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Mapping of Sequences in Pseudorabies Virus pUL34 That Are Required for Formation and Function of the Nuclear Egress Complex

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The nuclear egress complex (NEC) is required for efficient translocation of newly synthesized herpesvirus nucleocapsids from the nucleus to the cytosol. It consists of the type II membrane protein pUL34 which interacts with pUL31 at the inner nuclear membrane (INM). To map regions within pUL34 required for nuclear membrane targeting and pUL31 interaction, we constructed deletion/substitution mutations. Previously, we showed that 50 C-terminal amino acids (aa) of pseudorabies virus (PrV) pUL34, including the transmembrane domain, could be functionally replaced by cellular lamina-associated polypeptide 2β (Lap2β) sequences. In contrast, replacement of the C-terminal 100 aa abrogated complementation but not pUL31 interaction. To further delineate essential sequences within this region, C-terminal pUL34 truncations of 60, 70, 80, 85, and 90 aa fused to Lap2β sequences were generated. While truncations up to 85 aa were functional, deletion of the C-terminal 90 aa abrogated function, which indicates that the important region is located between aa 171 and 176. Amino acids 173 to 175 represent a motif suggested to mediate INM targeting. Mutagenesis to RQG revealed that the mutant protein exhibited pronounced Golgi localization, but a fraction still reached the INM. Deletion mutations in the N-terminal domain of pUL34 demonstrated that absence of the first 4 aa was tolerated, while removal of 9 or more residues resulted in a nonfunctional protein. In addition, mutation of three conserved cysteines did not abrogate pUL34 function, whereas alteration of a conserved glutamine/tyrosine sequence yielded a nonfunctional protein.

Herpesvirus capsids are assembled and viral genomes are packaged in the host cell nucleus while further virion maturation occurs in the cytosol. For transit from the nucleus to the cytoplasm, herpesvirus nucleocapsids bud at the inner nuclear membrane (INM), thereby acquiring a primary envelope which subsequently fuses with the outer nuclear membrane (ONM) to release the nucleocapsids into the cytosol. This process can be regarded as a vesicle (primary envelope)-mediated transport of cargo (nucleocapsids) through the nuclear envelope. Although mechanistic details of this process are not yet known, conserved herpesviral proteins homologous to herpes simplex virus 1 (HSV-1) pUL31 and pUL34 are required for efficient translocation (reviewed in references 1 to 3).

The pUL34 homologs constitute type II, tail-anchored membrane proteins which are efficiently targeted to the nuclear envelope. The pUL34 interaction partner pUL31 is diffusely distributed in the nucleoplasm in the absence of pUL34 but relocates to the INM in its presence, where both proteins form the heterodimeric nuclear egress complex (NEC). This complex recruits cellular and viral kinases to phosphorylate the nuclear lamina, a tight network of intermediate filaments underneath the INM, to partly dissolve or soften the lamina, and to permit access of nucleocapsids to the budding sites (reviewed in references 1, 3, and 4). Expression of only these two viral proteins is sufficient for the formation of vesicles from the INM which resemble primary envelopes, indicating that no other viral components are required for membrane bending, fusion, and fission during primary envelopment (5, 6).

How nucleocapsids are targeted to the budding site is unclear. Recently, an interaction between pUL31 and the capsid via pUL25 within the C-capsid specific component (CCSC; renamed capsid vertex-specific component, or CVSC) (7) has been shown for HSV-1 (8), and interactions between pUL31 and pUL25 have been demonstrated by yeast two-hybrid analyses of alpha herpesvirus homologs (8-10). However, pseudorabies virus (PrV) pUL25 seems not to be required for capsid binding of pUL31 (11). Moreover, HSV-1 and PrV nucleocapsids lacking pUL25 are often found close to the INM, indicating that they are able to dock at putative budding sites although primary envelopment does not ensue (12, 13). A direct link of pUL34 to the capsid via the major capsid protein has been suggested based on coprecipitation (14), but this requires confirmation.

