Functional characterization of the essential tail anchor of the herpes simplex virus type 1 nuclear egress protein pUL34

Melanie Ott, Georg Tascher, Sarah Haßdenteufel, Richard Zimmermann, Jürgen Haas, and Susanne M. Bailer

1Max von Pettenkofer-Institut, Ludwig-Maximilians-Universität München, Pettenkoferstr. 9a, 80336 München, Germany
2Technische Biochemie, Universität des Saarlandes, Saarbrücken, Germany
3Medizinische Biochemie und Molekularbiologie, Universität des Saarlandes, Homburg, Germany
4Division of Pathway Medicine, University of Edinburgh, UK

Correspondence
Susanne M. Bailer
Bailer@mvp.uni-muenchen.de

INTRODUCTION

Herpes simplex virus type 1 (HSV-1), a member of the subfamily Alphaherpesvirinae in the family Herpesviridae, can cause recurrent facial lesions and encephalitis. As in all herpesviruses, HSV-1 virions are characterized by a linear dsDNA genome, an icosahedral capsid, a protein layer called the tegument, and a host-derived envelope embedded with virally encoded membrane proteins. Morphogenesis of viral progeny starts in the infected host nucleus where a transcriptional cascade is initiated followed by viral DNA replication. Capsids newly assembled in the nucleus are 125 nm in diameter thereby exceeding the nuclear pore diameter for conventional nuclear export (Roizman et al., 2007). To egress from the nucleus, all herpesvirus subfamilies rely on a still poorly understood budding process occurring at the inner nuclear membrane (INM). Capsids equipped with a primary envelope gain temporary access to the perinuclear space, a subdomain of the endoplasmatic reticulum (ER) prior to fusion of their envelope with the outer nuclear membrane (ONM) or ER membrane and release to the cytoplasm. There secondary tegumentation and envelopment takes place followed by release of mature virions to the extracellular milieu (Mettenleiter et al., 2006, 2009; Baines, 2007).

The nuclear egress complex (NEC) which in HSV-1 is composed of pUL34, an integral membrane protein (Purves et al., 1992), and pUL31, a nucleoplasmically located protein (Chang & Roizman, 1993), is crucial for nuclear egress (Reynolds et al., 2001). Both proteins are essential for herpesvirus propagation (Ye & Roizman, 2000; Chang et al., 1997; Roller et al., 2000) and are conserved throughout the herpesvirus subfamilies (Chang & Roizman, 1993; Roller et al., 2000; Shiba et al., 2000; Zhu et al., 1999; Klupp et al., 2000; Fuchs et al., 2002; Muranyi et al., 2002; Lake & Hutt-Fletcher, 2004; Farina et al., 2000; Schnee et al., 2006; Santarelli et al.,...
Analysis of the essential tail anchor of HSV-1 pUL34

2008; Dal Monte et al., 2002; Camozzi et al., 2008). Full disruption of either of the two HSV-1 coding sequences results in capsids accumulating in the nuclear interior supporting a role of the NEC in capsid docking and budding at the INM (Chang et al., 1997; Roller et al., 2000). Two domains of pUL34 can clearly be discriminated: the N-terminal part exposed to the cyto- and/or nucleoplasm harbouring pUL31 interacting regions; and the hydrophobic C-terminal part which provides the membrane anchor (Shiba et al., 2000; Yamauchi et al., 2001; Bjerke et al., 2003; Roller et al., 2010).

Several morphological alterations at the nuclear envelope have been observed concomitant with nuclear egress. High resolution analysis revealed capsids docked at sites of the INM where membranes are negatively curved and associated with electron-dense material (Graznow et al., 2001; Mettenleiter, 2004; Baines et al., 2007). Capsids partially but tightly wrapped by membranes reaching into the perinuclear space coexist with enveloped capsids that based on a neck-like connection with the INM seem close to membrane scission. Inversely, capsids surrounded by envelopes open to the cytoplasmic space may represent fusion intermediates with the ONM in the process of release to the cytoplasm. While detailed mechanistic insight is still lacking, recent evidence provides the first access to understanding of nuclear egress. Coexpression of pseudorabies virus pUL34 and pUL31 in the absence of any other viral component is sufficient to generate membrane buds and vesicles reminiscent in size to capsids with primary envelopes (Klupp et al., 2007). Mutational analysis of both pUL34 and pUL31 revealed intricate interactions of these proteins required during membrane curving (Bjerke et al., 2003; Roller et al., 2010). Together these data led to the development of a model where pUL34 and pUL31 are central players during nuclear egress while cellular and probably other viral factors are expected to contribute to this multi-step process.

