The UL34 gene product of herpes simplex virus type 2 is a tail-anchored type II membrane protein that is significant for virus envelopment

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The UL34 gene of herpes simplex virus type 2 (HSV-2) is highly conserved in the herpesvirus family. The UL34 gene product was identified in lysates of HSV-2-infected cells as protein species with molecular masses of 31 and 32.5 kDa, the latter being a phosphorylated product. Synthesis of these proteins occurred at late times post-infection and was highly dependent on viral DNA synthesis. Immunofluorescence assays revealed that the UL34 protein was localized in the cytoplasm in a continuous net-like structure, closely resembling the staining pattern of the endoplasmic reticulum (ER), in both HSV-2-infected cells and in cells transiently expressing UL34 protein. Deletion mutant analysis showed that this colocalization required the C terminus of the UL34 protein. The UL34 protein associated with virions but not with A, B or C capsids. We treated virions, HSV-2-infected cells and cells expressing the UL34 protein with a protease in order to examine the topology of the UL34 protein. In addition, we constructed UL34 deletion mutant proteins and examined their intracellular localization. Our data strongly support the hypothesis that the UL34 protein is inserted into the viral envelope as a tail-anchored type II membrane protein and is significant for virus envelopment.

Introduction

Herpes simplex virus (HSV) is a large, enveloped DNA virus with a genome that encodes at least 74 different genes (Roizman & Sears, 1996; Dolan et al., 1998). Approximately half of the genes are essential for virus replication in cell cultures (Roizman, 1996).

The UL34 gene of HSV type 1 (HSV-1) and type 2 (HSV-2) is conserved in all human herpesviruses, and homologues can be found in alpha-, beta- and gammaherpesviruses including varicella-zoster virus (Davison & Scott, 1986), human cytomegalovirus (HCMV) (Chee et al., 1990), human herpesvirus-6 (Gompels et al., 1995), human herpesvirus-7 (Nicholas, 1996) and Epstein–Barr virus (Bear et al., 1984). These UL34 homologues all contain a hydrophobic stretch of 22 to 37 residues near the C terminus (Roizman & Sears, 1996; Dolan et al., 1998; Davison & Scott, 1986; Chee et al., 1990; Gompels et al., 1995; Nicholas, 1996; Bear et al., 1984; Albrecht et al., 1992).

In HSV-1, the UL34 gene product has been identified as a membrane protein which is either directly or indirectly phosphorylated by the US3 protein kinase (Purves et al., 1991, 1992; Daikoku et al., 1993, 1994) and whose transcription is also regulated by the US11 protein (Roller & Roizman, 1991). Recently, it has been reported that the UL34 gene product is essential for efficient envelopment of capsids (Roller et al., 2000) but the precise function of the UL34 protein is yet to be clarified. In an attempt to further characterize the UL34 protein, we raised in rabbits an antibody that specifically recognizes the protein. Through immunofluorescence studies of wild-type and deletion mutants of the UL34 protein along with biochemical approaches unveiling its topology, we suggest that the HSV-2 UL34 protein is a tail-anchored type II membrane protein involved in the envelopment of capsids in the endoplasmic reticulum (ER).
Methods

Cells and viruses. Vero cells, a stable line of African green monkey kidney cells, were grown in Eagle’s minimum essential medium (MEM) supplemented with 5% calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin and were used throughout this study. HSV-1 strain KOS, HSV-2 strain 186 and HSV-2 US3 mutant virus L1BR1 (Nishiyama et al., 1992) derived from a single plaque were propagated in Vero cells by infecting at a low multiplicity (0/01 p.f.u. per cell). Infected cells were harvested when almost all the cells exhibited cytopathic effect. After freezing and thawing three times and eliminating cell debris at 3000 r.p.m. for 10 min, virus was stored at −80 °C.