The nuclear envelope consists of two lipid bilayers, which are designated the inner and outer nuclear membranes. Both membranes are connected at multiple nuclear pores. Nevertheless, they possess unique protein compositions, and at least 100 different integral INM proteins have been identified (15). The ONM is contiguous and shares proteins with the rough endoplasmic reticulum (ER) but also contains a specific subset of polypeptides. How the selectivity of ONM and INM protein composition is realized is still not completely clear (16). Three different models are proposed to explain how membrane proteins specific for the INM reach their final destinations: (i) diffusion and retention, (ii) active transport using classical nuclear localization signals (NLS), and (iii) active trafficking via inner nuclear membrane sorting (INMS) motifs (17–21). Recent studies indicate that the SUN domain (Sad1p and UNC-84 homology domain) containing proteins of the INM contains several different localization signals throughout the proteins (16, 21, 22). Mammalian SUN2 carries
three different motifs: a classical NLS, a basic arginine-rich cluster which probably functions as a Golgi retrieval signal, and the SUN domain (22). The Caenorhabditis elegans homolog of mammalian SUN, Unc-84, contains four different trafficking signals including two classical NLS, an inner nuclear membrane-targeting signal, and the SUN domain (21). Only when all four signals are simultaneously deleted does Unc-84 completely fail to localize to the INM and to function, indicating that multiple mechanisms are used to ensure proper targeting.

HSV-1 and PrV pUL34 are targeted to the nuclear envelope independent of other viral proteins although the presence of the alphaherpesvirus-specific kinase pUS3 increases the efficiency (23, 24). While a putative NLS is predicted for HSV-1 pUL34 (PSort [http://www.psort.org]) (25) and HSV-1 pUL34 lacking its transmembrane domain efficiently localizes to the nucleoplasm (26), a similar motif is absent in PrV pUL34.

To define the regions necessary for pUL34 intracellular targeting and function, we (27) and others (28, 29) constructed chimeric proteins in which different parts of pUL34 were replaced by corresponding regions of either homologous viral proteins (human cytomegalovirus [HCMV] pUL50 and Epstein-Barr virus [EBV] BFRF1), other viral tail-anchored membrane proteins (HSV-1 pUS9 and pUL56), cellular proteins specific for the inner nuclear membrane (Emerin, Lap2β, and lamin B receptor), or ER-resident tail-anchored cellular proteins (Bcl and Vamp). These data indicated that the transmembrane domain can be functionally replaced by various alpha-helical membrane anchors with a length of at least 15 amino acids (aa) (27, 28). Deletion of the C-terminal 14 amino acids in HSV-1 pUL34, leaving only 15 hydrophobic amino acids at the C terminus, did not impair function (28), and extension of the luminal domain by at least 22 additional amino acids had no detectable effect on correct targeting and complementation, indicating that the C-terminal part serves solely to anchor the protein in the membrane. In PrV, the substituted region could be extended to 50 C-terminal amino acids without functional loss. However, when 100 C-terminal amino acids were replaced by Lap2β sequences, the chimeric protein was nonfunctional in complementation of a UL34-deleted PrV mutant (PrV-ΔUL34), although it still recruited pUL31 to the nuclear membrane into speckles indicative of vesicle formation (5, 27). These results localized a region which is required for function of pUL34 beyond pUL31 interaction to a stretch of 50 amino acids between aa 161 and 211 of the PrV protein.

The interaction domain of pUL34 homologs with pUL31 has been mapped in several herpesviruses, and important amino acids have been identified in HSV-1 (26, 29, 30), murine cytomegalovirus (MCMV) (31, 32), and HCMV (33). In PrV, yeast two-hybrid studies showed that the N-terminal 162 amino acids of pUL34 are sufficient for interaction with pUL31 (34), while in HSV-1 the interaction domain was further pinpointed to aa 137 to 181 (corresponding to aa 123 to 167 in PrV pUL34). This region mediated interaction with pUL31, necessary for colocalization of pUL31 and pUL34 and essential for function (35). However, sequences in the N-terminal part of HSV-1 pUL34 preceding the proposed interaction domain also mediated binding to pUL31 (29). A pUL34 mutant lacking amino acids 91 to 275 still interacted with pUL31 (29), whereas charged cluster mutants located outside the proposed interaction domain failed to bind pUL31 (26). In addition, data from MCMV and HCMV homologs also suggest that (nearly) the complete pUL34 N terminus is required for efficient binding to pUL31 homologs (aa 1 to 181 in HCMV) (33). Moreover, in HCMV, MCMV, and HSV-1, two amino acids within the conserved N-terminal part were found to be especially important for complex formation, represented by glutamate at position 53 and tyrosine at position 54 in PrV pUL34 (Fig. 1) (26, 31–33), indicating that in all herpesvirus pUL34 homologs, the complete N-terminal part may be involved in pUL31 binding.