The membrane anchor of HSV-1 pUL34 is essential for viral propagation (Bjerke et al., 2003). Characterized by a single hydrophobic region positioned at its C-terminal end pUL34 was suggested to be a tail-anchor (TA) protein (Shiba et al., 2000; Bjerke et al., 2003; Mettenleiter, 2004; Baines, 2007; Mettenleiter et al., 2009). Unlike transmembrane domains (TMDs) that are cotranslationally inserted into membranes, hydrophobic regions of TA proteins remain associated with the ribosomal tunnel until translation is complete (Borgese & Fasana, 2011). Consequently, TA proteins need to be released from the ribosome prior to post-translational insertion into mitochondrial, chloroplast, peroxisomal or ER membranes. With this study we aimed to analyse the requirements of the pUL34 TA for targeting to the INM as well as viral replication. Deletion analysis revealed that 15 residues of the hydrophobic region are required for targeting to the INM and viral function. While permutation of the pUL34 TA exhibited a high tolerance regarding sequence and hydrophobicity, it could not be replaced by an unrelated anchoring mechanism. This study provides the first mechanistic insights into the particular role of the TA of pUL34 in membrane curving and capsid egress from the host nucleus.

RESULTS

Membrane insertion of HSV-1 pUL34 occurs post-translationally

HSV-1 pUL34 is composed of 275 residues featured by a cytoplasmically exposed N-terminal domain (residues 1–252) and a TA region (residues 253–275; Fig. 1a). A 20 residue segment (residues 253–272) is predicted to form an α-helical TMD. To determine the mechanism pUL34 uses for membrane insertion, we established an in vitro assay (Fig. 1b). A reticulocyte lysate-based coupled transcription/translation reaction was applied to synthesize Strep-tagged pUL34 (Strep-pUL34). Following synthesis (S) for 30 min at 30 °C, translation was inhibited by addition of RNase A and cycloheximide and the translational machinery removed by centrifugation. Next, microsomes were added to the supernatant for post-translational insertion of pUL34 and after 30 min pelleted by centrifugation (M). To discriminate between proteins peripheral attached (W) and stably inserted into the membranes (P) the microsomes were treated with sodium carbonate and subsequently centrifuged. Analysis of the samples by SDS-PAGE and autoradiography revealed that after sodium carbonate treatment the majority of Strep-pUL34 was found in the membrane fraction (P) consistent with post-translational membrane insertion of pUL34 (Fig. 1c, top panel). In contrast, Strep-pUL34s263 (s263), a truncated version of pUL34 which retains merely 11 residues of the hydrophobic TMD, associated with membranes but was largely removed by carbonate treatment (Fig. 1c, bottom panel). In parallel, mammalian ERj1, a type I integral membrane protein known to be inserted into membranes in a cotranslational manner (Dudek et al., 2002, 2005), was analysed using the same assay. Under conditions that allow for cotranslational membrane insertion, a significant amount of the precursor preERj1 which carries an N-terminal signal peptide is processed to the mature ERj1 indicative of membrane insertion (Fig. 1d, top panel). In contrast, under conditions where post-translational insertion of pUL34 occurs, preERj1 appears unprocessed and is thus probably peripheral (Fig. 1d, bottom panel). Together our data support the conclusion that pUL34 is inserted into membranes in a post-translational manner.