DNA manipulations. The UL34 ORF is located between nucleotide positions 70119 and 70946 of the HSV-2 genome (Dolan et al., 1998). The UL34 coding sequences were cloned by PCR amplification from HSV-2 186 genomic DNA, using UL34-1 (5′ CCGGAATTCATGGCGGGATGCGGAGCC) as the forward primer and UL34-2 (5′ CCGGTGACCTCATTAGGGCCGCCCAACCGGCC) as the reverse primer. EcoRI and SalI sites were incorporated into the forward and reverse primers, respectively, to facilitate cloning. The PCR consisted of an initial 5 min denaturation step at 94 °C followed by 30 cycles of denaturation (94 °C, 1 min), annealing (58 °C, 2 min) and extension (72 °C, 3 min) and a final extension at 72 °C for 10 min. The PCR product was digested with EcoRI and SalI and cloned into the E. coli expression vector pGEX-4T-1 (Amersham Pharmacia) to give plasmid pGEX4-UL34. The expression of GST–UL34 protein is regulated by the IPTG-inducible vector pGEX-4T-1 (Amersham Pharmacia) to give plasmid pGEX4-UL34. Quantities of glutathione S-transferase (GST)–UL34 protein were expressed in cultured cells. Cleavage of pGEX4-UL34 with EcoRI and SalI sites were incorporated into the forward and reverse primers, respectively, to facilitate cloning. The PCR consisted of an initial 5 min denaturation step at 94 °C followed by 30 cycles of denaturation (94 °C, 1 min), annealing (58 °C, 2 min) and extension (72 °C, 3 min) and a final extension at 72 °C for 10 min. The PCR product was digested with EcoRI and SalI and cloned into the E. coli expression vector pGEX-4T-1 (Amersham Pharmacia) to give plasmid pGEX4-UL34. The expression of GST–UL34 protein is regulated by the IPTG-inducible lac operator and lac promoter. Translation is expected to terminate at the UL34 stop codon. Plasmid pGEX4-UL34 was transformed into E. coli strain JM101 which, following induction with IPTG, expressed large quantities of glutathione S-transferase (GST)–UL34 fusion protein.

The expression plasmid pGFP-UL34 was constructed for expression of the UL34 gene in cultured cells. Cleavage of pGEX4-UL34 with EcoRI and KpnI released the UL34 ORF and this DNA fragment was ligated into the multicloning site of pEGFP-C2 (Clontech) to give pEGFP-UL34, and the multicloning site of pGEX-4T-1 (Amersham) to give plasmid pGEX4-UL34. The PCR products were ligated into pcDNA3.1(−) (−).

Plasmid transfection. Cells were transfected by using Lipofectamine reagent according to the protocols recommended by the supplier (GibcoBRL). The PCR products were ligated into pcDNA3.1(−).

Preparation of polyclonal antiseras. Antisera were produced in two rabbits by immunization with an emulsion containing approximately 0.6 mg of E. coli-expressed GST–UL34 fusion protein in the MPL + TDL + CWS emulsion adjuvant system (RIBI ImmunoChem Research). Inoculation was by subcutaneous injection on the shaved back. The same adjuvant and 0.6 mg of the inclusion body preparation were used for subsequent boosts. A total of three booster injections was given at 3 week intervals after the primary injection. One week after the last immunization, blood was collected from the heart. Anti-UL34 polyclonal antiseras were also produced in rabbits by immunization with E. coli-expressed GST–UL34 fusion protein as described above.

Western blotting. At the times indicated, the denatured, solubilized polypeptides from lysates of mock-infected and HSV-infected cells were electrophoretically separated on SDS-polyacrylamide gels and electrophoretically transferred to Hybond PVDF membranes (Amersham). Non-specific protein binding was blocked by treating membranes at 4 °C overnight with Tris-buffered saline (TBS; 25 mM Tris–HCl, 150 mM NaCl, pH 7.5) containing 1% BSA and 0.05% Tween 20. The membranes were washed once with TBS and incubated at 37 °C for 1 h with a 1:5000 dilution of the UL34 antiserum in TBS containing 1% BSA and 0.05% Tween 20 at 37 °C for 1 h. After washing three times with TBS containing 0.05% Tween 20, the membranes were incubated with a 1:3000 dilution of goat anti-rabbit peroxidase-labelled second antibody at 37 °C for 1 h. The membrane was then washed three times with TBS containing 0.05% Tween 20, treated with ECL Western blotting detection system (Amersham), and exposed to Hyperfilm-ECL (Amersham).

