Herpes simplex virus type 2 UL34 protein requires UL31 protein for its relocation to the internal nuclear membrane in transfected cells

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Herpes simplex virus type 2 UL34 protein is expressed late in infection and is required for envelopment of nucleocapsids at the nuclear membrane and possibly at the endoplasmic reticulum (ER). It is a type II membrane protein with a C-terminal anchor that localizes mainly to the nuclear membrane in infected cells. However, in single transient expression, UL34 protein localizes predominantly to the ER. Relocation of UL34 protein from the ER to the internal nuclear membrane and the nucleus was observed in the presence of UL31 protein, a phosphoprotein known to interact physically with UL34. It is suggested here that interaction with UL31 protein is important for the nuclear targeting of UL34 protein and also that the trans-membrane region of UL34 protein is responsible for its localization at the internal nuclear membrane. The results also suggest possible sites for the interaction.

Herpes simplex virus (HSV) is an enveloped DNA virus that encodes at least 74 different genes (Roizman & Sears, 1996; Dolan et al., 1998). Approximately 40 of them, named core genes, are conserved in all families of herpesviruses (McGeoch, 1992). The UL34 gene homologues of HSV types 1 and 2 (HSV-1 and HSV-2) can be found in alpha-, beta- and gammaherpesviruses, and all these products contain a hydrophobic stretch of 22–37 residues near the C terminus (Davison & Scott, 1986; Chee et al., 1990; Gompels et al., 1995; Nicholas, 1996; Baer et al., 1984; Roizman & Sears, 1996; Dolan et al., 1998). Egress of HSV-1 capsids from the nucleus has been found to require UL34 protein (Roller & Roizman, 1991). The UL34 gene product of HSV-2 is identified as proteins with molecular masses of 31 and 32.5 kDa, the latter being the phosphorylated form and more susceptible to proteinase digestion. This implies a functional or structural change resulting from phosphorylation (Shiba et al., 2000). UL34 protein is detected in mature HSV-1 virions and has been proposed to interact with cytoplasmic dynein during virus entry, which should target incoming capsids to the nuclear membrane (Ye et al., 2000).

The UL31 gene is also one of the core genes, and the gene product of HSV is a largely insoluble, evenly dispersed nuclear phosphoprotein that co-fractionates with the nuclear matrix and is required for optimal processing and packaging of viral DNA into pre-formed capsids (Chang & Roizman, 1993; Yamada et al., 1998). Its nuclear distribution changes little during the course of infection and localization in transient expression assays is also nuclear (Zhu et al., 1999). Recently, the UL31 protein of HSV-1 has been found to depend on physical interaction with UL34 protein for its stabilization in infected cells (Ye & Roizman, 2000).

When expressed transiently, the C-terminal anchor of UL34 protein localizes the protein mainly to the endoplasmic reticulum (ER) and the nuclear membrane, orientating the N terminus in the cytoplasm (Shiba et al., 2000), which raises the question of how the primarily ER-associated fraction of the expressed protein localizes predominantly to the nuclear membrane, as in infection. One suggestion is that another virus protein is required.

In this study, we examined the effects of co-expression of UL31 and UL34 proteins on their intracellular localization in transfection assays. We found that the co-expression of UL31 protein was necessary for UL34 protein to localize predominantly to the nucleus and especially to the internal nuclear membrane. Furthermore, various deletion mutants of both proteins were co-expressed to estimate the regions required for each translocation step, (i) UL34 protein nuclear targeting and (ii) internal nuclear membrane targeting. The results suggest that the N terminus of UL34 and the C-terminal two-thirds of UL31 are possible sites for the interaction.

Plasmids expressing wild-type (wt) and various deletion mutants of UL34 and UL31 proteins were singly transfected...
with the Lipofectamine reagent (Gibco BRL) onto monolayers of Vero cells grown on coverslips. After 24 h, the cells were washed in PBS and fixed in cold acetone with the exception of GFP-expressing cells, which were fixed in PBS containing 4% formaldehyde. Cells were then analysed by indirect immunofluorescence with a laser scanning confocal microscope. Construction of and expression from plasmids pcDNA3-UL34, -UL34MC256/276, -UL34MN32/38, UL31wt, UL31MN31A1/110, UL31MC2A215/305 and UL31MN31A1/110-GFP has been described previously (Zhu et al., 1999; Shiba et al., 2000). The monoclonal mouse anti-lamin B1 antibody was obtained from ZYMED.

pcDNA3-UL34, pcDNA3-UL34MC256/276 or pcDNA3-UL34MN32/38 DNA was transfected onto Vero cells and detected after 24 h. UL34 protein showed a predominant ER pattern with nuclear membrane localization (Fig. 1b, field-view image in g). UL34MC256/276 showed dispersed localization throughout the cell but did not localize specifically to the nuclear membrane or the ER (Fig. 1c). This was thought to be due to the loss of 21 amino acids in the hydrophobic region of the C terminus. UL34MN32/38, in contrast, showed similar staining to that of the wild-type protein (Fig. 1d).