To further identify important functional domains and essential amino acids in PrV pUL34, we generated additional chimeric constructs and replaced sequentially either 60, 70, 80, 85, or 90 amino acids of the PrV pUL34 C terminus by Lap2β sequences as described previously (27). We show that pUL34 lacking the C-terminal 85 amino acids and fused to the Lap2β C terminus (pUL34-LapCT85) efficiently complemented the defect of PrV-ΔUL34, while pUL34-LapCT90 failed to, thus indicating that functionally important sequences were deleted. Recruitment of pUL31 was unimpaired, as described previously for pUL34-LapCT100 (27), but nucleocapsids were unable to leave the nucleus. Within this region, amino acid positions 173 to 175 are represented by RQR. The three-residue motif RXR has been shown to be present in the cytoplasmic domains of glycoproteins B in HSV-1 and HCMV as well as in the cellular lamin B receptor. Fused to a reporter protein (CD8), this signal was sufficient for INM localization (36, 37). To study the role of this motif in pUL34 localization, it was altered to RQG by site-directed mutagenesis. Moreover, to study requirements for pUL31 interaction in the N-terminal part of pUL34, N-terminal truncations were generated, and conserved amino acids were mutated and tested for pUL31 recruitment as well as functional complementation.

**MATERIALS AND METHODS**

**Cells and viruses.** Rabbit kidney (RK13) or porcine kidney (PSEK) cells were cultivated in Dulbecco’s modified Eagle’s minimum essential medium supplemented with 10% or 5% fetal calf serum, respectively. Wild-type PrV Kaplan (PrV-Ka) (38) was grown on RK13 or PSEK cells, whereas PrV-ΔUL34 was propagated on RK13-UL34 cells as described previously (23, 39).

**Generation of mutant pUL34.** Chimeric pUL34-LapCT proteins were generated as described recently (27) using primers listed in Table 1 and PrV-Ka DNA and plasmid pEGFP-Lap2β (40) as the templates. After fusion, PCR products were cloned into EcoRV-cleaved pcDNA3 (Invitrogen). Correct amplification and cloning were controlled by restriction enzyme cleavage and sequencing.

N-terminal truncations were introduced by PCR using the primers listed in Table 1 on pcDNA-UL34 as the template (39). PCR products were furnished with artificially introduced EcoRI and XhoI cleavage sites for cloning into pcDNA3. Correct amplification and cloning were verified by sequencing.

Point mutations were created by site-directed mutagenesis using an Agilent Technologies QuickChange II XL site-directed mutagenesis kit. pcDNA-UL34 served as the template for primers listed in Table 1. All plasmids were sequenced for correct amplification and cloning (data not shown).

**Generation of stable cell lines.** To generate cell lines which stably express mutant pUL34, RK13 cells were transfected by calcium phosphate precipitation (41). The transfected cells were selected in medium supplemented with 0.5 mg/ml G418 (Invitrogen). Resistant cell clones were picked, and pUL34 expression was examined by indirect immunofluorescence and Western blot analysis with a pUL34-specific polyclonal rabbit serum (39).

