Relevance of the Interaction between Alphaherpesvirus UL3.5 and UL48 Proteins for Virion Maturation and Neuroinvasion

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The UL3.5 and UL48 genes, which are conserved in most alphaherpesvirus genomes, are important for maturation of pseudorabies virus (PrV) particles in the cytoplasm of infected cells (W. Fuchs, B. G. Klupp, H. J. Rziha, and T. C. Mettenleiter, J. Virol. 70:3517–3527, 1996; W. Fuchs, H. Granzow, B. G. Klupp, M. Kopp and T. C. Mettenleiter, J. Virol. 76:6729–6742, 2002). In bovine herpesvirus 1 (BoHV-1), the homologous gene products pUL3.5 and pUL48 have been demonstrated to interact physically (N. Lam and G. Letchworth, J. Virol. 74:2876–2884, 2000). Moreover, BoHV-1 pUL3.5 partially complemented a pUL3.5 defect in PrV (W. Fuchs, H. Granzow, and T. C. Mettenleiter, J. Virol. 71:8886–8892, 1997). By using coimmunoprecipitation and yeast two-hybrid studies, we observed a similar interaction between pUL3.5 and pUL48 of PrV, as well as a heterologous interaction between the PrV and BoHV-1 gene products. The relevant domain could be confined to the first 43 amino acids of PrV pUL3.5. Unlike its BoHV-1 homologue, PrV pUL3.5 is processed by proteolytic cleavage, and only an abundant 14-kDa fragment consisting of amino acids 1 to ≥116 could be detected by peptide mass fingerprint analysis of purified wild-type PrV particles, which also contain the pUL48 tegument component. To determine the biological relevance of the protein-protein interaction, pUL3.5-, pUL48-, and double-negative PrV mutants were analyzed in parallel. All deletion mutants were replication competent but exhibited significantly reduced plaque sizes and virus titers in cultured rabbit kidney cells compared to wild-type and rescued viruses, which correlated with a delayed neuroinvasion in intranasally infected mice. Remarkably, the defects of the double-negative mutant were similar to those of pUL48-negative virus. Electron microscopy of cells infected with either deletion mutant revealed the retention of naked nucleocapsids in the cytoplasm and the absence of mature virus particles. In summary, our studies for the first time demonstrate the relevance of the pUL3.5-pUL48 interaction for secondary envelopment of an alphaherpesvirus, give a molecular basis for the observed trans-complementation between the PrV and BoHV-1 pUL3.5 homologs, yield conclusive evidence for the incorporation of a proteolytically processed pUL3.5 into PrV virions, and demonstrate the importance of both proteins for neuroinvasion and neurovirulence of PrV.

Pseudorabies virus (PrV), also designated suid herpesvirus 1, represents a member of the genus Varicellovirus within the Alphaherpesvirinae subfamily of the Herpesviridae (9). PrV causes Aujeszky’s disease in pigs and also leads to fatal neurological disorders in many other mammalian species, excluding higher primates and humans (55). Although PrV has been eradicated in several industrialized countries, it remains a problem in most parts of the world. Moreover, PrV has been developed into a helpful tool in neurophysiology as well as a valuable model for investigations of alphaherpesvirus gene functions for replication in tissue culture and for pathogenesis in laboratory animals (17, 56–58).

Most of the 72 genes identified within the 143-kbp genome of PrV (43) possess homologs in other alphaherpesviruses and partly also in beta- and gammaherpesviruses. This apparent conservation of gene and protein structure suggests a functional relationship. In particular, the genes required for viral DNA replication, capsid formation, and DNA encapsidation are highly conserved throughout the Herpesviridae (63). Furthermore, all hitherto-characterized members of the virus family possess two nonstructural proteins homologous to the UL31 and UL34 gene products of herpes simplex virus type 1 (HSV-1), which are involved in nuclear egress (54, 58).

In contrast, many structural components of tegument and envelope, which are acquired predominantly during virion morphogenesis in the cytoplasm, exhibit a higher degree of variability. Whereas several tegument proteins like those encoded by HSV-1 open reading frames (ORFs) UL7, UL11, UL16, UL21, UL36, UL37, and UL51 are conserved in all herpesvirus subfamilies, other major tegument components, encoded by UL41, UL46, UL47, UL48, and UL49, are apparently restricted to alphaherpesviruses (54, 56). Nevertheless, as shown by the generation of respective deletion mutants of HSV-1 and PrV, the presence of not only the conserved inner tegument components pUL36 and pUL37 but also the nonconserved pUL48 is crucial for final envelopment in the trans-Golgi region (11, 12, 23, 26, 42, 62, 70). Furthermore, the UL48 proteins of several alphaherpesviruses function as potent activators of immediate-early gene expression (1, 3, 23, 33, 61). However, whereas pUL48 has been shown to be required for efficient productive replication of HSV-1, PrV, and equid herpesvirus 1 (EHV-1), it proved to be dispensable for repli-
cation of varicella-zoster virus (VZV) and Marek's disease virus (MDV), and no UL48 homologue has been found in the genome of another avian alphaherpesvirus, psittacid herpesvirus 1 (PsHV-1) (5, 15, 23, 62, 69, 70).

