Effect of the pseudorabies virus US3 protein on nuclear membrane localization of the UL34 protein and virus egress from the nucleus

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The alphaherpesvirus UL34 protein is necessary for the primary envelopment of intranuclear capsids at the inner leaflet of the nuclear membrane. In herpes simplex virus type 1, the UL34 protein is exclusively phosphorylated by the protein kinase encoded by the non-essential US3 gene. To investigate the effect of the pseudorabies virus (PrV) US3 product on the intracellular localization of the UL34 protein and on virus morphogenesis, PrV US3 deletion mutants were isolated and characterized. Immunofluorescence analyses demonstrated that in the absence of the US3 protein, the localization of the UL34 polypeptide to the nuclear membrane was not as pronounced as that seen with US3, although immunoelectron microscopy indicated the presence of the UL34 protein in both leaflets of the nuclear membrane. Ultrastructurally, an accumulation of enveloped virions in the perinuclear space in large invaginations of the inner nuclear membrane was observed, which were shown by immunoelectron microscopy to contain the UL34 protein, but not glycoproteins gB or gC. Thus, the US3 protein appears to be involved in the de-envelopment of perinuclear virions by fusion with the outer leaflet of the nuclear membrane. Surprisingly, no difference in the phosphorylation of the PrV UL34 protein was observed in the presence or absence of the US3 kinase. Therefore, the observed effects of the PrV US3 protein on the intracellular localization of the UL34 protein and on virus morphogenesis are probably not due to the phosphorylation of the UL34 protein by the US3 kinase.

Introduction

Although still not unanimously accepted, there is increasing evidence for a two-step envelopment process during the maturation of herpes virions. In a first budding event at the inner nuclear membrane, intranuclear capsids acquire a primary envelope which is lost by fusion with the outer leaflet of the nuclear membrane. Secondary, final envelopment then occurs by the budding of intracytoplasmic capsids into vesicles in the trans-Golgi area after the acquisition of tegument proteins (Granzow et al., 1997, 2001). However, the molecular details of this complicated virion morphogenesis are still largely unclear.

So far, the only viral protein that has been unequivocally assigned a function in primary envelopment is the UL34 gene product of herpes simplex virus type 1 (HSV-1) and pseudorabies virus (PrV) (Klupp et al., 2000; Roller et al., 2000). In the absence of the UL34 protein, budding at the inner leaflet of the nuclear membrane is essentially blocked and capsids are only detectable dispersed in the nucleus. Therefore, the UL34 protein appears to play a crucial role in the primary envelopment process.

The UL34 polypeptide is a phosphoprotein, which has been demonstrated in HSV-1 to be the substrate for the US3 protein kinase (Purves et al., 1991). Moreover, in HSV-1-infected cells, US3 seems to be the only kinase capable of phosphorylating the UL34 protein (Purves et al., 1992). This striking specificity may suggest that phosphorylation of the UL34 protein by the US3 kinase is important for its biological role, possibly influencing intracellular localization and/or function. On the other hand, it seems paradox that, whereas deletion of UL34 results in severe growth defects in PrV and HSV-1 (Klupp et al., 2000; Roller et al., 2000), there is only little impairment of virus replication in the absence of US3 in cultured cells (Purves et al., 1987; Kimman et al., 1994). However, deletion of PrV US3 resulted in a significantly reduced virulence for the natural host.
of PrV, swine (Kimman et al., 1994). As observed by electron microscopy, enveloped virions appeared to accumulate in the perinuclear space in the absence of US3 and it was suggested that proteins that are phosphorylated by the US3 kinase are involved in fusion of perinuclear virions with the outer nuclear membrane (Wagenaar et al., 1995). There have also been reports of other mutations that result in the accumulation of enveloped virions in the perinuclear space, e.g. by the deletion of PrV gB (Peeters et al., 1992) or HSV-1 gK (Foster & Kousoulas, 1999). However, a similar phenotype could not be reproduced using either gB- (Granzow et al., 2001) or gK-deleted PrV (Dietz et al., 2000; Klupp et al., 1998), which, even in the absence of the respective glycoprotein, still exhibited normal virion maturation. Thus, we wanted to reanalyse the role of US3 in virion morphogenesis.

The PrV US3 gene is located immediately upstream of the gG gene; both the US3 gene and the gG gene are transcribed into 3′-coterminal mRNAs (Zhang & Leader, 1990). Two transcriptional start sites were mapped for the US3 mRNA: a minor transcript starting immediately upstream of the open reading frame (ORF) and a major transcript starting 64 nt upstream of an internal methionine codon. Consequently, two proteins with molecular masses of 41 and 53 kDa have been identified in infected cells (van Zijl et al., 1990). Whereas the majority of the US3 gene product(s) appeared to be localized in infected cells, one protein with a molecular mass of 38 kDa has also been detected in purified virions (Zhang et al., 1990).