**Laser scanning confocal microscopy.** To determine the localization of the mutated pUL34, RK13 cells were transfected with the different pcDNA-UL34 constructs by calcium phosphate precipitation. For colo-
calization studies of pUL34 and pUL31, RK13 cells were cotransfected with pcDNA-UL31 (34). At 48 h after transfection, cells were fixed with 3% paraformaldehyde for 20 min, washed with 50 mM NH₄Cl, and permeabilized with 0.5% Triton X-100. Immunostaining for pUL34 and/or pUL31 was performed with polyclonal rabbit anti-pUL31 (34) and polyclonal rabbit or mouse anti-pUL34 serum (5, 39), which was diluted 1:500 in phosphate-buffered saline (PBS), and incubated for 1 h at room temperature. Cell culture supernatant of hybridoma cell lines expressing anti-Golgi complex monoclonal antibody P3-b4-3 was used at a dilution of 1:10 (42, 43). Bound antibody was detected by Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 555 goat anti-mouse IgG (Invitrogen), which were diluted 1:1,000 in PBS. Fluorescence images were obtained using a confocal laser scanning microscope (LSM510 [Zeiss, Jena, Germany] and Leica SP5 [Wetzlar, Germany]).

**RESULTS**

**Localization of functionally important regions in the C-terminal part of pUL34.** Recently, we showed that deletion of 50 C-terminal amino acids of PrV pUL34 including the predicted transmembrane region and replacement with a corresponding region of the cellular Lap2/H9252 resulted in a functional chimeric protein (27). However, extension of the deleted region to 100 C-terminal amino acids retaining only amino acids 1 to 161 of pUL34 fused to the C terminus of Lap2/H9252 with the transmembrane region resulted in lack of complementation of the UL34 deletion mutant despite continuing pUL31 binding (27). To further delineate the functionally important sequences between amino acids 161 and 211, truncation mutants of pUL34 were generated lacking 60, 70, 80, or...
TABLE 1 Primers used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5′ to 3″)</th>
<th>Location in PrV-Ka (nt)</th>
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<td>31981–31998</td>
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</table>

a Restriction sites introduced for convenient cloning are underlined, artificial start codons are shown in bold, and mismatches for site-directed mutagenesis underlined and in italics.
b Positions correspond to GenBank accession number BK001744. nt, nucleotide.

90 C-terminal amino acids (Fig. 1) and fused to the same Lap2β sequences used for pUL34-LapCT50 (amino acids 374 to 452 of Lap2β). The corresponding chimeras pUL34-LapCT60, pUL34-LapCT70, pUL34-LapCT80, and pUL34-LapCT90 showed nuclear rim staining after transfection of the expression plasmids into RK13 cells and relocated coexpressed pUL31 to the nuclear boundary, indicating functional interaction as shown for pUL34-LapCT50 and pUL34-LapCT100 (Fig. 2) (27).

Cell lines stably expressing the different pUL34-Lap2β chimeric proteins were infected with either PrV-Ka or PrV-ΔUL34 at an MOI of 5 and harvested 30 h postinfection. As shown in Fig. 3A, pUL34-LapCT60, pUL34-LapCT70, and pUL34-LapCT80 complemented the defect of PrV-ΔUL34 to titers only approximately 5- to 10-fold lower than those of PrV-Ka on the same cells. However, PrV-ΔUL34 titers derivable from RK13-UL34-LapCT90 were similar to those from nontransfected RK13 cells (Fig. 3A). Titers of PrV-Ka from the different pUL34-Lap2β-expressing cell lines were above 10⁶ PFU/ml and similar to titers from nontransfected RK13 cells, indicating that none of the constructs exerted a significant dominant negative effect on virus replication.

Since these data indicated presence of a region important for pUL34 function independent of the pUL31 interaction between pUL34 amino acids 171 (pUL34-LapCT90) and 181 (pUL34-LapCT85), an additional chimeric protein was constructed lacking amino acids 176 to 262 of pUL34, designated pUL34-LapCT85. This protein localized to the nuclear rim, interacted with pUL34 (Fig. 2), and complemented PrV-ΔUL34 to a significant extent (Fig. 3A), delineating the functionally important domain between amino acids 171 and 176 of pUL34.

Besides its role in nuclear egress, pUL34 may also influence direct viral cell-to-cell spread (27, 45). Therefore, we tested the efficiency of plaque formation of PrV-ΔUL34 on the different pUL34-Lap2β-expressing cell lines. Wild-type-like plaque diameters were found on cells expressing pUL34-LapCT80, while plaque size was slightly reduced on RK13-UL34-LapCT60, RK13-UL34-LapCT70, and RK13-UL34-LapCT85 cells. RK13-UL34-LapCT90 failed to efficiently complement cell-to-cell spread of PrV-ΔUL34, and plaque diameters were only marginally larger than on RK13 cells (Fig. 3B), thereby mirroring the results of one-step growth analyses (Fig. 3A).

