Pseudorabies virus particles lacking tegument proteins pUL11 or pUL16 incorporate less full-length pUL36 than wild-type virus, but specifically accumulate a pUL36 N-terminal fragment

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Proteins of the virion tegument of alphaherpesviruses are involved in protein–protein interactions, which play important roles in virus morphogenesis. Seven single-gene deletion mutants of Pseudorabies virus were analysed for alterations in the overall composition of the virion beyond the loss of the targeted protein. The UL36 protein (pUL36) was present in equal amounts in wild-type virions and mutants lacking pUL21, pUL49, pUL51, pUS3 or pUS8. Virions lacking pUL11 or pUL16 incorporated less full-length pUL36 than wild-type particles, but contained increased amounts of an N-terminal fragment of pUL36 that is present only in traces in wild-type virus and the other mutants.

Homologues of the large tegument protein encoded by the UL36 open reading frame of herpes simplex virus (HSV) are present in all members of the Herpesviridae analysed so far. They constitute the largest herperviral gene products and are proposed to be part of the capsid–proximal inner tegument, a structure that resides between nucleocapsid and envelope. Pseudorabies virus (PrV) pUL36 is a 3084 aa protein that is strictly essential for virus replication (Fuchs et al., 2004). PrV pUL36 function is important for virion morphogenesis in the cytoplasm after nuclear egress. pUL36 interacts with pUL37, another conserved tegument protein (Klupp et al., 2002). However, removal of the N-terminally located pUL37-binding domain does not result in a complete loss of function of pUL36, indicating that the essential requirement for this protein is due to additional, still unknown, functions (Fuchs et al., 2004). Recently, a deubiquitinating proteolytic activity was demonstrated to reside in the very N terminus of herpesvirus pUL36 homologues (Kattenhorn et al., 2005). Thus, both hitherto assigned functional regions of pUL36 reside in the N-terminal part of the protein. It has been shown in a previous study that PrV particles devoid of the tegument proteins pUS3, pUL47 and pUL49 or pUS8 (the envelope glycoprotein E) incorporated the same amounts of several tegument proteins like pUL36, pUL37 and pUL47 as wild-type virions (Michael et al., 2006). This stoichiometric incorporation suggests that, like assembly of the capsid, the architecture of the tegument might, in part, be strictly controlled. On the other hand, amounts of other tegument proteins like pUL46, pUL48 and pUL49 varied to some extent among the different mutants, indicating also some degree of flexibility in tegument composition. The suggested interaction of pUL36 with pentonal sites on the capsid (Zhou et al., 1999), which are devoid of the small capsid protein pUL35 that decorates hexons, may partly explain this strict stoichiometry.

In a study to analyse single-protein-deleted PrV mutants for alterations in the composition of the virion beyond the loss of the deleted protein, structural proteins in virus particles were quantified by a procedure designated ‘stable isotope labelling by amino acids in cell culture’ (SILAC) developed by Ong et al. (2002), which was adapted for virions by our group (Michael et al., 2006).

Virus particles from infected porcine kidney cell cultures were purified by sucrose-density-gradient centrifugation (Karger et al., 1998). Deletion mutants were propagated on cells maintained in DME/F12 cell culture medium (Sigma-Aldrich D-9785) supplemented with conventional leucine, whereas wild-type PrV-Ka (Kaplan & Vatter, 1959) was harvested from cells that had been cultured in medium containing exclusively deuterated leucine (l-leucine-5,5,5-d3; Sigma-Aldrich, 99 atom% D), inserting a mass tag of 3 Da per leucine residue. Mutant and mass-tagged purified PrV-Ka were mixed at 1 : 1 protein ratios and proteins were separated by SDS-PAGE (Laemmli, 1970). Protein bands of interest were cut from Coomassie-stained gels (Neuhoff et al., 1988) and digested with trypsin for peptide mass fingerprint (PMF) analysis (Rosenfeld et al., 1992).