To reassess the role of US3 in the virus life cycle and to specifically analyse its influence on the intracellular localization and function of the UL34 protein, we isolated US3 deletion mutants that were isogenic to the various mutant viruses which we had prepared over the last 15 years and analysed it in our standardized cell culture system.

**Methods**

- **Viruses and cells.** All virus mutants used in this study were based on the laboratory strain PrV-Ka (Kaplan & Vatter, 1959). Viruses were grown on rabbit kidney cells (RK13) in Eagle’s minimum essential medium supplemented with 10% foetal calf serum. For isolation of US3-negative mutants, genomic BamHI fragment 10 was cleaved with either PstI, thereby deleting 114 bp of the US3 protein-coding sequences, or EcoRV/XhoI, resulting in a deletion of 778 bp that included both possible start codons (Fig. 1). Concomitantly, a green fluorescent protein (GFP) marker cassette was inserted (Jöns & Mettenleiter, 1997). The resulting
Fig. 2. Western blot of infected RK13 cells and purified virions. Purified virions of PrV-Ka (lanes 1) or PrV-ΔUS3 (lanes 2), as well as lysates of PrV-Ka-infected cells (lanes 3) and PrV-ΔUS3-infected cells (lanes 4) were separated by SDS–PAGE, blotted and incubated with antisera specific for US3 (A), UL34 (B), UL49 (C) or gB (D). The positions of the molecular mass marker for (A–C) are indicated on the left, whereas the positions of the molecular mass marker for (D) are indicated on the right.

Fig. 3. Immunofluorescence of infected RK13 cells. Cells were infected with wild-type PrV-Ka or the UL34- (ΔUL34) or US3-deletion (ΔUS3) mutants under plaque assay conditions (Klupp et al., 2000). Cells were fixed 1 day after infection with ethanol. Immunofluorescence was performed using polyclonal rabbit antisera specific for UL34 (α-UL34) or US3 (α-US3). Assay results were recorded by laser scanning microscopy.

Plasmid was cotransfected using calcium–phosphate coprecipitation (Graham & van der Eb, 1973) with wild-type PrV DNA into RK13 cells. Green fluorescent plaques were picked and purified until homogeneity. The correct deletion of US3-specific sequences and the insertion of the GFP marker sequence were verified by Southern blotting (data not shown). In all analyses, both mutants behaved identically. Therefore, representative results using either of the two mutant viruses, designated PrV-ΔUS3, are shown. PrV-ΔUL34B, in which the UL34 gene was substituted by the gB gene of bovine herpesvirus type 1 in a gB-negative PrV background, has been described previously (Klupp et al., 2000).
Fig. 4. Electron microscopy of RK13 cells infected with PrV-Ka. Cells were infected at an m.o.i. of 0-5 and analysed 14 h after infection, as described previously (Klupp et al., 2000). Cells show all stages of virus morphogenesis (A), including budding at the inner nuclear membrane (B), as well as intracytoplasmic and extracellular virions (C). Immunogold labelling using an anti-UL34 serum demonstrates the presence of UL34 in both leaflets of the perinuclear membrane (D), as well as in perinuclear primary enveloped virions (E). (A, C) Bars, 1 µm. (B, D and E) Bars, 150 nm.

GFP-expressing UL34-negative PrV mutant was also constructed for better comparison with the GFP-expressing US3 mutants (data not shown).

■ Preparation of monospecific US3 antiserum. To obtain an antiserum specific for the US3 protein, the predicted US3 ORF was amplified by PCR using platinum Pfx DNA polymerase (Life Technologies) and the primers US3FOR, 5′ CACAGAATTCTGAATTGCCGGCAATGC 3′ (nt 2921–2940, accession no. D00676), and US3REV, 5′ CACAGAATTCTAAGTGGCCGAGCGGACC 3′ (nt 3950–3931, accession no. D00676). Both primers contained an EcoRI site (italics) for convenient cloning. The resulting 1 kb fragment was cloned into the mammalian expression vector pcDNA3 (Invitrogen) and, for expression in Escherichia coli, in-frame into the bacterial expression vector pGEX-4T-2 (Amersham Pharmacia). A 63 kDa glutathione S-transferase (GST) fusion protein was purified after SDS–PAGE by electroelution and used for the immunization of a rabbit.