These data indicate that pUL34 sequences preceding amino acid 176 (present in pUL34-LapCT85) (Fig. 1) are required for pUL34 function during viral replication. Since amino acids 1 to 162 were shown to be sufficient for pUL31 interaction (27, 34), this result points to a function beyond recruitment of pUL31 to the INM.

The RQR motif does not specifically target pUL34 to the INM. The pUL34 amino acid sequences present in functional pUL34-LapCT85 but lacking in noncomplementing pUL34-LapCT90 comprise the tripeptide RQR at amino acids 173 to 175 (Fig. 1). This RQR motif has been shown to be sufficient for targeting HCMV and HSV-1 glycoprotein B and lamin B receptor as well as a CD8 reporter protein to the INM (36, 37). To study the role of this sequence on PrV pUL34 localization, RQR was altered

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to RQG by site-directed mutagenesis, and cell lines were isolated which stably expressed pUL34RQG. Indirect immunofluorescence analyses with the rabbit anti-pUL34 serum (39) revealed a perinuclear pattern characteristic for Golgi localization in addition to nuclear rim staining (Fig. 4A). Double fluorescence staining using a Golgi complex-specific monoclonal antibody (42, 43) verified colocalization in these punctate structures in the cytosol but not at the nuclear rim, indicating that pUL34RQG, but not wild-type pUL34, was present in the Golgi apparatus (Fig. 4A). However, after cotransfection with pcDNA-UL31, nuclear speckles were still observed in which both proteins colocalized, demonstrating that, despite its prominent Golgi localization, a fraction of pUL34RQG also reached the INM to recruit nucleoplasmic pUL31 (Fig. 4B). These data suggest that the RQR motif is not essential for pUL34 targeting to the nuclear membrane but might act as a Golgi retrieval signal to relocate Golgi compartment-localized molecules back into the ER and from there into the nuclear membrane.

Despite its enhanced Golgi localization, pUL34RQG partially complemented the defect of PrV-ΔUL34 in one-step growth kinetics, with approximately 25-fold reduced titers (Fig. 3A) and reduced plaque diameters (ca. 60% compared to PrV-Ka) (Fig. 3B), correlating with the continuing, but reduced, presence of pUL34RQG at and recruitment of pUL31 to the INM (27). Therefore, to further delineate the pUL31 interaction domain, N-terminal truncations of 4, 9, 19, 29, or 39 N-terminal amino acids were created (designated pUL34-N5, -N10, -N20, -N30, and -N40, respectively). For all constructs, an artificial start codon was added by the primers used for amplification (Table 1; Fig. 1). Transfection of the pcDNA3 expression constructs revealed that all proteins localized to the nuclear rim as did native pUL34 (data not shown). However, after cotransfection with pcDNA-UL31, only pUL34-N5 showed the typical punctate nuclear rim pattern, with costaining of both anti-pUL34 and anti-pUL31 sera indicating interaction and vesicle formation (5) (Fig. 5). In contrast, all other N-terminally truncated pUL34 proteins were unable to recruit pUL31 to the INM (exemplarily shown for pUL34-N10) (Fig. 5) since the protein was found diffusely distributed in the nucleoplasm as in cells expressing only pUL31 (34). These results demonstrate that deletion of as few as nine N-terminal pUL34 amino acids abolishes pUL31 recruitment to the INM.

To test for complementation, cell lines stably expressing pUL34-N5, pUL34-N10, pUL34-N20, and pUL34-N30 were generated and tested after infection with either PrV-Ka or PrV-ΔUL34. Only RK13-UL34-N5 complemented PrV-ΔUL34 to wild-type-like titers and plaque diameters while all the others failed, with values similar to those found on nontransfected RK13
Conserved amino acids in the N-terminal domain of pUL34.