Indirect immunofluorescence. Vero cells were grown on coverslips and were either mock-infected or infected with HSV-2 at a multiplicity of 3 p.f.u. per cell. At various times after infection, the cells were fixed in cold acetone. Coverslips were then incubated for 1 h at room temperature with 20% human serum in PBS to reduce background levels produced as a consequence of the affinity binding of rabbit immunoglobulin to the Fc receptor formed by glycoproteins E and I. The cells were reacted with anti-UL34 serum diluted 1:50 in PBS containing 1% BSA at 30 min at 37 °C, washed in excess PBS and then reacted with a 1:50 dilution of FITC-conjugated goat anti-rabbit immunoglobulin in blocking solution for 1 h at 37 °C. Fluorescent images were viewed with a Zeiss laser scanning microscope LSM510, or with the Bio-Rad MRC 1024 confocal imaging system.

Immunocytochemistry. Vero cells were grown on coverslips and were transfected with plasmid pcDNA3-UL34 by using Lipofectamine. At 24 h post-transfection, the cells were fixed in cold methanol. Coverslips were then incubated for 30 min at 37 °C with 10 μM Dicam (3) (Molecular Probes) (Terasaki et al., 1984; Terasaki & Jaffe, 1991; Lukas et al., 1998) and washed in excess PBS. The cells were reacted with anti-UL34 serum diluted 1:50 in PBS containing 0.1% BSA for 30 min at 37 °C, washed in excess PBS and then reacted with a 1:50 dilution of FITC-conjugated goat anti-rabbit immunoglobulin in blocking solution for 1 h at 37 °C. Fluorescent images were viewed with a Zeiss laser scanning microscope LSM510.

Fractionation of intracellular viral capsids. Vero cells were infected with HSV-2 at a multiplicity of 3 p.f.u. per cell and incubated at 34R (CGCGGTACCTCATAGGGCCGCCCAACCGGCC/KpnI). The PCR products were ligated into pcDNA3.1(−).
37 °C for 15 h. Infected cells were harvested by centrifugation and washed three times with PBS. Cell lysates were resuspended in 1 ml of lysis buffer (20 mM Tris–HCl, 0.1 M NaCl, 1 mM EDTA, 1% Triton X-100, pH 7.5), disrupted by sonication and the debris was pelleted at 3000 r.p.m. for 10 min. The supernatant was layered onto a 12 ml linear gradient of 10–50% (w/v) sucrose in buffer and centrifuged at 24000 r.p.m. for 40 min in a Hitachi PRS 40 rotor. Fractions (500 μl) were collected from bottom to the top and the position of virus capsids was determined by SDS–PAGE followed by silver staining.

- **Virion purification.** Monolayers of Vero cells cultured in roller bottles (850 cm²) were infected with HSV-2 at a multiplicity of 3 p.f.u. per cell. After adsorption for 1 h at 37 °C, maintenance medium containing 5% serum was added. HSV-2 virions were harvested from the extracellular medium at 36 h post-infection (p.i.). After removal of cell debris by low-speed centrifugation, virions were pelleted by centrifugation at 87000 g for 1 h. The virus suspension was layered onto a continuous 10–50% sucrose gradient, followed by centrifugation at 20000 r.p.m. for 1 h at 4 °C. The peak virion-containing fractions were collected as described above, diluted in PBS and pelleted again by centrifugation at 87000 g for 1 h. The virions were purified by a second cycle of sucrose-gradient centrifugation.