Matrix-assisted laser desorption/ionization time-of-flight mass spectra were registered on an Ultraflex Instrument
To assay for a potential variation in pUL36 incorporation in different wild-type PrV strains, virions of PrV-Ka, PrV-Becker (Robbins et al., 1984) or PrV-NIA-3 (Baskerville, 1973) were analyzed for pUL36 incorporation. The protein migrating at the calculated molecular mass of 330 kDa for the full-length pUL36 (Fig. 1) was identified as pUL36 by PMF and by immunoblot analysis (Towbin et al., 1979) with a pUL36-specific antiserum (Fig. 1b; Klupp et al., 2002). No difference was observed between the three different wild-type PrV strains in the level of incorporation as deduced from the gel or in the size of the packaged pUL36.

SDS-PAGE analysis of PrV mutants deleted in single tegument components as well as glycoprotein E (gE) produced an unexpected result. In mutants lacking pUS3, pUL21, pUL49, pUL51 or gE, pUL36 appeared as it does in PrV-Ka, whereas an additional protein of approximately 220 kDa was rather prominent in PrV-ΔUL11 and PrV-ΔUL16 virions in Coomassie-stained gels (Fig. 2a). Silver staining with prolonged development (Fig. 2b) revealed that mutants lacking pUS3, pUL21, pUL49, pUL51 or gE contained only minute amounts of proteins migrating in this region of the gel (Fig. 2b). In immunoblot analysis, the additional approximately 220 kDa protein found in PrV-ΔUL11 and PrV-ΔUL16 reacted with a pUL36-specific antiserum (Fig. 2c), but after prolonged film exposure, faint reactivity in this region of the blot was also found in PrV-Ka and the other mutants, indicating that pUL36-derived proteins of the same approximate size were also present, although much less abundantly.

Using PMF, the approximately 220 kDa protein was identified as an N-terminal fragment of pUL36. Tryptic peptides representing the pUL36 N-terminal fragment partially covered aa 118–3081 of the pUL36 sequence (SWISS-PROT Q8UZ11, 3085 aa), whereas tryptic peptides originating from the full-length form partially covered the sequence aa 118–3081. The N-terminal fragment of pUL36 found in PrV-ΔUL16 was slightly smaller than that in PrV-ΔUL11, but this size difference did not translate into different peptide coverages in the PMF analysis. Thus, it remains unclear whether the different apparent molecular masses result from additional truncations of pUL36 found in PrV-ΔUL16 virions or from differential post-translational modifications. Although present in PrV-Ka only in very small amounts, relative levels of the pUL36 N-terminal fragments in PrV-ΔUL11 and PrV-ΔUL16 could be determined by the SILAC technique from mixtures of mutant virus particles with mass-tagged PrV-Ka. Levels of the pUL36 N-terminal fragment were elevated 3- to 4-fold in PrV-ΔUL11 and 5- to 6-fold in PrV-ΔUL16. Concomitant with the appearance of the N-terminal pUL36 fragment in the UL11 and UL16 deletion mutants, relative amounts of full-length pUL36 were reduced in both mutants, but were at wild-type levels in all the other mutants tested (Fig. 3). Colorimetric evaluation of the Coomassie-stained gels (AIDA software package; Raytest) confirmed the quantitative MS data. The pUL36 N-terminal fragment was found to represent 26-4 % (PrV-ΔUL11) and 32-9 % (PrV-ΔUL16).
of the molar amounts of the respective full-length products and, thus, compensates for a significant part of the mean loss of 37% (PrV-DUL11) and 41% (PrV-DUL16) of the full-length pUL36 (Fig. 3). In all other mutants tested, amounts of the pUL36 N-terminal fragments were minimal and were 1–4% of the full-length product.