Since truncations in the N terminus, besides deletion of the first 4 amino acids, resulted in nonfunctional proteins, we aimed to identify functionally important amino acids in the conserved N-terminal part of PrV pUL34. Similar studies have been performed for pUL34 homologs of HSV-1 (charge cluster mutants) (26), MCMV (linker insertions) (31), and HCMV (point mutations) (33). We first focused on the conserved cysteine residues. These had not been addressed in other studies but might play an important role for pUL34 structure. To this end, cysteine residues at positions 36, 67, and 141 of PrV pUL34 (Fig. 1) were changed to alanines by site-directed mutagenesis. While cysteine at position 36 is strictly conserved also positionally within pUL34 homologs in all herpesvirus subfamilies, the cysteines at positions 67 and 141 are found at similar, though not identical, positions (Fig. 1) (33).

Interaction between the mutated pUL34 constructs and pUL31 was tested after cotransfection of the corresponding expression plasmids with pcDNA-UL31 into RK13 cells, followed by coimmunostaining at 2 days posttransfection. As shown in Fig. 5, all cysteine mutants still localized to the nuclear rim and recruited pUL31 into nuclear speckles, indicating continuing interaction.

The ability to functionally complement PrV-ΔUL34 was again
assayed on cell lines stably expressing the mutated pUL34 proteins (Fig. 6A). While RK13-UL34-C36A complemented PrV-ΔUL34 to wild-type-like titers, infectious progeny derived from RK13-UL34-C67A and RK13-UL34-C141A reached lower titers than PrV-Ka on the corresponding cell lines, but titers were still well above those of the nontransgenic control cells. However, plaque diameters formed by PrV-ΔUL34 after infection of RK13-UL34-C67A and RK13-UL34-C141A were similar to those found after infection of nontransfected RK13 cells (Fig. 6B), indicating that these residues are important for a step beyond nuclear egress.

Lastly, we wanted to validate our assay system by mutating amino acids which had already been shown to be important for pUL34 function in other herpesviruses. In the HCMV and HSV-1 pUL34 homologs, amino acids corresponding to glutamate 53 and tyrosine 54 in PrV pUL34 have been shown to be important for pUL31 interaction and protein function (26, 33). To test for their role in PrV, both amino acids were simultaneously altered to alanines, giving rise to pUL34-E53AY54A (Fig. 1). In transient expression assays, pUL34-E53AY54A localized to the nuclear rim but was unable to relocate pUL31 (Fig. 5), indicating that these two amino acids play an important role for pUL31 interaction also in PrV pUL34. On transgenic cells stably expressing pUL34-E53AY54A, titers and plaque diameters of PrV-ΔUL34 were similar to those from nontransfected RK13 cells (Fig. 6), demonstrating that these two conserved amino acids of pUL34 play an important role in herpesvirus replication, presumably by direct involvement in interaction with pUL31.

**DISCUSSION**

Conserved herpesvirus proteins designated pUL34 and pUL31 in PrV and HSV-1 physically interact to form the NEC, which is required for efficient nuclear egress, by mediating primary envelope formation, fusion, and fission (reviewed in references 1 to 3). While the N-terminal part is well conserved between the pUL34 homologs throughout the herpesvirus subfamilies, the C-terminal half including the membrane anchor region of these type II membrane proteins is more divergent (31, 33, 45). The N-terminal domain is required for interaction with and recruitment of pUL31 to the INM as a prerequisite for primary envelopment during nuclear egress (27, 29, 31, 33, 34).

In the absence of a crystal structure for the NEC, several mutagenesis and/or deletion analyses have been performed to identify regions in either protein required for complex formation and function. Although the results improved our understanding of the NEC, the picture is far from being complete. Thus, in this study we extend previous analyses on PrV pUL34 by creating truncated and site-specifically mutated versions of the protein and testing them for pUL31 interaction and for functional complementation of a pUL34 deletion mutant.