Besides pUL36, pUL37, and pUL48, several other PrV proteins are involved in secondary envelopment and virus egress. Virion formation of deletion mutants lacking the tegument proteins encoded by UL7, UL11, UL16, UL21, UL47, or UL51 was less efficient or delayed compared to that of wild-type PrV (27, 45, 46, 48, 49), whereas deletion of the interacting alphaherpesvirus-specific membrane proteins encoded by UL20 and UL53 (glycoprotein K) affected the release of enveloped particles from Golgi membrane-derived vesicles into the extracellular space (13, 20, 39). Drastic egress defects associated with huge aggregations of unenveloped capsids and associated tegument proteins as well as membrane dilations in the cytoplasm of infected cells have been observed after the simultaneous deletion of several individually nonessential proteins, including the conserved glycoprotein gM (pUL10) and membrane-associated pUL11, or gM and the alphaherpesvirus-specific glycoproteins gI (pUS7) and gE (pUS8) (2, 50).

An even less-conserved gene, UL3.5, has also been demonstrated to play a very important role during final PrV envelopment in the trans-Golgi region (19). UL3.5 has originally been considered a unique gene of PrV (10), but careful reinvestigations identified homologous ORFs at corresponding genome positions of many other alphaherpesviruses, including bovine herpesvirus 1 (BoHV-1), EHV-1, VZV, avian infectious laryngotracheitis virus, and MDV (8, 18, 34, 36, 67). In contrast, HSV genomes do not contain a corresponding ORF (14, 54). Moreover, unlike deletion of PrV UL3.5, deletion of the homologous ORF57 of VZV did not significantly affect virus replication in cultured cells (6). Other alphaherpesvirus UL3.5 deletion mutants have not been described up to now. However, replication of BoHV-1 was severely impaired in cells expressing a C-terminally truncated BoHV-1 pUL3.5 (52), and UL3.5-negative PrV could be partly rescued by insertion of the UL3.5 gene of BoHV-1 (21). These findings indicated that functions of pUL3.5 in PrV and BoHV-1 might be related. Furthermore, for BoHV-1, a physical interaction between pUL3.5 and pUL48 has been demonstrated (51); this interaction, if also present in PrV, would explain the similar phenotypes of UL3.5 and UL48-negative virus mutants (19, 23). However, a direct interaction between the two PrV proteins seemed plausible, since in our previous studies only pUL48, but not pUL3.5, could be detected in purified viruses (19, 23), whereas both proteins have been identified as abundant components of mature BoHV-1 virions (65). However, an apparently pUL3.5-related polypeptide of 14 kDa was recently detected in purified PrV virions whose origin remained obscure (59).

To investigate this finding in more depth, proteome analyses of highly purified PrV particles were repeated and refined, including analysis of a virus mutant expressing a functional UL3.5 gene product which had been tagged with enhanced green fluorescent protein (EGFP). Yeast two-hybrid and co-immunoprecipitation studies were performed to unravel possible interactions between PrV pUL3.5 and pUL48. Furthermore, isogenic UL3.5-, UL48-, and double-negative virus recombinants were generated and analyzed in vitro and in vivo.
was added, and incubation was continued at 37°C. One hour after the temperature shift, nonpenetrated virus was inactivated by low-pH treatment, and after 3, 6, 9, 12, 24, 48, and 72 h, cells were scraped into the medium and lysed by freezing (−H1100°C) and thawing (37°C) (49). Progeny virus titers were determined by plaque assays using RK13 cells which were fixed at 48 h postinfection (p.i.) for 30 min with a 1:1 mixture of methanol and acetone at −20°C. Plaques were visualized by indirect immunofluorescence with a gC-specific monoclonal antibody (40) and Alexa Fluor 488-conjugated anti-mouse secondary antibodies (Invitrogen) at dilutions of 1:100 and 1:1,000, respectively. The mean titers for four parallel kinetics studies per virus were calculated. Furthermore, the diameters of 30 plaques per virus were measured, and average plaque sizes as well as standard deviations were plotted.

Western blot and radioimmunoprecipitation. For Western blot analyses, RK13 cells were harvested 16 h after infection with PrV-Ka, PrV-ΔUL48, PrV-ΔUL3.5F, PrV-ΔUL3.5/48, PrV-UL3.5F, or PrV-UL3.5G at a multiplicity of infection (MOI) of 5. Samples of infected and noninfected cells as well as of PrV virions were prepared as described previously (13), separated by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and electrotransferred to nitrocellulose membranes (Mini-Protean II and Transblot SD cell; Bio-Rad). Blots were blocked with 5% low-fat milk in Tris-buffered saline (TBS-T; 150 mM NaCl, 10 mM Tris-HCl [pH 8.0], 0.25% Tween 20), and incubated for 1 h with monospecific rabbit antisera against pUL3 (44), pUL3.5 (19), pUL48 (23), and pUL49 (2) or EGFP (kindly provided by C. Höhle and G. M. Keil, Insel Riems, Germany) at dilutions of 1:20,000 to 1:100,000 in TBS-T. Bound antibody was detected with peroxidase-conjugated anti-rabbit antibodies (Dianova) and visualized by chemiluminescence (SuperSignal, Pierce) recorded on X-ray film.