- **Phosphatase treatment.** Monolayers of Vero cells were infected with HSV-2 at a multiplicity of 3 p.f.u. per cell. At 24 h p.i., the cells were washed and suspended in buffer 1 (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% NP-40) of which 50 μl was incubated for 30 min at 30 °C with 1000 U of lambda protein phosphatase (New England Biolabs) per ml in 1 × buffer (50 mM Tris–HCl, pH 7.5, 0.1% dithiothreitol, 0.1% EDTA, 1M DTT, 0.01% Brij 35) and 2 mM MgCl₂. Samples were analysed by Western blotting after SDS–PAGE.

- **Protease treatment.** Isolated virions and HSV-2-infected cells, pcDNA3-UL34- or pEGFP-UL34-transfected cells were treated with proteinase K (TaKaRa) in the presence or absence of 1% NP-40. After incubation for 30 min at room temperature, PMSF was added to each sample to a final concentration of 2 mM to inhibit further proteolysis. Samples were then separated by SDS–PAGE and analysed by Western blotting with UL34 or GFP antisera.

### Results and Discussion

#### Preparation and specificity of anti-UL34 rabbit antiserum

We generated anti-UL34 rabbit antisera by using a recombinant HSV-2 UL34 fusion protein as antigen. As described in Methods, plasmid pGEX4T-UL34 was constructed and the UL34 fusion protein was overexpressed in E. coli after induction with IPTG (Fig. 1a, lane 2). The UL34 fusion protein was then purified with the Prep Cell system (Bio-Rad) (Fig. 1a, lane 3) and the purified protein was used to immunize two rabbits. The antisera produced were analysed by Western blotting to determine their reactivity and specificity (Fig. 1b). One of the antisera reacted strongly to proteins with apparent molecular masses of 31 and 32.5 kDa in lysates of HSV-2-infected Vero cells (Fig. 1b, lane 6). The antisera also reacted to a 30 kDa protein in lysates of HSV-1-infected cells (Fig. 1b, lane 5) but no protein bands were observed in lysates of mock-infected cells (Fig. 1b, lane 4). There was no significant change in the reactivity of the antisera after pre-adsorption with a control E. coli lysate (Fig. 1b, lane 8) although antiserum reactivity was clearly eliminated by pre-adsorption with an E. coli lysate expressing UL34 fusion protein (Fig. 1b, lane 10). Preimmune rabbit serum did not recognize any proteins in lysates of HSV-1- or HSV-2-infected cells (Fig. 1b, lanes 2 and 3). These results indicated that the antiserum specifically detected the double-banded UL34 proteins in HSV-2-infected cells, and therefore was used for further experiments in our attempt to characterize the HSV-2 UL34 gene product.

### Expression of the UL34 product in HSV-2-infected cells

The kinetics of UL34 protein expression in HSV-2-infected Vero cells were analysed by Western blotting. At various times after infection, cell lysates were subjected to electrophoresis, transferred to PVDF membranes and reacted with the UL34 antiserum. As shown in Fig. 1(c), the UL34 protein was first detectable as a single band of 31 kDa at 6 h p.i. and increased in amount until 9 h p.i. On the other hand, the 32.5 kDa protein was detected from 9 h p.i. and increased in amount until 12 h p.i.

To determine the dependence of UL34 protein production on viral DNA synthesis, infected cells were maintained for various times after a 1 h adsorption period in the presence or absence of phosphonoacetic acid (PAA) at 300 μg/ml. In the presence of PAA, no UL34 protein was produced even at 15 h p.i. (Fig. 1c, lane 9). This indicated that UL34 protein synthesis was highly dependent on viral DNA synthesis, suggesting that the UL34 protein is a late gene product.