A minimal length of 15 residues is sufficient for stable membrane anchoring of pUL34

To determine the minimal requirement of the TA region for membrane insertion and anchoring, constructs were generated encoding pUL34 mutants where the TA region was consecutively shortened (Fig. 2a). Mutants were transiently expressed in Hep2 cells and analysed by indirect immunofluorescence (Fig. 2b). The TA mutants
pUL34 (residues 1–269) and pUL34 (residues 1–267) showed an ER-like distribution with a considerable amount of pUL34 at the nuclear periphery comparable to the wild-type (wt) pUL34. Upon coexpression with pUL31, pUL34 wt and both pUL34 mutants were recruited to the nuclear periphery. Deletion of two additional residues of the TMD (pUL34 residues 1–265) led to an exclusive nucleoplasmic localization of the protein comparable to the pUL34 mutant carrying a more drastic TMD deletion (residues 1–256; Fig. 2b). Thus neither of these mutant proteins was able to stably associate with nuclear membranes as was also confirmed by the in vitro membrane insertion assay (Fig. 1c, bottom panel; and data not shown). Together we conclude that 15 residues of the TMD are sufficient for stable membrane insertion of pUL34 and NEC formation at the nuclear periphery.

**Minimal requirements of the pUL34 TA region**

For genetic analysis of the TA region, bacterial artificial chromosome (BAC) mutagenesis was performed based on the pHSV1(17+)lox BAC (Fig. 3a). First, the coding region of UL34 was replaced by a galK-kan (galK-kn) cassette using traceless homologous recombination. In a second homologous recombination step, the selection cassette was exchanged for a wt UL34 gene or various TA mutants. Restriction pattern analysis and DNA sequencing of the generated BAC mutants was used to control for correct
insertion. Transfection of the HSV-1 ΔUL34/galK-kn BAC DNA into Vero cells and subsequent indirect immunofluorescence analysis showed that no viral progeny was generated consistent with the previously reported essential function of UL34 (Fig. 3e; Roller et al., 2000).

To determine the minimal requirements of pUL34 TA in the viral context, the HSV-1 ΔUL34/galK-kn BAC was tested for complementation by various TMD deletions. HSV-1 reconstituted from BACs where the ΔUL34/galK-kn was replaced by UL34 wt (residues 1–275), the UL34s269 (residues 1–269) or UL34s267 (residues 1–267) coding sequences showed wt-like behaviour (Fig. 3b, c). In contrast, deletion of two additional residues (pUL34s265) or more (pUL34s251) resulted in a BAC mutant unable to produce viral progeny as indicated by the absence of viral plaques (Fig. 3b, e). Indirect immunofluorescence analysis using pUL34-specific antibodies revealed a Lamin A/C-like nuclear rim staining upon infection with the wt or the UL34s269 and UL34s267 mutant viruses (Fig. 3d). Upon BAC transfection of the UL34s265 or UL34s251 mutants where the TMD was further shortened, pUL34 was detected in the nucleoplasm 20 h post-transfection (p.t.) (Fig. 3e).

Thus, functional complementation correlated well with localization of pUL34 to the nuclear periphery. We conclude that 15 residues of the TA are sufficient and required for NEC formation and function in the viral context.

Mutational analysis of the transmembrane segment of pUL34

To decipher the sequence information provided by the pUL34 TA, we generated chimera where the C-terminal part of pUL34 (residues 252–275) was replaced by orthologous regions of human cytomegalovirus (HCMV) pUL50 (residues 363–397) or Epstein–Barr virus (EBV) pBFRF1 (residues 318–336). In addition, chimeric proteins were engineered where the corresponding regions of the HSV-1 TA proteins pUL56 (residues 211–234) and pUS9 (residues 69–90), both known to be located to the trans-Golgi network (TGN), were fused to the C-terminal region of pUL34 (residues 1–272). Finally, fusion proteins were generated where the pUL34 TA was permuted by the respective regions of the cellular protein Bcl-2 (residues 218–239), an apoptotic factor, or Vamp2 (residues 95–116), a protein of the secretory pathway. All pUL34 TA chimera were expressed in vitro with the expected length and stably inserted into microsomes as determined by the in vitro membrane insertion assay (data not shown). To determine the functionality of the UL34 chimera, the galK-kan cassette of the HSV-1 ΔUL34/galK-kn BAC was replaced by the respective sequences (Fig. 4a). Vero cells infected with the reconstituted viral mutants were analysed 14 h post-infection (p.i.) by immunofluorescence using anti-pUL34 antibodies in conjunction with anti-Lamin A/C antibodies. All pUL34 chimera showed a wt-like localization to the nuclear periphery similar to Lamin A/C (Fig. 4b). To test the generated mutants for functional complementation, one-step growth curves were done following infection of Vero cells (Fig. 4c). The onset of virus production was comparable for wt and mutants indicating that the pUL34 TA can be replaced by TAs of proteins with various functions.