■ Metabolic labelling and immunoprecipitation. RK13 cells were infected at an m.o.i. of 5 with the respective viruses, in medium...
Pseudorabies virus US3 protein containing either $^{[35]S}$methionine/cysteine (ICN) or $^{[32]P}$orthophosphate (ICN). At 16 h after infection, cells were lysed and radioactively labelled proteins were precipitated, as described previously (Lukács et al., 1985).

- Immunofluorescence and electron microscopy. These techniques were performed as described previously (Granzow et al., 2001; Klupp et al., 2000).

Results and Discussion

Purified virions of wild-type PrV-Ka (Fig. 2, lanes 1) or PrV-ΔUS3 (Fig. 2, lanes 2), as well as RK13 cells infected with PrV-Ka (Fig. 2, lanes 3) or PrV-ΔUS3 (Fig. 2, lanes 4), were analysed by immunoblotting using the monospecific anti-US3 serum (Fig. 2A), an anti-UL34 serum (Fig. 2B; Klupp et al., 2000), an anti-UL49 serum (Fig. 2C; Brack et al., 1999) or an anti-gB monoclonal antibody (MAb) (Fig. 2D). The anti-US3 serum detected 41 and 53 kDa proteins in lysates of PrV-Ka-infected cells and a 41 kDa protein in purified wild-type virions, which parallels earlier studies (van Zijl et al., 1990; Zhang et al., 1990). No reactivity was detected in PrV-ΔUS3 virions or infected cells. For control, the UL34 protein was detected similarly in cells infected by either virus, but was absent from purified virions (Klupp et al., 2000). In contrast, the UL49 protein was present in all preparations, as was gB. These data demonstrate that our antiserum specifically recognizes the PrV US3 protein.
Fig. 6. Cells were infected with PrV-ΔUS3 at an m.o.i. of 0.5 and analysed by immunoelectron microscopy 14 h after infection, as described previously (Klupp et al., 2000). Virions in the perinuclear space (A, C, F), the trans-Golgi area (B, D, G) and the extracellular space (B, E, H) are shown labelled with anti-UL34 serum (A, B), anti-gB serum (C–E) or anti-gC MAb (F–H). Bars, 250 nm.
and that deletion of US3 has no effect on the intracellular steady-state level of UL34. Also, it did not lead to the incorporation of UL34 into mature extracellular virions.

To analyse whether deletion of US3 has an effect on the intracellular localization of UL34, RK13 cells were infected with wild-type PrV or UL34- or US3-deleted PrV mutants and analysed by confocal laser scan microscopy using monospecific sera directed against either the UL34 gene product or the US3 gene product. As shown in Fig. 3, in wild-type PrV-infected cells, UL34 was primarily detected in a perinuclear rim, whereas US3 showed diffuse staining that totally covered the infected cells. In the absence of UL34, US3 was still detected in the cytoplasm and nuclei of infected cells. In contrast, the absence of US3 altered the intracellular localization of UL34, resulting in diffuse staining with strongly reduced perinuclear accumulation. Thus, deletion of US3 from the viral genome influences the intracellular localization of UL34, whereas deletion of UL34 apparently did not affect the localization of US3. To assess whether the observed effect can be reproduced by transient expression, expression plasmids for UL34 and US3 were transfected into RK13 cells either singly or in combination. Unfortunately, the expression of US3 proved to be detrimental to the cells and we were unable to reproducibly differentiate specific effects from the drastically altered morphological appearance of dying cells (data not shown).

Previously, the absence of several proteins, including PrV gB (Peeters et al., 1992), HSV-1 gk (Foster & Kousoulas, 1999) and PrV US3 (Wagenaar et al., 1995), has been suggested to result in the accumulation of enveloped virions in the perinuclear space. In exhaustive studies, we were not able to see a similar defect in our gB- or gK-deleted PrV mutants (Dietz et al., 2000; Granzow et al., 2001; Klupp et al., 1998). Thus, up to now, the relevance of these previous findings is unclear. To ascertain whether the results on US3 could be reproduced, cells infected with wild-type PrV-Ka (Fig. 4) or PrV-US3 (Fig. 5) were analysed by electron microscopy. As shown in Fig. 5(A–C), we observed an accumulation of enveloped virions within large invaginations of the inner leaflet of the nuclear membrane; this was not detected after wild-type PrV-Ka infection (Fig. 4A–C). Therefore, the US3 deletion mutant is, so far, the only PrV mutant that reproducibly exhibited a defect at this stage of virion morphogenesis.