Two bands of the UL34 protein were confirmed by Western blotting. Phosphorylation of the UL34 protein has been reported previously (Purves et al., 1991, 1992) and taking this into consideration the 32.5 kDa protein could be the phosphorylated form. If the 32.5 kDa protein was a result of phosphorylation, it is almost certain that phosphatase treatment would change its mobility. To examine this hypothesis, lysates of HSV-2-infected cells were treated with lambda protein phosphatase and then applied to SDS–PAGE and analysed by Western blotting with the UL34 antiserum. The mobility of the 32.5 kDa protein was altered by treatment with the phosphatase, whereas the mobility of the 31 kDa protein was not (Fig. 1d). These results indicated that the 32.5 kDa protein was indeed a phosphorylated product of the UL34 protein.

### Intracellular localization of the UL34 product in HSV-2-infected cells

The intracellular localization of the UL34 protein was examined by indirect immunofluorescence. Vero cells were mock infected or infected with HSV-2 and infected samples were fixed in cold acetone at 9 and 15 h p.i. The cells were then treated with human serum to block nonspecific staining and...
Fig. 1. (a) Induction of the GST–UL34 fusion protein. *E. coli* cells harbouring pGEX4T-UL34 were grown in the absence (1) or presence (2) of IPTG, and the UL34 fusion protein was purified using the Prep Cell system (Bio-Rad) (3). (b) Reactivity and specificity of rabbit polyclonal antiserum against the GST–UL34 fusion protein. Vero cells were mock-infected (1, 4, 7, 9) or infected with HSV-1 (2, 5) or HSV-2 (3, 6, 8, 10) and harvested at 15 h post-infection (p.i.). Proteins were separated by SDS–PAGE and electrically transferred to Hybond PVDF membranes (Amersham). The membranes were incubated with preimmune rabbit serum (1–3), the UL34 antiserum (4–6), the UL34 antiserum pre-adsorbed with an *E. coli* lysate expressing the UL34 fusion protein (9, 10). (c) Production of the UL34 protein in HSV-2-infected cells. Vero cells were mock-infected (1) or infected with HSV-2 (2–9) at a multiplicity of 3 p.f.u. per cell. The cells were cultured in the absence or presence of 400 µg/ml PAA (9) and harvested at 3 (2), 6 (3), 9 (4), 12 (5), 15 (6 and 9), 18 (7) or 24 (8) h p.i. Proteins were separated by SDS–PAGE and analysed by Western blotting with the UL34 antiserum. (d) Lambda phosphatase treatment of the UL34 protein. Lysates of HSV-2-infected cells were incubated in either the absence or presence of lambda phosphatase. The samples were then separated by SDS–PAGE and analysed by Western blotting with the UL34 antiserum. Positions of molecular mass markers (kDa) are indicated on the left.

Fig. 2. Localization of the UL34 protein in HSV-2-infected cells. Mock-infected (a) and HSV-2-infected (b–d) Vero cells were fixed with cold acetone at 9 (a–c) and 15 (d) h p.i. and stained with the UL34 antiserum (a, c, d) or with preimmune serum (b).

reacted with the UL34 antiserum (Fig. 2 a, c, d) or with preimmune serum (Fig. 2 b). As shown in Fig. 2(c, d), the UL34 protein localized in a continuous net-like structure in the cytoplasm and clustered strongly in the perinuclear region. No specific staining was seen in mock-infected cells (Fig. 2a).

**Association of the UL34 product with virions**

In order to determine whether the UL34 protein was a component of HSV-2 virions, extracellular virions were harvested from culture media at 36 h p.i. Virus particles were pelleted by centrifugation at 87000 g for 1 h and purified by sucrose density-gradient centrifugation (see Methods); fractions were collected from the bottom to the top of the gradient. The fractions were subjected to electrophoresis and silver stained to identify the peak of virion-containing fractions (5–8) (Fig. 3 a). All the fractions were also analysed by Western blotting with the UL34 antiserum, and the 31 and 32–35 kDa proteins were detected in fractions corresponding to the peak virion-containing fractions (Fig. 3 b). These results suggested that the UL34 protein was a component of HSV-2 virions.