Previously, it has been shown that replacement of the C-terminal transmembrane domain of pUL34 by corresponding regions of other cellular INM proteins or of other related or unrelated viral polypeptides was functionally tolerated, whereas deletion of the transmembrane domain resulted in a nonfunctional protein.
Thus, the presence of a membrane anchor in pUL34 but not its viral origin is necessary for function since it can be replaced by a variety of alpha-helical sequences of at least 15 amino acids (27–29). We showed recently that even the exchange of 50 C-terminal residues of PrV pUL34 with Lap2β/H9252 sequences resulted in a functional protein which efficiently mediated nuclear egress and infectious virus formation (27). However, extension of the substituted region to 100 amino acids, retaining only amino acids 1 to 161 of the 262 amino acids of PrV pUL34, resulted in a nonfunctional protein although it was still able to recruit pUL31. To further delineate this functionally important region, we sequentially deleted sequences from the C terminus of PrV pUL34 and fused the truncated proteins with the membrane anchor domain of cellular Lap2β (27). We show here that extension of the deletion up to amino acid 177 of PrV pUL34 did not impair pUL31 interaction and NEC function. In contrast, deletion of an additional 5 amino acids resulted in a nonfunctional protein which was, however, still able to recruit pUL31. Thus, most of the less-conserved C-terminal region of pUL34 is actually not required for protein function. This suggests that this part of the protein could simply act as a “stalk” exposing the N-terminal pUL31 interaction domain for recruitment of the complex partner. Apparently, heterologous sequences are able to substitute for authentic pUL34 sequences to provide this feature.

The 5 amino acids which differ between functional pUL34-LapCT85 and nonfunctional pUL34-LapCT90 contain the tripeptide RQR. This motif bears striking resemblance to the RXR sequence shown to locate glycoproteins B of HSV-1 and HCMV as well as nonviral reporter CD8 to the INM (36, 37). To test the influence of this arginine-rich motif on pUL34 localization, we altered its sequence to RQG. After transient as well as stable expression, a fraction of the mutated protein still located to the nuclear rim and interacted with pUL31. However, a major fraction of the mutated pUL34 was found to colocalize with a Golgi complex-specific protein in perinuclear structures, indicative of localization to the Golgi apparatus. Thus, after mutation of the RQR sequence, a fraction of pUL34 does not reach the nucleus but may leak into the cellular secretory pathway to the Golgi compartment. This indicates that the RQR motif may function as a Golgi retrieval signal relocating pUL34 from the secretory pathway. Similar results were obtained for the cellular SUN2 protein in which a cluster of arginines mediates Golgi retrieval (22).

RXR motifs have been described on ER-resident proteins. They were first identified as ER retention signals for type II membrane proteins (46). Protein kinase A and C phosphorylation sites flank the RXR sequence, suggesting phosphorylation-controlled ER export of RXR-containing proteins as a quality control mechanism (47). Frequently, a dileucine endocytic sorting motif is located in the vicinity (48). In PrV pUL34 the sequence comprising the RQR motif is predicted as a phosphorylation site (http://elm.eu.org/) with a preceding dileucine (both in boldface) sequence (SQTQRDLDDRAMEQRQSRSP) (Fig. 1). Further experimentation is needed to test whether pUL34 is phosphorylated at this position and to elucidate the influence of phosphorylation as well as the importance of the dileucine motif on pUL34 localization and function. Interestingly, a linker insertion within the dileucine sequence in the MCMV homolog was detrimental to protein function (31).

Whereas the C-terminal, less-conserved part of pUL34 shows little sensitivity toward alterations, the well-conserved N terminus is highly sensitive. Only the amino-terminal four amino acids could be deleted without impairing pUL34 function and interaction with pUL31. Extending the truncation to nine amino-terminal amino acids resulted in a nonfunctional protein. In this instance loss of pUL34 function correlated with loss of pUL31 interaction, which again emphasizes the importance of complex

FIG 5 Interaction of N-terminally truncated and site-specifically mutated pUL34 with pUL31. To test whether the N-terminally truncated pUL34 and mutated constructs were able to interact with pUL31, plasmids expressing the indicated proteins were cotransfected with pcDNA-UL31. Two days later cells were fixed and stained with pUL34-specific murine serum and pUL31-specific rabbit serum. Bound antibodies were visualized after incubation with the corresponding secondary antibodies under the confocal laser scanning microscope (LSM510; Leica SP5). Scale bar, 10 μm.
formation for nuclear egress. These data support results of other investigators (29, 31, 33), demonstrating that interaction of pUL34 with pUL31 requires more than the interaction domain mapped by Liang and Baines (35). Most likely, the complete well-conserved amino terminal part of pUL34 is necessary for complex formation.

