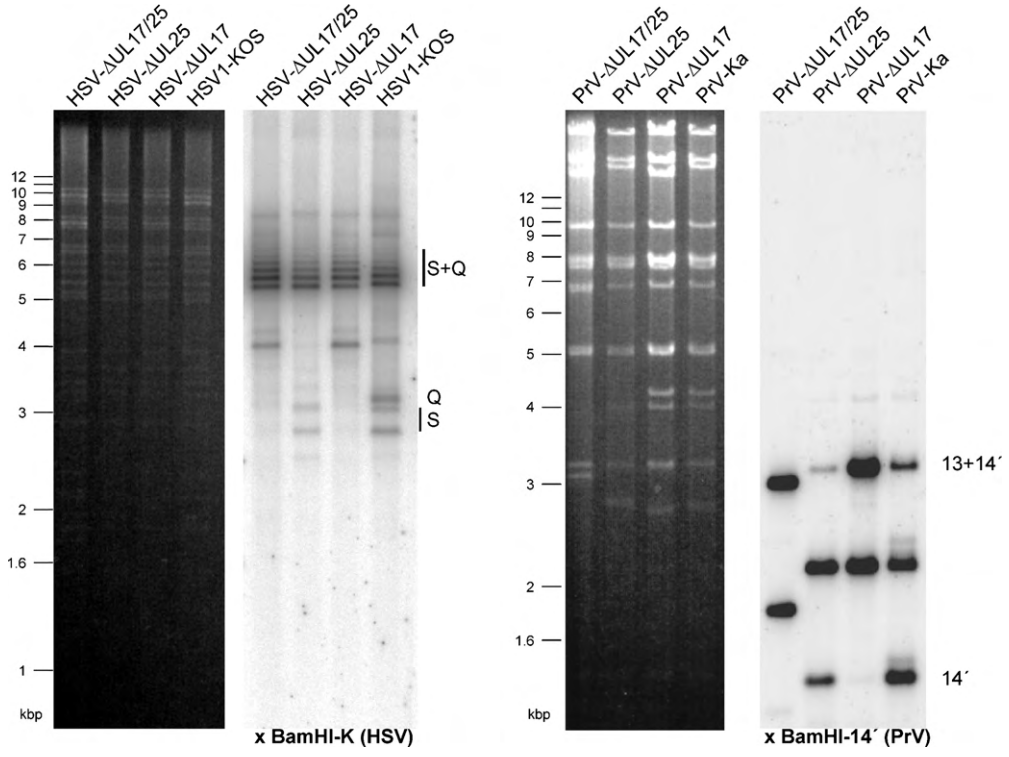

Fig. 8. Cleavage of concatemeric viral DNA. RK13 cells were infected at an MOI of 1 with the different parental viruses and corresponding single and double deletion mutants for 16 h and whole-cell DNA was isolated, cleaved with BamHI and analyzed by Southern blotting with labeled BamHI-fragments K (for HSV-1) or 14′ (for PrV). Corresponding Ethidiumbromide stained agarose gels are shown on the left. Terminal fragments Q and S (for HSV-1) and 14′ (for PrV) as well as fusion fragments SQ (for HSV-1) and 13 + 14′ (for PrV) are indicated. During isolation of PrV-ΔUL17/25 the size of BamHI-fragment 14′ has decreased, probably due to loss of repeated sequences located in this genomic region. Additional hybridizing fragments are of unknown provenance (Stow, 2001). The sizes of marker DNAs are indicated.

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viruses, whereas they are absent in cells infected by the pUL17 and pUL17/UL25 deleted viruses. Thus, the HSV1- or PrV-ΔUL17/UL25 double deletion mutants show a defect similar to the HSV1- or PrV-ΔUL17 mutants.

4. Discussion

Homologs of the UL17 and UL25 gene products of HSV-1 are capsid-associated proteins essential for virus replication. However, their exact role is still unclear. Recently, a complex of these two proteins has been identified specifically on mature C-capsids and has, therefore, been designated as C-capsid specific component, CCSC (Trus et al., 2007). However, defects in either of the two complex partners produces different phenotypes. Whereas DNA packaging still occurs in the absence of pUL25 (Klupp et al., 2006; Kuhn et al., 2008; Stow, 2001), it is abolished in the absence of pUL17 (Klupp et al., 2005; Salmon et al., 1998; Thurlow et al., 2005; this study) indicating different functions for these two proteins. It could be assumed that association of pUL17 with capsids occurs during genome packaging (Klupp et al., 2005), whereas pUL25 is added later (Thurlow et al., 2006) completing the capsid maturation process and preparing the capsid for nuclear egress (reviewed in Mettenleiter et al., 2009).