**Association of the UL34 product with intracellular capsids**

To examine the association of the UL34 protein with intracellular capsids, cell lysates were prepared from HSV-2-
infected cells at 15 h p.i. and subjected to sucrose density-gradient centrifugation; fractions were collected from the bottom to the top. Each fraction was then analysed by SDS–PAGE and silver stained to identify the positions of A, B and C capsids. It is known that A and B capsids lack viral DNA, while C capsids contain viral DNA. Moreover, A and C capsids differ in protein composition from B capsids in that they lack the scaffolding protein VP22a (Rixon, 1993). The peak fraction of B capsids was identified by the presence of VP22a (40 kDa) (Fig. 3c). Judging from the abundance of the major capsid protein VP5 (155 kDa) and the presence of VP22a, the peak positions of A, B and C capsids seemed to be fractions 13, 11 and 6, respectively. Each fraction from the same preparation was also examined by Western blotting with the UL34 antiserum. The UL34 protein was not detected in any of the capsids, but only in the top fraction of the sucrose density-gradient (Fig. 3d). These results suggest that the UL34 protein was an envelope component rather than a capsid/tegument component.

Intracellular localization of the UL34 protein in singly expressing cells

To observe the intracellular localization of the UL34 protein when transiently and singly expressed, we constructed the expression plasmid pcDNA3-UL34, which expresses the UL34 gene under the control of the HCMV promoter. Vero cells were transfected with pcDNA3-UL34 and examined by indirect immunofluorescence. At 24 h post-transfection, the UL34 protein was observed as a continuous net-like structure in the cytoplasm and especially strong staining was seen in the perinuclear region. As the localization pattern was very similar to the staining patterns of the ER, we took advantage of an ER-specific stain, DilC16(3), to carry out double-staining with the UL34 antiserum (Fig. 4). The merged image in Fig. 4(c) suggested that the UL34 protein localized in the ER.

Topology of the UL34 protein

Analysis of the predicted sequence of the UL34 protein suggests that it has no obvious N-terminal signal sequence characteristic of a type I membrane protein, but contains a hydrophobic domain near the C terminus. The UL34 protein also possesses a microbody targeting motif (ARL) in its last three amino acids.

To determine if either the N terminus or the hydrophobic C terminus is significant for localization of the UL34 protein, we constructed N-terminal and C-terminal deletion mutant plasmids (Fig. 5a). MCA274/276, MCA241/276, MCA251/276 and MCA256/276 have C-terminal deletions of 3, 22, 27 and 37 amino acids. MNA2/23, MNA2/28 and...
Fig. 5. (a) Construction of the UL34 deletion mutants. All the plasmids constructed were finally inserted into pcDNA3.1(−) as in Methods. Deleted amino acids are shown by thin lines. (b) Expression of the UL34 mutant proteins. Vero cells were transfected with pcDNA3-UL34 (1), MCΔ274/276 (2), MCΔ241/276 (3), MCΔ251/276 (4), MCΔ256/276 (5), MNΔ2/23 (6), MNΔ2/28 (7) or MNΔ2/38 (8). Cells were harvested at 24 h post-transfection. Proteins were separated by SDS–PAGE and analysed by Western blotting with the UL34 antiserum.

MNΔ2/38 have N-terminal deletions of 22, 27 and 37 amino acids. Vero cells were transfected with pcDNA3-UL34 -MCΔ274/276, -MCΔ241/276, -MCΔ251/276, -MCΔ256/276, -MNΔ2/23, -MNΔ2/28 or -MNΔ2/38. At 24 h post-transfection, cell lysates were analysed by Western blotting (Fig. 5b) and it was confirmed that these deletion mutants were constructed as expected.