For immunolabeling, RK13 cells were infected with PrV-Ka or PrV-UL3.5G at an MOI of 5 and incubated for 2 h in MEM without methionine and cysteine prior to the addition of 3.7 MBq/ml [35S]methionine and [35S]cysteine (Tran35S-Label; MP Biochemicals). Sixteen hours after infection, the cells were lysed, and lysates were precipitated with monospecific rabbit antisera against gH (40), pUL48, or EGFP as described previously (53). Samples were separated by SDS-PAGE, and labeled proteins were detected in dried gels by radioluminography (FLA-3000; Fuji).

Proteome analyses of PrV particles. Virions were purified and separated by one-dimensional SDS-PAGE as described previously (59). Protein bands were isolated from Coomassie brilliant blue-stained gels and, after in-gel tryptic digestion, mass spectra of the obtained peptides were registered with a Bruker Ultraflex instrument. Proteins were identified using flexAnalysis 2.0 software (Bruker) with an in-house database of the PrV proteome, which had been deduced from the published genome sequence (43).

Yeast two-hybrid studies. A Matchmaker LexA two-hybrid system (Clontech) was used for investigation of interactions between pUL48 and UL3.5 of PrV and BoHV-1. Yeast cells containing the reporter plasmid pGAL-lacZ were simultaneously transformed with pB42-UL48 and one of the pLex-UL3.5 constructs shown in Fig. 1C. Furthermore, cells transformed with the empty vectors (pLexA, pB42AD) and pLexA-Pos were used as negative or positive controls, respectively. Yeast clones were selected on plates lacking amino acids which required plasmid-encoded genes for synthesis, and the presence of the plasmids was verified by PCR amplification and DNA sequencing of the insert fragments. According to the manufacturers’ protocols, liquid cultures of two clones per plasmid combination were assayed for reporter gene expression using o-nitro-
phenyl-β-D-galactopyranoside (ONPG) as substrate, and β-galactosidase activities were calculated (60).

Electron microscopy. RK13 cells were infected with 1 PFU per cell of PrV-ΔUL3.5F, PrV-ΔUL3.5/48, or PrV-UL3.5R. After 1 h on ice and an additional hour at 37°C, the inoculum was replaced by fresh medium, and incubation was continued for 13 h at 37°C. Fixation and embedding were performed as described previously (41), and counterstained ultrathin sections were analyzed with an electron microscope (Tecnai 12; Philips).

Animal experiments. The relevance of pUL48 and pUL3.5 for virulence and neuronal spread of PrV was investigated by infection of 10^7-week-old CD1 mice, each with PrV-ΔUL3.5F, PrV-ΔUL48, PrV-UL3.5/48, or PrV-Ka. After bilateral intranasal instillation of 5 µl of virus suspensions containing 10^6 PFU per ml, the animals were observed three times a day for clinical signs. Mean survival times were determined, and standard deviations were calculated. Single mice were necropsied every 24 h after inoculation, and viral spread in the trigeminal circuit was analyzed by immunohistochemistry of paraffin sections of the brains by using an antiserum against the major capsid protein (pUL19) of PrV as described previously (37).

RESULTS

Protein expression of virus mutants. The previously described UL3.5 mutants of PrV contain large foreign gene insertions encoding either gB of BoHV-1 (19) or β-galactosidase of E. coli (21). To exclude any possible effects of these heterologous sequences on other PrV genes or viral DNA replication, a novel gene deletion mutant was generated by BAC mutagenesis in bacteria. The resulting recombinant PrV-ΔUL3.5F lacks the major part (codons 7 to 199) of the UL3.5 ORF of PrV-Ka, which is translated into a 224-amino-acid protein (Fig. 1B). Since the deleted sequence was replaced by only 36 bp of foreign DNA, representing an FRT site, an impairment of mRNA stability or expression rate of the coterminaly transcribed upstream genes UL1, UL2, and UL3 (10) was unlikely. This could be confirmed by Western blot analyses of infected cell lysates, which revealed that pUL3 is expressed at similar levels by wild-type PrV-Ka and PrV-ΔUL3.5F (Fig. 2C). In contrast, neither the described 30-kDa pUL3.5 (19) nor smaller products of this protein were detectable in cells infected with PrV-ΔUL3.5F (Fig. 2A).