Anchoring of proteins in the INM or lamina is achieved by diverse mechanisms. Prelamin A, the precursor of Lamin A, one of the major components of the nuclear lamina contains a CAAX motif at its C terminus (Corrigan et al., 2005). For integration into the lamina, a series of post-translational modifications including isoprenylation and carboxyl-methylation at the CAAX motif followed by endoproteolytic cleavage at residue 646 of Lamin A are required (Fig. 4d). HSV-1 pUL34 was previously reported
to interact with Lamins and emerin (Reynolds et al., 2004; Leach et al., 2007; Mou et al., 2008) and thus could use an analogous pathway for INM targeting. We therefore generated a BAC mutant where the pUL34 TA residues 252–275 were replaced by the last 20 residues of Prelamin A including the CAAX motif and the endoproteolytic cleavage site but lacking a TMD. The capacity of this mutant to form plaques was analysed 3 days p.t. of Vero cells (Fig. 4d). We found that the pUL34-PreA mutant was unable to generate viral progeny while plaques were readily visible following transfection of the pUL34 wt BAC. Thus, while pUL34 tolerates a TMD varying in sequence and hydrophobicity, insertion into the INM appears to be essential for viral function.

**DISCUSSION**

Based on its domain organization, pUL34 was postulated to be a type II membrane protein with the N-terminal domain exposed to the cytoplasm/nucleoplasm (Shiba et al., 2000; Bjerke et al., 2003; Mettenleiter, 2004; Baines, 2007; Mettenleiter et al., 2009). We developed an *in vitro* membrane insertion assay which shows unequivocally that membrane insertion of pUL34 occurs post-translationally and that its C-terminal hydrophobic part is the major region for membrane association. Interestingly, since post-translational insertion of TA proteins can occur into various target membranes including the outer mitochondrial, peroxisomal or the ER membrane, our data point to another possibility where insertion into the INM occurs subsequent to nuclear import of a TA protein.

How pUL34 is transported to the INM and where the pUL34/pUL31 complex is formed is currently not known. Functional permutation of the pUL34 TA with that of ER and TGN resident proteins showed that this region of pUL34 does not carry information for INM targeting. Instead, the nuclear localization of pUL34 (residues 1–256) in the absence of any other viral protein supports an intrinsic nuclear import activity within the N-terminal part (this study; Bjerke et al., 2003). Indeed, a potential nuclear localization sequence has been predicted using the program PSORT (http://psort.hgc.jp/) between residues 178 and 194 of the pUL34 cytoplasmic domain. Nuclear import signals have also been described for HSV-2 pUL31 and its murine cytomegalovirus orthologue (Zhu et al., 1999; Lotzricht et al., 2006) suggesting that each of the two NEC proteins can reach the nucleus independent of other viral factors. We recapitulate previous data where deletion of most or all of the hydrophobic part of HSV-1 pUL34 led to a mutant protein mislocalized to the nuclear interior and unable to complement virus function (Roller et al., 2000; Bjerke et al., 2003). Our detailed functional analysis revealed that HSV-1 pUL34 carrying 15 TMD residues behaved in a wt-like manner while removal of two additional residues resulted in a mutant unable to grow. Close inspection showed that the pUL34 TMD mutant (residues 1–267) was targeted to the nuclear periphery both in transfected and in infected cells while the shorter version located to the nuclear interior. Thus functional complementation and targeting to the nuclear periphery correlate well suggesting that the TA region of pUL34 is particularly important for anchoring of pUL34 and thus the NEC at the INM. This notion is supported by mutants of emerin, a cellular member of the TA protein family which like pUL34 executes its function at the INM. Emerin has gained great attention due to its genetic association with various muscular dystrophies (Foissner, 2001; Wheeler & Ellis, 2008). One particular disease allele carries a six codon deletion resulting in a TA composed of 12 residues and a mutant emerin mislocalized to the nuclear interior (Fairley et al., 1999). Our results are thus consistent with a minimal length of 15 TMD residues required for function and membrane integration.