Next, in order to examine the localization of these mutant proteins, transfected cells were examined by indirect immunofluorescence with the UL34 antiserum. The MCΔ274/276 mutant protein localized in the cytoplasm in a reticular staining pattern similar to that of the wild-type protein (Fig. 6a). However, the MCΔ256/276 and the MCΔ251/276 mutant proteins distributed rather diffusely throughout the cytoplasm (Fig. 6b, c). On the other hand, the MCΔ241/276 mutant protein was distributed in both the cytoplasm and the nucleus (Fig. 6d). The localization of the N-terminal mutant proteins was similar in all cases to that of the UL34 wild-type protein (Fig. 6e–g) which implies a distinct difference in the roles of each terminus. These results suggest that the ER localization of the UL34 protein required the C-terminal hydrophobic domain but not necessarily the N terminus.

Type II membrane proteins are defined as integral membrane proteins having an N terminus exposed to the cytosol. It is also known that many C-terminal-anchored membrane proteins orient their N termini in the cytoplasm (Kutay et al., 1993). Combined with our observations, it seems likely that the UL34 protein is a C-terminal-anchored type II membrane protein. If this is the case, it would be predicted that the short C-terminal domain of the UL34 protein faces the lumen of the ER or the virus envelope whereas the much longer N-terminal domain is found in the cytoplasm or in the tegument of the CEAC.
The UL34 protein synthesized in cells will therefore be sensitive to proteases in the presence of a detergent such as NP-40. First, we treated UL34 protein-expressing cells with proteinase K. As a control, UL34 protein-expressing cells were treated with or without NP-40 in the absence of the protease to verify that the detergent had no effect on protein stability or migration. Treated/un-treated cells were then analysed by Western blotting with the UL34 antiserum. The UL34 protein turned out to be sensitive to proteinase K in both the presence and absence of NP-40 (Fig. 7a, lanes 7 and 8). Thus, the protease sensitivity of the UL34 protein suggests that the majority of the UL34 protein faces the cytoplasm.

In addition, the following assays were carried out to determine the topology of the UL34 protein. First, we constructed pEGFP-UL34, which expresses a GFP-tagged version of the UL34 protein, as described in Methods. Vero cells were transfected with pEGFP-UL34 and examined by confocal microscopy. At 24 h post-transfection, the localization of the GFP-UL34 fusion protein (Fig. 6h) was similar to that of the UL34 protein expressed alone, showing that fusion of GFP to the N terminus of the UL34 protein did not interfere with its localization in the ER.

We next treated the GFP–UL34 fusion protein with proteinase K, followed by Western blotting with the GFP antiserum. The GFP–UL34 fusion protein was detected as a 59 kDa band (Fig. 7b, lanes 1 and 2); the faint bands detected in these samples are thought to be degradation products of the GFP–UL34 fusion protein. The GFP–UL34 fusion protein was sensitive to proteinase K in both the presence and absence of NP-40 (Fig. 7b, lanes 7 and 8); the 27 kDa band detected irrespective of the presence of NP-40 was thought to be a GFP immunoreactive protease-resistant fragment (Fig. 7b, lanes 3–8). These results suggest that the GFP–UL34 fusion protein was oriented as a type II membrane protein.

In order to investigate whether the N-terminal domain of the UL34 protein was located on the surface of the virus envelope or on the tegument, isolated virions were mock-treated or treated with proteinase K and analysed by Western blotting with the UL34 antiserum. The UL34 protein was completely resistant to 100 µg/ml proteinase K in the absence of NP-40 (Fig. 7c, lane 9) whereas it was highly sensitive to the protease in the presence of NP-40 (Fig. 7c, lane 10). The results indicated that the major part of the UL34 protein was not present on the surface of the virus envelope. The previous data showed that the UL34 protein did not associate with intracellular capsids. Considering these results, it now seems clear that the UL34 protein associated with the envelope and that the N-terminal domain of the UL34 protein faced the tegument.

We next investigated how the UL34 protein is present in infected cells. Interestingly, protease sensitivity of the 31 and 32.5 kDa proteins differed (Fig. 7d). The 32.5 kDa protein was digested by the protease regardless of the presence of NP-40 (Fig. 7d, lanes 3, 5, 7 and 9). However, the 31 kDa protein was more sensitive to the protease in the presence of NP-40 than in its absence (Fig. 7d, lanes 4, 6, 8 and 10). These results suggest that the majority of the 32.5 kDa protein was present as a protease-sensitive form in infected cells. In addition, our series
of protease treatment assays showed that protease sensitivity in infected cells was different from that in transfected cells.