Three additional mutants were derived from PrV-ΔUL3.5F: double-deletion mutant PrV-ΔUL3.5/48, rescuant PrV-UL3.5R, and PrV-UL3.5G (Fig. 1B). As expected, the double mutant did not also express any detectable pUL3.5, whereas pUL3.5 of the rescuant and of the previously described mutant PrV-ΔUL48 (23) were undistinguishable from that of wild-type PrV (Fig. 2A). PrV-UL3.5G expresses an EGFP-UL3.5 fusion gene under control of the PrV UL3.5 promoter, which consists of codons 1 to 238 of EGFP, five originally nonexpressed codons, and the complete UL3.5 ORF of 225 codons (Fig. 1B). The deduced 467-amino-acid protein has a predicted molecular mass of 51 kDa. This roughly fits the apparent mass of ca. 55 kDa of the largest protein found by Western blot analyses with pUL3.5-specific (Fig. 2A) or EGFP-specific (Fig. 2D) antisera. However, like the native pUL3.5, the EGFP-UL3.5 fusion protein appears to be highly unstable, since numerous smaller protein fragments were detectable.

The insertion of PrV-UL3.5G also affects the 3' end of the overlapping UL3 gene, since it replaces its last 5 codons with 21 codons from the pEGFP-N1 polylinker region (Fig. 1B).
Thus, the size and molecular mass of the deduced protein increased from 237 to 253 amino acids and from 25.6 to 27.6 kDa, respectively. Consequently, the apparent mass of pUL3 of PrV-UL3.5G was slightly increased compared to the respective gene products of all other investigated viruses (Fig. 2C).

In PrV-ΔUL48, the UL48 ORF had been replaced by a tetracycline resistance gene (23), whereas PrV-UL3.5/48 contains a kanamycin resistance gene at the corresponding genome position (Fig. 1B). However, both mutants possess identical deletions of a viral NcoI/NheI DNA fragment comprising codons 58 to 365 of UL48. In Western blot analyses, a UL48-specific antiserum did not react with lysates of cells infected with PrV-UL48 or PrV-UL3.5/48 (Fig. 2B), whereas all other investigated viruses expressed multiple forms of pUL48 with apparent masses of ca. 53 to 57 kDa, as has previously been shown for wild-type PrV (23).

**Virion localization of PrV pUL3.5.** In the past, pUL3.5 of PrV has been considered a nonstructural protein (19), since repeated Western blot analyses with the available monospecific antiserum did not detect any UL3.5 gene products in purified particles of PrV-Ka or PrV-UL3.5G (Fig. 2A). Whereas pUL3 and pUL31 were also absent from virion preparations (Fig. 2C and F), as had been described previously (22, 44), tegument components like pUL48 and pUL49 were efficiently incorporated (Fig. 2B and E). However, an EGFP-specific specific antiserum detected a protein of ca. 35 kDa in PrV-UL3.5G virions (Fig. 2D). Although this polypeptide did not react with the UL3.5-specific antiserum (Fig. 2A), it appeared to be the most abundant form of the fusion protein also in infected cells (Fig. 2D). The different reaction patterns of the two antisera can be explained by the pronounced instability of the full-length pUL3.5 (see above) and by the fact that the anti-UL3.5 serum had been prepared against the C terminus (amino acids 137 to 223) of the protein (Fig. 1D) (19), whereas EGFP had been fused to its N terminus (Fig. 1C). To clarify which part of the EGFP-UL3.5 fusion protein targets its virion incorporation, a PrV recombinant expressing native EGFP was included in the present study. The 27-kDa EGFP was detectable only in cells infected with PrV-ΔUL43G (47), not in purified virus particles (Fig. 2D). Thus, virion incorporation is dependent on the presence of an N-terminal fragment of the UL3.5 protein, which was not detectable by our monospecific antiserum.

This finding was confirmed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry of one-dimensionally separated proteins from PrV particles, which identified an abundant 14-kDa virion component of PrV-Ka as the N-terminal part of the UL3.5 gene product. In contrast, only traces of the 30-kDa full-length protein were detectable. Peptides representing pUL3.5 amino acids 7 to 116 were identified after tryptic digestion of the 14-kDa fragment as well as of the 35-kDa EGFP-UL3.5 fusion protein from PrV-UL3.5G (Fig. 1D). However, the precise C terminus of the EGFP-UL3.5 protein present in virions and the responsible processing mechanism remain to be determined.

**Physical interactions between pUL3.5 and pUL48.** Since a direct interaction between pUL3.5 and pUL48 of BoHV-1 has been demonstrated (51), yeast two-hybrid studies were performed to test whether this also applies to the respective PrV homologs. For this purpose, an expression plasmid was constructed in which the complete UL3.5 ORF of PrV had been fused in-frame to the gene of the sequence-specific DNA-binding LexA protein (pLex-UL3.5) (Fig. 1C). A second plasmid contained the entire UL48 ORF fused to the coding sequence of a transcription-activating protein (pB42-UL48; Fig. 1C). Cotransfection of yeast cells with these two plasmids led to pronounced expression of a lacZ reporter gene under control of an inducible promoter containing LexA-binding elements. Using ONPG as a substrate, β-galactosidase activities of 1,200 to 1,400 Miller units per ml in liquid cultures could be determined (Fig. 3). This level of activity was similar to that obtained with a positive control plasmid provided by the manufacturer (Fig. 3, pLexA-pos). However, if the UL3.5 or UL48...
plasmid was replaced by the empty vector pLexA or pB42AD, respectively, $\beta$-galactosidase expression was almost completely abolished (Fig. 3). These findings strongly indicated that a physical interaction between the UL3.5 and UL48 parts of the fusion proteins was responsible for reporter gene activation.