Congruent with their essential functions, both proteins are conserved throughout the *Herpesviridae*. This conservation of sequence may parallel conservation of function also across different herpesviruses. We found partial complementation of a PrV pUL25 deletion mutant by the homologous HSV-1 protein indicating overlapping functions of the two proteins (Kuhn et al., 2008). However, the reverse experiment did not show any complementation. Since pUL17/pUL25 form a complex it was reasonable to assume that coexpression of both complex partners may allow or increase complementation. Thus, we set out to construct pUL17/pUL25 double deletion mutants of PrV and HSV-1 as well as HSV-1 and PrV pUL17/pUL25 double expressing cell lines.

Proper gene expression in the cell lines was verified by indirect immunofluorescence with monospecific antisera (data not shown), as well as by Western blot analyses. Both cell lines constitutively coexpressed the respective pUL17 and pUL25 proteins at the appropriate molecular mass and at levels sufficient to fully complement the respective single deletion mutants. Both double deletion mutants were also able to replicate productively on cells co-expressing the homologous proteins demonstrating that they expressed the respective proteins in functional form and levels sufficient for compensating either the pUL17 or the pUL25 defects in either virus. Although the HSV-1 pUL17 and pUL25 co-expressing cells complemented the single and double HSV-1 mutants to similar, wild-type-like levels in one-step growth and plaque sizes, the cells co-expressing the PrV homologs fully complemented the PrV single mutants, but complemented the double mutant only at reduced levels. Titers were approximately 10-fold reduced compared to wild-type PrV-Ka or the single deletion mutants, and plaque sizes were decreased up to ca. 30%. The reason for this difference is unclear at present. Full complementation of the single mutants indicates that functional proteins are expressed in the cell line and that their expression level is sufficient for full complementation. Thus, the PrV pUL17/pUL25 double mutant, during the somewhat complicated double mutagenesis, may have acquired additional defect(s) which cannot be compensated for by the constructed cell line.

Despite the homologous complementation observed by the respective cells, heterologous complementation was again only observed in a combination of HSV-1 pUL25 expression and PrV pUL25 deletion mutant. This has been observed before for a cell line expressing only HSV-1 pUL25 (Kuhn et al., 2008) and has now been verified by the pUL17/pUL25 co-expressing cells (see Fig. 4). However neither the reciprocal experiment, i.e. infection of PrV pUL17/pUL25-expressing cells by HSV1-ΔUL25, nor any attempt to detect complementation of the pUL17/pUL25 double mutants on cells expressing the heterologous proteins was successful. Thus, coexpression of both CCSC partners from a homologous background is not sufficient to complement the defect induced by absence of CCSC in a heterologous virus. Most likely, either incorporation of the heterologous proteins into the maturing capsid per se, or incorporation in a functionally active form may be impaired leading to a loss of function.

Ultrastructural analyses of cells infected with the deletion mutants demonstrated a dominance of the pUL17 defect, i.e. only capsids devoid of viral genome due to lack of DNA cleavage and encapsidation, over the pUL25 defect in the doubly-deficient viruses. This result supports the hypothesis that requirement for pUL17 precedes pUL25 function, and is congruent with the fact that pUL17 is present in the virion prior to and, at least partially, independent of pUL25 (Sheaffer et al., 2001; Thurlow et al., 2005), but is required for subsequent pUL25 binding (Thurlow et al., 2006). The absence of A-capsids in cells infected with either the pUL17- or the pUL17/pUL25-deleted viruses indicates that the packaging reaction had indeed not been initiated in the absence of pUL17 (Klupp et al., 2005; Salmon et al., 1998).

Interestingly, HSV-1 pUL17 was found on the outside of the capsid shell and also in tegument (Salmon et al., 1998; Thurlow et al., 2005), whereas PrV pUL17 appears to be an internal capsid protein, incorporated into capsids concomitant with genomic DNA, but is not a prominent component of the tegument (Klupp et al., 2005). Different locations of pUL17 in the capsid may also influence positioning of pUL25. Whereas in PrV, pUL25 has only been detected on mature C-capsids but not on immature A-, and B-capsids by immunoelectron microscopy (Klupp et al., 2006), it has been shown on all HSV-1 capsid forms, although in decreasing amounts with the highest level present on C-capsids (Sheaffer et al., 2001; Thurlow et al., 2005). In addition, PrV C-capsids seem to have higher pUL25 occupancy than HSV-1 as judged by cryo-EM reconstruction (Conway et al., 2010).

From our studies it can be concluded that coexpression of the two components of the CCSC from the same virus does not overcome the functional barriers exerted by the role of the individual proteins. In all cases analyzed the results from trans-complementation follows that observed from the single mutants and single protein expressing cell lines. This may reflect different functions of the individual complex partners or the inability to interact with the heterologous capsid during its maturation. In fact, PrV and HSV-1 capsids differ in electron microscopical appearance and, so far, no capsid precursors prior to angularization have been observed in PrV as compared to HSV-1 (Granzow et al., 1997; Newcomb et al., 1996). Thus, the observed failure in trans-complementation may actually reflect differences in capsid architecture. In contrast, the complementation of pUL25-deficient PrV by HSV-1 pUL25 (Kuhn et al., 2008) points to a set of overlapping functions, which has been confirmed in this study.

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