To test for the importance of specific amino acids in this region, we mutated three conserved cysteine residues to alanines. Whereas Cys36 of PrV pUL34 is positionally identical in pUL34 homologs of other herpesviruses, Cys67 and Cys141 are slightly offset in pUL34 homologs of HCMV, MCMV, and EBV (Fig. 1) (33). All of these mutants still interacted with pUL31, resulting in nuclear rim localization. However, different phenotypes in functional complementation were observed. pUL34-C36A efficiently complemented the replication and cell-to-cell spread defect of PrV-ΔUL34. In contrast, whereas titers found after infection of RK13-UL34-C67A and RK13-UL34-C141A were only slightly reduced, direct cell-to-cell spread was nearly abrogated, and plaque diameters were similar to those found after infection of nontransgenic RK13 cells. This unexpected result points to an important function of PrV pUL34 Cys 67 and Cys 141 in a step beyond nuclear egress.

Alteration of the highly conserved dipeptide Glu53/Tyr54 of PrV pUL34 to alanine abolished pUL31 interaction and function of pUL34. These amino acids have already been shown to be required for function of pUL34 homologs in HSV-1 (26) and HCMV (33). Moreover, a linker insertion between them in MCMV pUL34 resulted in a dominant negative protein (32). A

FIG 6 Complementation of PrV-ΔUL34 by N-terminally truncated and site-specifically mutated pUL34. (A) Cell lines stably expressing N-terminally truncated or site-specifically mutated pUL34 were infected at an MOI of 3 with PrV-Ka or PrV-ΔUL34 and harvested after 24 h. Infectious progeny was titrated on RK13-UL34 cells. Given are mean values of three independent assays with the corresponding standard deviations. (B) To test for complementation of cell-to-cell spread, cell lines were infected with PrV-Ka or PrV-ΔUL34 under plaque assay conditions. Plaque diameters of 30 plaques each were measured microscopically in three independent assays. Values of PrV-Ka on the corresponding cell line were set at 100%, and diameters of PrV-ΔUL34 plaques were calculated accordingly.
cell line stably expressing HSV-1 pUL34Y68A, which is equivalent to PrV Tyr54, replicated a corresponding pUL34 deletion mutant to approximately 60-fold lower titers than the parental protein and complemented direct viral cell-to-cell spread only very inefficiently. In pUL34Y68A-expressing cells the nuclear envelope showed blebs, presumably as the result of disconnecting nuclear envelope from the underlying lamina, pointing to an exaggerated lamina disruption. In addition, trafficking of gE was inhibited in this cell line after infection with the UL34 deletion mutant, but interaction of pUL34Y68A with pUL31 was not tested (45). Collectively, these data identify a conserved dipeptide motif in herpesvirus pUL34 homologs which is highly important for function of the NEC.

In summary, our data show that most of the C-terminal portion of pUL34, which shows little to no conservation between the herpesvirus pUL34 homologs, can be deleted and replaced by heterologous sequences if a transmembrane anchor is provided. Thus, this portion of the protein may primarily, if not solely, act to anchor pUL34, and, consequently, the NEC, in the INM and provide a stalk for presenting the N-terminal domain for interaction with pUL31. In contrast, the well-conserved N-terminal part of pUL34 is essential for NEC function during virus replication and nuclear egress although conserved cysteine residues, in contrast to a similarly conserved EF motif, are not required. Amino acids 5 to 161 of PrV pUL34 are sufficient for pUL31 binding, while the precise termini of this interaction domain remain to be identified, preferably by elucidation of the crystal structure of the NEC. However, sequences up to amino acid 176 also play a fundamental role for viral replication independent of pUL31 binding. In this region, trafficking signals such as the presumptive Golgi retrieval motif RQR could modulate the intracellular location of pUL34 and, therefore, impact its function in nuclear and extranuclear events of herpesvirus replication.

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