To investigate the interaction in more detail, three truncated pLexA-UL3.5 plasmids containing UL3.5 codons 1 to 139, 1 to 43, or 20 to 224 were generated (Fig. 1C). Coexpression with pB42-UL48 revealed that the N terminus of the UL3.5 protein (aa 1 to 20) is essential for transactivation, whereas amino acids 44 to 223 are dispensable (Fig. 3). Remarkably, the N-terminal part is highly conserved between the UL3.5 proteins of different alphaherpesviruses (18); therefore, it was conceivable that the homologues of other virus species might also interact with the UL48 gene product of PrV. This could be confirmed for the 126 codon UL3.5 ORF of BoHV-1, which also interact with the UL48 gene product of PrV. This could be confirmed for the 126 codon UL3.5 ORF of BoHV-1, which was also cloned and expressed as LexA fusion protein (pLexA-UL3.5B; Fig. 1C) and which induced $\beta$-galactosidase expression by interaction with PrV pUL48 as efficiently as the autologous constructs (Fig. 3).

We also attempted to localize the interacting domain of pUL48. However, all tested 5′- or 3′-terminal truncations of the insert of pB42-UL48 completely abolished transactivation (results not shown). Thus, for interaction with pUL3.5, a discontinuous sequence motif and/or a proper tertiary structure of pUL48 might be required. It was also impossible to confirm the yeast two-hybrid interactions by reversal studies using a LexA-UL48 fusion protein, since this construct induced reporter gene expression on its own. Most likely, this was a consequence of the known transcription-activating properties of the UL48 gene products of different herpesviruses, including that of PrV (23).

To provide further evidence for the interaction between pUL3.5 and pUL48 of PrV, immunoprecipitation experiments with [35S]methionine- and [35S]cysteine-labeled lysates of infected cells were performed. Since our UL3.5-specific antiserum is not suitable for these studies, the virus mutant expressing the EGFP-UL3.5 fusion protein (PrV-UL3.5G) and an EGFP-specific serum were used. This serum precipitated two proteins of ca. 35 and 37 kDa, which likely represent truncated EGFP-UL3.5 fusion proteins, but also three proteins of 53 to 57 kDa, which correspond to different forms of pUL48 (Fig. 4), as shown by precipitation by an anti-UL48 serum but not by a gH-specific control antiserum (Fig. 4). The 53- to 57-kDa UL48 proteins were also precipitated from lysates of cells infected with wild-type PrV-Ka, but the 35- and 37-kDa proteins were not coprecipitated by the anti-UL48 serum (Fig. 4), which confirmed that they indeed represent EGFP-UL3.5 fusion proteins. Remarkably, there was no clear evidence for coprecipitation of the full-length wild-type pUL3.5. However, since the anti-EGFP serum did not specifically precipitate any proteins from PrV-Ka (Fig. 4), the coprecipitation of pUL48 from PrV-UL3.5G-infected cells was apparently caused by its interaction with the EGFP-UL3.5 fusion protein. Immunofluorescence analyses of RK13 cells infected with PrV-UL3.5G revealed a cytoplasmic colocalization of the EGFP-UL3.5 and pUL48, which was not seen in cells infected with PrV mutants expressing native EGFP (data not shown). Thus, taken together, our studies provide strong evidence for the existence of a pUL3.5-pUL48 complex in PrV-infected cells, which is also incorporated into virions.

**In vitro growth properties of UL3.5- and UL48-negative PrV mutants.** PrV mutants lacking either pUL3.5 or pUL48 have already been described (19, 21, 23). However, these mutants originating from distinct parental viruses had never been investigated in parallel under identical conditions. For the present study, the growth properties of isogenic mutants derived from the BAC-cloned PrV- strain Ka were determined in rabbit kidney cells. Furthermore, an UL3.5 and UL48 double-deletion mutant was analyzed for the first time.

In agreement with the results of earlier investigations, plaque diameters measured 2 days after infection of RK13 cells with UL3.5- or UL48-negative PrV were significantly reduced, by 50 or 65%, respectively, compared to the plaque diameters for wild-type PrV-Ka (Fig. 5A). Rescuing PrV-UL3.5R as well as PrV-UL3.5G exhibited wild-type-like plaque sizes, demonstrating that the observed defect of the deletion mutant was indeed caused by the absence of the UL3.5 gene and that the EGFP-UL3.5 fusion protein can functionally substitute for the native pUL3.5 (Fig. 5A). This was also confirmed by one-step growth studies, in which maximum titers of PrV-∆UL3.5F and PrV-ΔUL48 were reduced ca. 15-fold and 100-fold, respectively, whereas the two rescuing reached wild-type-like titers (Fig. 5B). A rescuing of PrV-ΔUL48 was not tested in the present study, since it had already been shown that the defects of this mutant can be fully complemented by pUL48 (23).