Despite its effect on intracellular localization of UL34 and on the accumulation in the perinuclear space of enveloped virions, the absence of US3 only moderately impairs virus replication, resulting in an approximately 10-fold decrease in final titres (Kimman et al., 1994; unpublished observations). Therefore, if the presence of UL34 in the nuclear membrane is required for primary envelopment and subsequent de-envelopment, it must localize to this subcellular compartment, even in the absence of US3. To analyse this in detail, we used our monospecific anti-UL34 serum in immunoelectron microscopy. UL34 was indeed detectable by immunogold labelling in the nuclear membrane in wild-type PrV-Ka (Fig. 4D) as well as in PrV-US3-infected cells (Fig. 5D). This parallels earlier findings of nuclear membrane localization of UL34 in transfected cells that only express the UL34 protein (Klupp et al., 2000). The UL34 protein could also be demonstrated in enveloped virus particles of PrV-Ka (Fig. 4E) and PrV-US3 (Fig. 6A) in the perinuclear space. As observed before, intracytoplasmic and extracellular enveloped virions were not labelled by the UL34 serum (Fig. 6B; Klupp et al., 2000). The particular phenotype seen in the US3 deletion mutants, i.e. accumulation of virions in the perinuclear space, provides the opportunity to analyse more easily the composition of these virions by immunoelectron microscopy. As shown in Fig. 6(C–E), a polyclonal antiserum against gB labelled intracytoplasmic (Fig. 6D) and extracellular virions (Fig. 6E), but failed to react with perinuclear virions (Fig. 6C). Similar results were obtained using a MAb against gC (Fig. 6F–H). Both, the antiserum and the MAb recognize mature and immature forms of the glycoproteins (data not shown). This is the first direct evidence to indicate that the glycoprotein
composition in the primary envelope is different from that of mature virions, which is most easily explained by the de-/re-
envolution model. In correlation with these results, we have
demonstrated previously that virion morphogenesis proceeds
undisturbed in the absence of gH or gB (Granzow et al., 2001).
In the absence of the US3 protein kinase, the intracellular
localization of the UL34 protein is altered, which correlates
with an accumulation of enveloped virions in the perinuclear
space. Thus, any effect of US3 on the UL34 protein does not
preclude the primary envelopment of intranuclear capsids.
However, the loss of the primary envelope by fusion with the
outer leaflet of the nuclear membrane appears to be less
efficient in the absence of US3. This may be due to a difference
in the phosphorylation of UL34, which, in HSV-1, has been
shown to be the substrate for the US3 kinase (Purves et al.,
2001). To confirm that the PrV UL34 protein is also a substrate
for the PrV US3 kinase, cells infected with PrV-Ka (Fig. 7,
lanes 1, 4 and 7), PrV-AU3 (Fig. 7, lanes 2, 5 and 8) or PrV-AU3 (Fig. 7,
lanes 3, 6 and 9) were metabolically labelled with either
[35S]methionine/cysteine (Fig. 7A) or [32P]orthophosphate
(Fig. 7B) and immunoprecipitated using antibodies against gB
(Fig. 7, lanes 1–3), UL34 (Fig. 7, lanes 4–6) or US3 (Fig. 7, lanes
7–9). Comparison of the resulting autoradiographs after
SDS–PAGE demonstrates phosphorylation of the uncleaved
precursor and large proteolytic cleavage product of gB,
irrespective of the absence or presence of US3. The smaller
cleavage product did not appear to be phosphorylated. In PrV-
Ka- and PrV-AU3-infected cells, the US3 protein is also
phosphorylated, whereas it is completely absent in PrV-AU3-
infected cells, as expected. Surprisingly, the UL34 protein was
also phosphorylated to a similar extent irrespective of the
absence or presence of US3 (Fig. 7, lanes 4 and 5). The identity
of the precipitated protein could be confirmed by its absence in
PrV-AU3-infected cells (Fig. 7, lane 6). We conclude that PrV
UL34 represents a phosphoprotein similar to its homologue in
HSV-1, but, unlike the situation in HSV-1, it is not or not
exclusively phosphorylated by the US3 kinase. Comparing the
amino acid sequences of the HSV-1 and the PrV UL34 proteins,
this lack of US3-mediated phosphorylation correlates with the
absence of the identified consensus sequence for US3
phosphorylation (Purves et al., 1986), which is localized in a
region of the HSV-1 UL34 protein that is not conserved in
alpha herpesvirus UL34 homologues, including PrV UL34.
In conclusion, the observed effects are not due to differential
phosphorylation of UL34 in the presence or absence of US3,
but may correlate with a reduction in the amount of UL34
present in the nuclear membrane, as indicated by our
immunofluorescence studies. In either case, virion morpho-
genesis proceeds quite efficiently, even in the absence of US3,
whereas the absence of UL34 drastically impairs virus
replication (Klupp et al., 2000).

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Pseudorabies virus US3 protein


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