Considering a role of the UL34 protein in virus replication and its association with virions, it is thought that a part of the 31 kDa protein is surrounded by the envelope or is in the ER membrane. An envelopment model that has been previously reported showed that virions acquire their envelopes from the inner nuclear membrane, and then are unenveloped by fusion with the outer nuclear lamellae followed by retrieval of their envelopes from the ER and/or the Golgi complex. (Nii et al., 1968; Stackpole, 1969; Whealy et al., 1991; Roizman & Sears, 1996). Another more recent paper (Roller et al., 2000) reported that in cells infected with a recombinant UL34 deletion virus, no enveloped virus particles in the cytoplasm or at the surface of infected cells were observed, suggesting an essential role for the UL34 protein in efficient envelopment of capsids. Putting this together with the implications of our protease assays, we have produced a schematic model of how the UL34 protein might be involved in the envelopment process (Fig. 8). In this model, free UL34 proteins in the ER are sensitive to protease digestion. In contrast, UL34 proteins involved in budding at the ER or those surrounded with envelopes are protected from protease digestion. We predict that the UL34 protein plays an important role when unenveloped nucleocapsids acquire their envelope by budding at the ER membrane.

In the present study, the UL34 protein localized not only in the cytoplasm but also in the perinuclear region (Fig. 4a). It is thought that nucleocapsids acquire envelopes at both the inner nuclear membrane and the ER membrane (Nii et al., 1968; Stackpole, 1969; Nii, 1992; Whealy et al., 1991; Roizman & Sears, 1996) and it is probable that the UL34 protein may participate in envelopment at both these sites. This concept is also consistent with the report that in cells infected with a baculovirus expressing the UL34 protein, the UL34 protein localized in the perinuclear region and the outer nuclear membrane was dissociated from the inner membrane forming arcs that gave it a wavy appearance (Ye et al., 2000).

Type II membrane proteins have been identified in several herpesviruses. The UL45 gene product of HSV-2 (Cockerell & Muggeridge, 1998), the US9 gene product of pseudorabies virus (Brideau et al., 1998) and the Bcl-2 homologue (BHFR1) of Epstein–Barr virus (Pfitzner et al., 1987) have all been identified as type II membrane proteins. We demonstrated that the UL34 protein is a tail-anchored type II membrane protein (Kutay et al., 1993; Linstedt et al., 1995; Whitley et al., 1996). These proteins have no N-terminal signal sequence but instead possess a hydrophobic segment near the C terminus that orients the N terminus in the cytoplasm (Kutay et al., 1993). In this topology, the major part of the UL34 protein is not exposed on the surface of the viral particle but instead is within the tegument region. As the UL34 protein is likely to have no direct association with capsids, we predict that it interacts with tegument proteins or the tails of other envelope proteins and that such interactions may facilitate UL34 protein-escorted envelopment.

It is not clear how and when the 31 and 32–5 kDa proteins act in the envelopment process. Judging from the results of our protease assays, we assume that the 31 kDa protein may participate in envelopment prior to its phosphorylated 32–5 kDa counterpart. Also, the 31 and 32–5 kDa proteins may have different roles or may also be subject to conformational change during the process. A very interesting issue lies in the fact that the 32–5 kDa species is highly susceptible to protease digestion, implying that it may be a dead-end product ready to undergo digestion after fulfilling a role as a 31 kDa protein, but further study will be needed to be certain. Phosphorylation is a general strategy for regulating enzymes (Frangioni et al., 1992), and it is attractive to broaden our view to a hypothesis.
in which phosphorylation may regulate envelopment via the UL34 protein in a way comparable to enzyme regulation.

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