Membrane curving is a dynamic process where the energetically unfavourable bending is supported by the assembly of coat proteins and lipid asymmetries (Fig. 5; McMahon & Gallop, 2005; Lundmark & Carlsson, 2010; Graham & Kozlov, 2010). Numerous studies support a role of the NEC proteins pUL34 and pUL31 in membrane curving and vesicle formation (Klupp et al., 2007; Roller et al., 2010). To assist in this process, pUL34 and pUL31 could form homo- or heterooligomers reminiscent of BAR domain proteins (Fig. 5a; Suetsugu et al., 2010) while stabilization by capsids is
dispensable (Klupp et al., 2007). Alternatively, the NEC could recruit cellular and/or viral transmembrane proteins as suggested before (Fig. 5b; Baines et al., 2007; Wills et al., 2009). In addition, ER luminal proteins could energetically favour bud formation, based on our data however physical interaction with the pUL34 TA region is not required for it (Fig. 5c, d). An important feature of curved membranes is lipid asymmetry in the two leaflets. The NEC could recruit proteins that alter lipid heads enzymically or enable lipid flipping from one leaflet to the other (Fig. 5e). In addition, local perturbations caused by α-helical transmembrane regions could facilitate lipid exchange between the leaflets (Sanyal & Menon, 2009). Thus, with an α-helical domain spanning three-quarters of the double membrane, the functional TA of pUL34 could – in addition to merely anchoring the NEC – enable lipid transfer to the convex side of the bud.

Our data show that viral propagation is independent of the last six residues of pUL34. Thus, the luminal part of pUL34 is not only dispensable for budding into the perinuclear space but also during the de-envelopment process where the primary virion envelope contacts and fuses with the ONM. This process therefore probably requires the presence of a luminally exposed fusogenic domain provided by proteins other than pUL34. It is even possible that an extension of the luminal part of pUL34 would be rather inhibitory during fusion of the primary envelope with the ONM. In summary, our detailed analysis of the TA region of pUL34 revealed features relevant for membrane insertion, anchoring and function in the viral context, thereby providing novel insights into molecular details of nuclear egress.

**METHODS**

**Cells, viruses and general cloning.** Hep2 (ATCC-No. CCL-23) and Vero cells (ATCC-No. CCL-81) were grown as described previously (Schmidt et al., 2010). HSV-1 strain 17+ (provided by Beate Sodeik) was used for all experiments. The HSV1 BAC clone pHSV1(17+)lox is based on the pHSV1(17+)blueLox described previously (Nagel et al., 2008). Compared with pHSV1(17+)blueLox, the reporter lacZ gene has been removed, and the gene UL23 that encodes the viral thymidine kinase has been repaired (B. Sodeik). HSV-1 thymidine kinase has been repaired (B. Sodeik, personal communication; to be published elsewhere). HSV-1 propagation, titration and kinetics were done as described by Schmidt et al. (2010). Plasmid transfection was performed using Effectene Transfection Reagent (Qiagen), while BAC transfection was done using Lipofectamine 2000 (Invitrogen).

**Plasmids.** To generate UL34 mutants with TMD truncations, coding regions were amplified by PCR and cloned into the pEXPR-IBA5 vector (IBA Biotechnology) using the restriction enzymes XhoI and EcoRI. Alternatively, a stop codon was introduced at the site of interest by QuickChange Site-directed Mutagenesis (Stratagene) using the plasmid pEXPR-IBA5-UL34 as template. The UL31 coding sequence was introduced into the Gateway compatible pCR3-N-myc vector according to the manufacturer’s protocol (Invitrogen). To generate the pUL34- TA chimera, a vector was generated where the Strep-UL34 (residues 1–251) coding region was flank by HindIII and AgeI restriction sites leading to an amino acid exchange of alanine to valine at position 252 (A252V) which was functionally irrelevant just proximal to the region encoding the TMD. The AgeI site was subsequently used to permute the coding region of the pUL34 TA. To this end, the regions encoding the respective TA domains of HCMV pUL50 (residues 363–397), EBV pBPRF1 (residues 318–336),
HSV-1 pUL56 (residues 211–234), HSV-1 pUS9 (residues 69–90) or the cellular protein Bcl-2 (residues 218–239) and Vamp2 (residues 95–116) were amplified by PCR introducing Agel and EcoRV sites and together with the Strept-pUL34 (residues 1–251) coding region ligated into the HindIII/EcoRV-digested pcDNA3 vector (Invitrogen). All primers and plasmids are described in Supplementary Tables S1, S2 and S3 (available in JGV Online) and Tables 1 and 2, respectively.