PrV-ΔUL3.5/48, which was derived from PrV-ΔUL3.5/48 by the additional deletion of UL48, exhibited replication defects similar to those of PrV-ΔUL48 (Fig. 5A and B). Thus, the functions of pUL3.5 and pUL48 during replication of PrV are obviously not independent of each other, since the presence or absence of pUL3.5 is no longer relevant after the deletion of pUL48. However, the functions may not be completely identical, since the defects of pUL3.5-negative PrV are less pronounced than those of pUL48-negative mutants.
Electron microscopy of RK13 cells which were fixed during the late replication phase of PrV-ΔUL3.5/48 (Fig. 6A to C) or PrV-ΔUL3.5F (Fig. 6D) revealed very similar phenotypes. Nucleocapsid formation in the nucleus (Fig. 6A and D) and nuclear egress by sequential envelopment and deenvelopment at the inner and outer lamellae of the nuclear membrane (Fig. 6B) were not detectably affected. In contrast, secondary envelopment of cytoplasmic nucleocapsids was almost completely blocked (Fig. 6A, C, and D). Remarkably, the infected cells exhibited no tight aggregations of capsids or electron-dense tegument material, as had been described for several other PrV mutants possessing egress defects (56). Unlike PrV-ΔUL48 (23), neither the double mutant nor PrV-ΔUL3.5F showed an enhanced release of tegument-containing but capsidless light (L) particles from the cells (Fig. 6A and D). As expected from the restoration of plaque formation and virus replication, the release of mature particles and all preceding steps of virion morphogenesis of the rescue mutant PrV-UL3.5R (Fig. 6E) appeared similar to those observed for cells infected with wild-type PrV-Ka (30). Taken together, these results indicate that pUL3.5 and pUL48 of PrV are relevant for secondary envelopment of virus particles in the cytoplasm of infected cells, and, coinciding with the physical interaction of the proteins, their functions are interdependent.

Relevance of pUL3.5 and pUL48 of PrV for in vivo neurovirulence. Previous studies revealed that the deletion of the UL48 gene severely affected the kinetics of neuroinvasion of PrV after intranasal infection of mice (37). To investigate the in vivo relevance of pUL3.5, similar animal experiments including PrV-ΔUL3.5F and the double-deletion mutant PrV-ΔUL3.5/48 were performed. In these studies, the first clinical symptoms like depression, anorexia, cowering in a hunched position, and scratching could be observed 3 to 4 days after infection with either of the deletion mutants, which was approximately 48 h later than in mice infected with wild-type PrV-Ka (Table 1). Taken together, these results indicate that pUL3.5 and pUL48 of PrV are relevant for secondary envelopment of virus particles in the cytoplasm of infected cells, and, coinciding with the physical interaction of the proteins, their functions are interdependent.

DISCUSSION

The salient findings of this study are the following. (i) As previously observed for the homologous BoHV-1 proteins (51), pUL3.5 and pUL48 of PrV physically interact directly. (ii) pUL3.5 of BoHV-1 forms a complex with pUL48 of PrV, which constitutes the molecular basis for the observed functional complementation (21). (iii) PrV pUL3.5 is proteolytically processed, and only the processed 14-kDa N-terminal portion is incorporated into virions, explaining our previous failure to detect pUL3.5 in virions by probing with an antisem directed against the C terminus (19). (iv) The interaction between pUL3.5 and pUL48 is important for virion formation, since deletion mutants for either gene and a dou-
ble-deletion mutant exhibit similar phenotypes. (v) The interaction between both proteins is also critical for neuroinvasion and neurovirulence.

Despite the important roles of pUL3.5 and pUL48 during efficient envelopment of PrV nucleocapsids in the trans-Golgi region, these proteins are not conserved throughout the Herpesviridae but are restricted to members of the subfamily Alphaherpesvirinae (9). Moreover, no UL3.5 homologues have been found in HSV genomes (14, 54), and a recently sequenced avian alphaherpesvirus, PsHV-1, possesses none of the two genes (68). In contrast, in the genomes of most other alphaherpesviruses of the genera Varicellovirus (e.g., VZV, BoHV-1, EHV-1), Mardivirus (MDV-2), and Iltovirus (avian infectious laryngotracheitis virus), UL3.5 and UL48 homologues have been identified (8, 10, 18, 34, 36, 67). However, these proteins apparently differ with respect to their importance for viral replication. pUL3.5 and pUL48 are dispensable for replication of VZV in cell culture (5, 6), as is pUL48 of MDV (15). Remarkably, in vitro these two viruses do not form infectious extracellular particles at detectable levels but, rather, spread only directly from cell to cell. Thus, the secondary envelopment of VZV capsids as observed in the cytoplasm of cultured cells (32) is obviously mediated, at least partly, by other viral proteins. This is in stark contrast to PrV and BoHV-1, which form extracellular infectious virions at high levels. Although it is unclear whether expression of a pUL3.5-like protein in VZV or a pUL48 homolog in MDV would enhance the formation of extracellular virions, our studies clearly show that these proteins and their interaction are crucial for efficient virion morphogenesis of BoHV-1 and PrV.