The UL31 ORF is located between nucleotide positions 66932 and 67849 of the HSV-2 genome. pFLAG-UL31, which expresses a FLAG-tagged HSV-2 UL31 protein, was constructed by using primers UL31F (5′ TACAAAGCTTATGATGACATGCCCAGCCCG 3′; HindIII site in italics and initiation codon in bold) and UL31R (5′ TACAGGATCCCGGCGGAGGAACTCGT 3′; BamHI site in italics). pFLAG-UL31 was transfected to observe the localization of UL31 protein and showed localization in the nucleus and nucleolus but not the nuclear membrane (Fig. 1e). Singly expressed UL31MN31A1/110-GFP showed diffuse intracellular localization and formed clusters in some places (Fig. 1f).

In order to examine the effect of co-expressing UL34wt and UL31wt proteins, pcDNA3-UL34 and UL31 were transfected and detected after 24 h. In these co-transfected cells, the localization of UL34 protein was greatly altered: UL34 protein was predominantly nuclear and localized at what seemed to be the internal nuclear membrane (Fig. 1h).

To determine the localization of UL31wt protein, pcDNA3-UL34 and pFLAG-UL31 were co-transfected and double stained (Fig. 2a–c). As expected, the proteins showed co-localization in the nucleus and especially the nuclear membrane. Furthermore, double staining of UL34 protein with lamin B1, a component of the internal nuclear membrane, showed clear co-localization (Fig. 2d–f). Thus, UL34 protein was found to be localized predominantly in the internal nuclear membrane when co-expressed with UL31. In the same fashion, UL34 mutant proteins were co-expressed with UL31wt protein (Fig. 2g–i). Interestingly, in co-expressing cells, UL34MC256/276 was located in the nucleus and nucleolus in co-localization with UL31 protein but excluded from the nuclear membrane (Fig. 2g–i). On the other hand, UL34MN32/38 was predominantly cytoplasmic and localized at the ER, but localization of UL31 protein was identical to its singly expressed state (Fig. 2j–l), suggesting that UL31 did not influence the N-terminally truncated UL34 protein.

Next, UL31MN31A1/110-GFP was co-expressed with UL34 protein and double stained. We found that N-terminally truncated UL31 and UL34wt proteins co-localized strongly at a perinuclear cytoplasmic area (Fig. 2m–o). This suggests that the N-terminal domain of UL31 was not important for its interaction with UL34, but was important for the nuclear targeting of the two proteins. These co-expression experiments and the intracellular localization patterns observed for each of the UL31 and UL34 wt or deletion-mutant proteins are summarized schematically in Fig. 3 (a–d). Quantification of the fraction of cells showing predominantly nuclear localization of UL34 protein for each experiment is shown in Fig. 3 (e).

Ye et al. (2000) reported recently that UL34 protein interacts with both cytoplasmic dynein intermediate chain and UL31 protein in HSV-1-infected cells and also that UL31 protein depends on UL34 protein for its stability (Ye & Roizman, 2000). The principal site of localization of UL34 protein in infected cells is the nuclear membrane. In transiently expressing cells, however, it localizes predominantly to the ER as a type II membrane protein (Shiba et al., 2000). This observation led to our chief interest in how the protein is translocated, in the presence of other viral proteins, to the nuclear membrane and, in particular, to the internal nuclear membrane. A natural candidate was UL31 protein, a largely insoluble nuclear protein that binds to the nuclear matrix (Chang & Roizman, 1993).