**BAC mutagenesis.** For BAC mutagenesis the UL34 gene together with homologous sequences upstream and downstream of the coding region was amplified by PCR using the pLoCMV-VP1/3 DNA (AG Sodeik/Nagel) containing the complete HSV-1 ORFs UL32 to UL36 with homologous sequences upstream and downstream of the coding region ligated into the HindIII/EcoRV-digested vector. PCR was performed using the HotStar HiFidelity Polymerase kit (Qiagen) and the XhoI-UL34-BAC for and HindIII-UL34-BAC rev primers (Supplementary Table S2). The PCR product was cloned into pBlueScript (Stratagene) using the authentic Sf I-XhoI and the newly introduced 3' HindIII restriction site resulting in the pBS-UL34 BAC vector. For subcloning of different UL34 constructs carrying mutations within the coding region of the TA, a silent mutation leading to an SfiI site was inserted at the stop codon region of UL34 using QuickChange site-directed mutagenesis (Stratagene) and the primers SfiI-UL34 BAC for and SfiI-UL34 BAC rev (Supplementary Table S2). An authentic NcoI site at the start region of UL34 was used for subsequent cloning. Several UL34 mutants were amplified by PCR using the indicated plasmids as templates and primers listed in Supplementary Table S2. Fragments consisting of wt or mutant UL34 and additional 385 upstream and 556 downstream homologous nucleotides were cleaved at authentic XhoI and BstBI sites and used for BAC mutagenesis. All constructs were verified by DNA sequencing.

HSV-1 UL34 mutants were engineered using the BAC pHSV1(17+)Lox and a modified version of the galK-based BAC recombination cassette (Warming et al., 2005). First, the coding region of UL34 was replaced by a galK-kan targeting cassette (Dölkken et al., 2010) which was amplified using the pgalK-kan plasmid as template (kindly provided by Z. Ruzsics; GenBank accession no. FR832405) and the primers H3-UL34/galK-ko and H3-UL34/galK-kan with 50 bp homologies upstream and downstream of the UL34 gene (Supplementary Table S3). This cassette was introduced into pHSV1(17+)Lox by homologous recombination using induced SW102 bacteria carrying the wt BAC. Successful primary targeting conferred resistance to kanamycin alongside the chloramphenicol resistance encoded by the BAC cassette. Double-resistant single colonies were picked and analysed for galK functionality. To replace the marker regions, an XhoI/BstBI fragment excised from the pBS-UL34 TA mutants was introduced into the galK-kan labelled BACs by a second round of homologous recombination. Here the correct recombinants were selected by loss of the galK gene by plating the transformants on M63 minimal plates containing 2% 2-deoxygalactose (Sigma). Successful replacement of the galK-kan cassette resulted in a pHSV1(17+)Lox UL34 TA mutant. At every targeting level the generated BAC was confirmed by restriction pattern analysis and sequencing of the target site prior to viral reconstitution by transfection into Vero cells.

**Indirect immunofluorescence analysis.** Indirect immunofluorescence analysis of transfected or infected cells was done as described in Schmidt et al. (2010). As primary reagents, the mouse mAbs anti-Strep-tag II (IBA), anti-Lamin A/C (Santa Cruz), anti-ICP0 (Santa Cruz), anti-ICP8 (R. Heilbronn, Charité, Berlin) as well as the rabbit polyclonal antibodies against bacterially expressed and purified HIS6-pUL34 residues 2–252 (this study) and rabbit polyclonal anti-myc antibodies (Cell Signalling) were used. Goat anti-rabbit or anti-mouse antibodies coupled to Alexa488, goat anti-mouse or anti-rabbit antibodies coupled to Alexa594 (all Invitrogen) were used as secondary reagents. Cells were examined using a Leica confocal laser scanning microscope TCS SP5.