To assay whether preferential incorporation of the pUL36 N-terminal fragment in PrV-ΔUL11 and PrV-ΔUL16 resulted from overexpression in infected cells, expression levels were assayed by immunoblot analysis of rabbit kidney (RK13) cells infected with PrV-Ka, PrV-ΔUL11 or PrV-ΔUL16 (Fig. 2e). Cells were extracted 16 h after infection with PBS containing 1% Triton X-100 and a protease inhibitor cocktail (Complete; Roche) for 10 min on ice with occasional shaking. Twenty micrograms of the clarified extract (15 000 g, 15 min, 4°C) were analysed after electrophoretic separation on 5% polyacrylamide gels (Laemmli, 1970). Expression patterns and ratios between the full-length form and the N-terminal fragment (marked by arrows) were similar after infection of RK13 cells with any virus and no excessive expression of the pUL36 N-terminal fragment was observed after infection with PrV-ΔUL11 or PrV-ΔUL16. Molecular masses are given in kDa.
Fig. 3. Quantification of full-length pUL36 and the N-terminal fragment. Relative amounts of the full-length form of pUL36 (a) and the N-terminal fragment (b) were assayed in two independent experiments (filled and open bars). Results are expressed in multiples of the amount of the respective protein present in PrV-Ka, i.e. values above 1.0 indicate that larger amounts of the respective protein are present in the mutant than in PrV-Ka virions and values below 1.0 indicate smaller amounts in the mutant viruses. In PrV-ΔUL11 and PrV-ΔUL16, a deficiency in full-length pUL36 and an increased incorporation of the N-terminal fragment were observed. Error bars represent the SD of the isotope ratios of peptides that have been selected for quantification.

Fragment was observed after infection with PrV-ΔUL11 or PrV-ΔUL16, indicating that incorporation of the pUL36 N-terminal fragment in mutant viruses did not simply reflect its amounts in infected cells. Immunoblot analysis of purified PrV-ΔUL11 or PrV-ΔUL16 virions that had been propagated on pUL11- or pUL16-expressing recombinant cells, respectively (RK13-UL11, RK13-UL16 in Fig. 2d; Kopp et al., 2003; Klupp et al., 2005a) showed that the pUL36 N-terminal fragment was not incorporated into virus particles after phenotypic complementation of PrV-ΔUL11 and PrV-ΔUL16, although it was well expressed in the respective infected cells (Fig. 2e). These results indicate that the presence of pUL11 and pUL16 is required to preclude any pUL36 N-terminal fragment present from incorporation into the mature virion. The origin and potential function of the pUL36 N-terminal fragment is unclear. Since two functional motifs mediating pUL37 interaction and proteolytic deubiquitinating activity are located in the N terminus of pUL36 (Klupp et al., 2002; Fuchs et al., 2004; Kattenhorn et al., 2005), these domains are most likely still functional in the N-terminal pUL36 fragment. Thus, its incorporation into virions could be mediated in the network of protein-protein interactions by its complex formation with pUL37. However, most interesting is the specific correlation of incorporation of the N-terminal pUL36 fragment with the lack of pUL11 and pUL16. In both mutants, incorporation of the N terminus of pUL36 into virions was accompanied by a significant loss of full-length pUL36, suggesting that full-length pUL36 was partly replaced by the N-terminal fragment. This could mean that the requirement for constant amounts of pUL36 in an intact tegument can be accomplished by incorporation of an N-terminal fragment and that the stoichiometric interaction of pUL36 with other virion components is mediated by the N terminus rather than the C terminus. Although neither UL11 nor UL16 is essential for the replication of PrV in cell culture, an impairment in secondary envelopment has been shown in a UL11 mutant (Kopp et al., 2003) in electron microscopy studies. Interestingly, the absence of pUL36 also results in a defect in cytoplasmic virion morphogenesis, whereas deletion of UL36, UL11, UL16 or UL11 + UL16 does not influence the intranuclear stages of virus morphogenesis (Fuchs et al., 2004; Klupp et al., 2005a). Loomis et al. (2003) have demonstrated that pUL11 and pUL16 of HSV-1 form a complex and that pUL11 of PrV can interact with pUL16 of HSV-1. Therefore, formation of a complex between pUL11 and pUL16 of PrV seems likely. From the data presented here, it is hypothesized that pUL11 and pUL16 or a potential complex of both proteins play an important role in the exclusive incorporation of full-length pUL36 into the tegument of mature PrV virions excluding any N-terminal fragments of pUL36 that are present in infected cells.

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References