Our present study suggests that the UL31–UL34 interaction is required for translocation of UL34 protein into the nucleus and enrichment at the internal nuclear membrane. UL34 protein, when co-expressed with UL31 protein, exhibited either smooth, fine patterns (Fig. 2b) or larger, dotted accumulations (data not shown) in the nucleus. UL31 protein co-localized with UL34 protein in both cases. It was suggested that UL34 protein required UL31 protein for its targeting to the internal nuclear membrane and vice versa. We thus inferred that UL34 protein provides a link for UL31 protein between the internal nuclear membrane and the nuclear matrix. In fact, the deletion of 21 amino acids at the C terminus of UL34 protein eliminated its membrane localization and smooth nuclear patterns and subsequently that of UL31 protein. These findings suggest that the C-terminal region of UL34 protein may be important for nuclear membrane targeting of both proteins. However, this speculation apparently conflicts with a recent report that an HSV-1 mutant lacking sequences encoding the 31 C-terminal residues of UL34 is able to produce a significant number of virus particles in the extracellular space, although this mutant forms plaques and replicates at levels approximately 10-fold lower than those of the wild-type or repaired viruses (Ye & Roizman, 2000). These observations
taken together suggest that the loss of the C terminus may be compensated for to some extent by other factors and also that, although not essential, the C terminus is important in the lifecycle of HSV.

Truncating the N terminus of UL31 protein or the C terminus of UL34 protein greatly altered the outcome of their intracellular localization in cells co-expressing the full-length UL34 or UL31 protein, respectively. Most interestingly, when some of the mutant proteins were co-expressed with the other full-length protein, distribution of the former was greatly affected and resembled the staining patterns of the latter. These data suggest that the regions required for interaction between the UL31 and UL34 proteins, or at least the regions that interact more readily, are the non-deleted areas; the N terminus of UL34 protein and the C-terminal two-thirds of UL31 protein. It was reported recently that a mutant virus lacking sequences encoding amino acids 3–119 of UL34 replicated very poorly in rabbit skin cells (Ye & Roizman, 2000). Our study implies that this may have been due to the loss of interaction with UL31 protein.

In mammalian cells infected with a recombinant baculovirus expressing UL34 protein, the majority of the protein co-localizes with the nuclear membranes and is also detected in the perinuclear region or the cytoplasm in a cell line-dependent manner (Ye et al., 2000). In transfected Vero cells, UL34 protein localized at the nuclear membrane but predominantly at the ER. This association with the ER was reduced greatly by co-expression of UL31 protein, which resulted in enrichment at the internal nuclear membrane. We propose that UL34 protein is primarily targeted to the ER, but UL31 protein is able to target it to the nucleus, whereby the hydrophobic C-terminal sequence of UL34 binds to the internal nuclear membrane. How the hydrophobic tail is masked or inactivated until it passes through the nuclear pores into the nucleoplasm is unknown. This effective targeting to the internal nuclear membrane most likely assists the virus in efficient envelopment of capsids.

The role of this interaction in infection is under investigation, but another interesting issue is how the US3 protein kinase is related. In simultaneous triple expression of UL31, UL34 and US3 proteins, the fine nuclear pattern of UL34 protein was disrupted (data not shown), so the environment surrounding UL34 protein and the timing of expression seem to be crucial. A US3-deletion mutant of pseudorabies virus has been observed to accumulate enveloped virus particles in the perinuclear space. In the absence of UL34, budding at the inner nuclear membrane does not occur, whereas, in the absence of US3 (and possibly a lack of phosphorylation of UL34), de-envelopment at the outer nuclear membrane seems to be blocked (Klupp et al., 2000). The majority of phosphorylated UL34 protein (32–5 kDa) is present in infected cells as a protease-sensitive form, suggesting that the unphosphorylated 31 kDa protein participates in envelopment before the 32–5 kDa protein (Shiba et al., 2000). The role of US3 protein in the phosphorylation of UL34 protein is also a future aspect of our research.
Fig. 2. Co-expression of versions of UL31 and UL34 proteins shows various targeting patterns. (a)–(c) A cell expressing UL31wt-FLAG and UL34wt proteins, showing co-localization in the nucleus. Localization at the internal nuclear membrane is emphasized by closed arrowheads (b). (d)–(f) Such cells were also detected with anti-UL34 antibody and anti-lamin B1 monoclonal antibody and showed co-localization at the internal nuclear membrane. (g)–(i) A cell co-expressing UL31wt-FLAG and UL34MC256/276 proteins shows that they co-localize in the nucleus and nucleolus (large nuclear aggregates) but are excluded from the internal nuclear membrane. This is emphasized by open arrowheads (h). (j)–(l) A cell co-expressing UL31wt-FLAG and UL34MN2/38 proteins displays that distribution of each protein is similar to that in their singly expressed state. (m)–(o) A cell co-expressing UL34wt and UL31MN3Δ1/110-GFP proteins shows co-localization in an ER-like area in the cytoplasm. Nu, Nucleus.
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References
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