### Table 1. Expression vectors used in this study

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<th>Cloning strategy</th>
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*Triple ligation with † and ‡ in HindIII/EcoRV-digested vector pcDNA3.
†HindIII/Agel-digested fragment.
‡Agel/EcoRV-digested fragment.
Table 2. Cloning vectors used in this study

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<td>26</td>
<td>pBS-UL34-Bcl-2tail*</td>
<td>Primer no. 32/41, template pcDNA3-StrepUL34-Bcl-2tail†</td>
</tr>
<tr>
<td>27</td>
<td>pBS-UL34-Vamp2tail*</td>
<td>Primer no. 32/42, template pcDNA3-StrepUL34-Vamp2tail†</td>
</tr>
<tr>
<td>28</td>
<td>pBS-UL34-prelaminAtail*</td>
<td>Primer no. 32/43 (1st PCR), primer no. 32/44 (2nd PCR), template pEXPR-IBA5-UL34†</td>
</tr>
</tbody>
</table>

*Triple ligation with † and ‡ in NcoI/BstBI-digested vector pBS.
†NcoI/SfiI-digested fragment.
‡556 bp fragment generated by SfiI/BstBI digestion of pBS-UL34 BAC (SfiI).

while plates were analysed using a Leica DM4000B fluorescence microscope. Images were recorded using the Leica Application Suite AF6000 Software and processed using Adobe Photoshop.

Coupled in vitro transcription/translation and membrane insertion assay. Synthesis of HSV-1 pUL34 was carried out in rabbit reticulocyte lysate in the presence of 35S-methionine (Amersham Biosciences) by the TNT coupled in vitro transcription/translation kit according to the manufacturer’s instructions (Promega). The plasmid pEXPR-IBA5-UL34 was used as the template. Following 30 min of protein synthesis, translation was stopped by addition of RNase A and cycloheximide and an aliquot removed for gel analysis (S, synthesis). To remove the translation machinery, the sample was centrifuged at 225 000 g for 20 min at 4 °C. Canine pancreatic microsomes prepared as described previously (Watts et al., 1983) were added to allow for post-translational insertion of pUL34. Following incubation for 30 min at 30 °C the reaction was divided in two parts both of which were centrifuged at 225 000 g for 20 min at 4 °C. One of the pellet fractions was subsequently analysed by SDS-PAGE (M, microsomes) while the second one was treated with 100 mM sodium carbonate for 30 min on ice followed by centrifugation at 225 000 g for 20 min at 4 °C to separate peripherally associated proteins (W, wash) from proteins stably inserted into the membranes (P, pellet). All samples were boiled in Laemmli buffer for 5 min, separated by SDS-PAGE and visualized by autoradiography.

To test the membrane insertion of ERj1 (formerly called Mtj1), a type I membrane protein of the mammalian ER, was synthesized as described before (Dudek et al., 2002, 2003) either in the presence (cotranslational transport) or absence of microsomes for 30 min. After synthesis in the absence of microsomes, the translation was stopped by addition of RNase A and cycloheximide prior to 30 min continued incubation of the protein in the presence of microsomes (post-translational transport). The precursor ERj1 (preERj1) is processed to ERj1 by signal peptidase upon insertion into the microsomal membrane.

We note that reticulocyte lysate contains residual amounts of ER membranes that led to minute amounts of mature ERj1 during synthesis of preERj1 (Fig. 1d, lower panel). Furthermore, carbonate extraction is not perfect, some fraction of the membrane proteins is artificially removed with the wash (Fig. 1c and d, mature ERj1 and wt UL34 in the upper panels) while some non-membrane protein is found in the pellet (preERj1 and s263 in the lower panels), probably due to aggregation of the protein. Aggregated protein present in the wash may be SDS resistant and thus not separated by electrophoresis leading to disproportionate amounts of protein in microsomes (M) over combined wash (W) and pellet (P) fractions. After all, this is just an operational definition of membrane proteins. However, a significant enrichment of a protein in the P fraction over the W strongly suggests a real bilayer integrated protein (wt in Fig. 1c, upper panel; ERj1 and possibly unprocessed preERj1 in Fig. 1d, upper panel).

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REFERENCES