In contrast, HSV-1 also readily forms extracellular virions without a pUL3.5 homolog. Thus, pUL3.5 function must have been adopted by another protein of HSV-1, perhaps by pUL48 itself. Like PrV pUL48, the HSV-1 homolog not only exhibits
transcription-activating functions but also is relevant for virion morphogenesis (1, 3, 23, 62, 70). For closer cognates of PrV, like BoHV-1 and EHV-1, the functional relevance of the UL3.5 gene has not yet been investigated. A UL48-negative EHV-1 mutant has been described and shown to be defective in viral egress, like the corresponding PrV and HSV-1 mutants (69). To assess the role of pUL3.5 in these viruses, construction and characterization of corresponding deletion mutants is required, as has been done for PrV (19; this study).

The UL48 gene products of several alphaherpesviruses, including PrV, have been identified as abundant tegument proteins (23, 63). pUL3.5 was also detected in mature BoHV-1 virions (65); full-length pUL3.5 had not been found in PrV virions (19), although a smaller product was recently detected (65); full-length pUL3.5 had not been found in PrV or HSV-1 (65). To assess the role of pUL3.5 in these viruses, construction and characterization of corresponding deletion mutants is required, as has been done for PrV (19; this study).

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The hitherto-identified UL3.5 genes of other alphaherpesviruses are significantly smaller than the UL3.5 ORF of PrV, and detectable amino acid sequence homologies are limited to the N-terminal 57 residues (18). However, our yeast two-hybrid studies revealed that the conserved amino acids 1 to 43 of PrV pUL3.5 are sufficient for interaction with pUL48, whereas the interaction was completely abolished after deletion of the N-terminal 19 residues. In similar truncation analyses, amino acids 1 to 86 but not amino acids 21 to 126 of the BoHV-1 UL3.5 protein interacted with pUL48 in pull-down experiments (51). Thus, the conserved N-terminal domains of other alphaherpesvirus UL3.5 proteins might function in similar specific protein interactions. Interestingly, our yeast two-hybrid studies further demonstrated a strong interaction between pUL3.5 of BoHV-1 and pUL48 of PrV. This interaction explains the previously described heterologous cis complementation of UL3.5-negative PrV by the corresponding gene of BoHV-1 (21). To date, it is unclear whether the unique C-terminal part of PrV pUL3.5, which is removed during processing, possesses any biological relevance. In earlier studies, a PrV mutant expressing a truncated UL3.5 gene product consisting of the first 158 amino acids exhibited no detectable in vitro replication defects compared to the parental virus expressing the complete protein (19). However, in vivo virulence of this mutant has not been investigated.

To determine more precisely the biological role of the interaction between the N-terminal part of pUL3.5 with pUL48, a PrV-mutant lacking UL3.5 codons 7 to 199 was investigated in parallel with UL48-negative virus (23) and a double mutant carrying deletions of both genes. Electron microscopy of rabbit kidney cells infected with either mutant revealed similar defects during virus morphogenesis. As in earlier studies with single deletion mutants (19, 23), capsid formation, DNA-encapsulation, and nuclear egress were not detectably affected. In contrast, the secondary envelopment of nucleocapsids in the cytoplasm was blocked in the absence of UL48, UL3.5, or both. An inhibition of final envelopment was also observed with several other gene deletion mutants of PrV. However, semicrystalline aggregations of cytoplasmic capsids like those observed in the absence of pUL37 (42) were not detectable with UL3.5 or UL48 mutants. The deletion of UL3.5 and/or UL48 also failed to induce electron-dense accumulations of the pUL46, pUL47, and pUL49 tegument components in the cytoplasm, or pronounced dilations of Golgi membranes, which had been observed after deletion of gM in combination with gE and gl (2), or gM and pUL11 (50). Whereas from cells infected with these double and triple mutants no virions or virus-like particles were secreted at detectable levels, UL48-negative PrV has been shown to release a high amount of enveloped, tegument-containing but capsidless particles (23). Such L particles were not produced at similar levels by UL3.5-negative, or UL3.5 and UL48 double-negative PrV, which was the only marked difference detected by electron microscopic analyses between these deletion mutants. Nevertheless, our results indicate closely related functions of pUL3.5 and pUL48.

### Table 1. Virulence in mice

<table>
<thead>
<tr>
<th>Virus</th>
<th>Mean time to death (SD)</th>
<th>Clinical symptoms at indicated day p.i.</th>
<th>Immunohistochemistry (day p.i.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Pvu-Ka</td>
<td>49.1 (1.58)</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>Pvu-UL3.5</td>
<td>125.7 (3.26)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pvu-UL3.5F</td>
<td>117.7 (9.93)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pvu-UL3.5/UL48</td>
<td>127.7 (6.42)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* a Average times to death (in hours) after intranasal infection with 10⁴ PFU of the indicated virus were calculated for 10 animals for each virus.
* b Clinical symptoms were scored as follows: -, clinically inconspicuous; +, slight depression, hunched position, and ruffled hair coat; +++, severe attacks of excitation, self-mutilation, skin erosions, and heavy dyspnea; †, animals moribund or dead.
* c Time in days p.i. of first UL19 antigen detection at the subsequent levels of the trigeminal pathway: nasal cavity (respiratory mucosal epithelium), first-order neurons (trigeminal ganglion), second-order neurons (spinal trigeminal nucleus, Sp5), third-order neurons (ectochoral cortex). ND, not detected.
during an egress step which occurs after primary tegumentation of cytoplasmic nucleocapsids but that is independent of targeting of at least several other tegument components to Golgi-derived vesicles, which has been suggested to be mediated by interactions between, e.g., the UL49 tegument protein and envelope glycoproteins gE and gM (24, 56).

Although almost no mature virus particles were detectable in or around noncomplementing cells infected with UL3.5, UL48, or double-negative PrV mutants, plaque assays and one-step growth analyses revealed that none of the deletions caused a complete inhibition of direct cell-to-cell spread or formation of infectious progeny virus. It is still unclear whether this apparent discrepancy can be explained by the insensitivity of electron microscopy or by the infectivity of subviral structures like nucleocapsids, primary enveloped virions, or even naked virus DNA. Irrespective of the precise reason, UL3.5 and UL48 of PrV are, strictly speaking, nonessential either singly or in combination.

In rabbit kidney cells, plaque diameters of UL3.5-negative PrV were reduced by ca. 50%, and maximum virus titers were ca. 15-fold lower than those of wild-type PrV or rescue mutants. Previous studies performed with African green monkey (Vero) cells indicated much more pronounced replication defects of other UL3.5 deletion mutants of PrV (19, 21). It remains to be investigated whether these differences are exclusively cell type dependent or are caused partly by the large foreign gene insertions at the UL3.5 gene locus of the previous mutants, which might have affected transcription efficiency or mRNA stability of the coterminally transcribed upstream genes UL1, UL2, and UL3 (10). Remarkably, UL1, which encodes gL, is known to be essential for formation of infectious virions and for cell-to-cell spread (38). However, since the mutant used in the present study contains only an FRT site upstream genes proved to be unaffected, the observed replication defects could be attributed exclusively to the deletion of UL3.5.

Plaque diameters of UL48-negative PrV in RK13 cells were reduced by ca. 65%, and titers were 100-fold lower than those of wild-type or rescued viruses. Thus, deletion of UL48 caused more severe in vitro replication defects than deletion of UL3.5. This could indicate that the functions of pUL48 and pUL3.5 during viral egress are overlapping but distinct, despite the similar ultrastructural patterns of the deletion mutants. On the other hand, PrV pUL48 is at least bifunctional (23), having transcription-activating properties in newly infected cells. For this function, the presence of pUL3.5 is dispensable. Thus, in the absence of pUL48, pUL3.5 is no longer relevant, as shown by almost identical in vitro replication deficiencies of the double-deletion mutant and UL48-negative PrV.

The observed in vitro growth defects correlate with significantly delayed neuroinvasion in vivo, leading to prolonged survival times after intranasal infection of mice. Again, UL3.5-negative PrV was slightly less impaired than the UL48 and double-deletion mutants. Therefore, it appears unlikely that the two proteins function during independent steps of virion morphogenesis and neuronal spread or that they possess redundant functions, since in both cases the defects caused by the two mutations should be additive or synergistic, like those observed for several other PrV mutants with multiple deletions of tegument and/or envelope proteins involved in viral egress (2, 25, 50).

Thus, most likely, pUL3.5 and pUL48 execute functions during the same process, which requires their physical interaction. Fractionation of PrV-infected cells indicated a tight association of pUL3.5 with cytoplasmic membranes, although the nature of this interaction remained unclear (19). For HSV-1 pUL48, physical and functional interactions with other tegument proteins encoded by UL41, UL46, UL47, and UL49 have been reported (16, 66, 71). Similar interactions of the PrV homolog, together with its binding to the membrane-associated pUL3.5, might be a prerequisite for proper assembly of virion components at the future budding site in the trans-Golgi region. In HSV-1, another membrane-associated protein might execute the role of the missing UL3.5 gene product. In this context, the proposed interactions of HSV-1 pUL48 with envelope glycoproteins gB, gD, and gH should be considered (31, 72).

In summary, we show here that pUL3.5 and pUL48 of PrV interact physically and demonstrate for the first time the importance of this interaction for virion formation of an alphaherpesvirus. Heterologous complex formation between the BoHV-1 pUL3.5 and PrV pUL48 explains the observed trans-complementation of UL3.5-deficient PrV by the BoHV-1 homolog. In contrast to other pUL3.5 homologs, the PrV protein is proteolytically processed prior to incorporation into virions, retaining only the N-terminal portion which is responsible for complex formation. Thus, the pUL3.5-pUL48 complex apparently plays a prominent role during cytoplasmic virion morphogenesis of these alphaherpesviruses.

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