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

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Release of herpes simplex virus type 1 (HSV-1) nucleocapsids from the host nucleus relies on the nuclear egress complex consisting of the two essential proteins pUL34 and pUL31. The cytoplasmically exposed N-terminal region of pUL34 interacts with pUL31, while a hydrophobic region followed by a short luminal part mediates membrane association. Based on its domain organization, pUL34 was postulated to be a tail-anchor (TA) protein. We performed a coupled in vitro transcription/translation assay to show that membrane insertion of pUL34 occurs post-translationally. Transient transfection and localization experiments in mammalian cells were combined with HSV-1 bacterial artificial chromosome mutagenesis to reveal the functional properties of the essential pUL34 TA. Our data show that a minimal tail length of 15 residues is sufficient for nuclear envelope targeting and pUL34 function. Permutations of the pUL34 TA with orthologous regions of human cytomegalovirus pUL50 or Epstein–Barr virus pBFRF1 as well as the heterologous HSV-1 TA proteins pUL56 or pUS9 or the cellular TA proteins Bcl-2 and Vamp2 revealed that nuclear egress tolerates TAs varying in sequence and hydrophobicity, while a non-α-helical membrane anchor failed to complement the pUL34 function. In conclusion, 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.

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.,...
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 (Granzzow 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 a-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 peripherally 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). 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

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**Fig. 2.** A minimal transmembrane region of 15 residues is sufficient for stable membrane insertion of pUL34. (a) A schematic diagram of C-terminal deletion mutants to determine the minimal transmembrane region required for membrane insertion and function of pUL34. (b) Strep-pUL34 wt or mutants s269, s267, s265 and s256 were transiently expressed in Hep2 cells either alone or in combination with myc-tagged pUL31. Their localization was determined by staining with anti-Strep-tagII mAbs while polyclonal rabbit anti-myc antibodies were used to localize pUL31. Nuclei were visualized by DAPI staining.
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-PrelA 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; Lotzertich 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 hydrophilic 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

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**Fig. 3.** Generation of HSV-1 pUL34 TA mutants. (a) The schematic diagram of the pHSV1(17+)Lox genome shows the strategy to replace the UL34 gene with a selection cassette which in turn is replaced by wt UL34, or mutants s269, s267, s265 and s251. (b) The generated HSV-1 BACs where the selection cassette was replaced by wt UL34 or UL34 mutants s269, s267, s265 and s251 are graphically depicted. Their ability to generate viral progeny is summarized on the right. To this end, Vero cells were transfected with BAC DNA and monitored for cytopathic effects for 3 days post-transfection (p.t.) or longer. (c) To determine the growth properties of HSV-1 wt compared to the mutants s269 and s267, Vero cells were infected at an m.o.i. of 0.1, the supernatant was harvested at the indicated time points and titrated on Vero cells in triplicates. (d) To monitor the localization and expression of pUL34 wt and pUL34 mutants s269 and s267, Vero cells were infected with the recombinant virus (m.o.i. of 1) and analysed by indirect immunofluorescence 14 h post-infection (p.i.) using polyclonal anti-pUL34 and monoclonal anti-Lamin A/C antibodies. (e) Deletion of UL34 in HSV-1 (ΔUL34/galK-kr) and replacement with wt UL34 (ΔUL34/wt) or TA truncation mutants (ΔUL34/s265, ΔUL34/s267) was monitored by transfection of BAC DNA into Vero cells followed by indirect immunofluorescence analysis 20 h p.t. using polyclonal rabbit anti-pUL34 antibodies and mAbs to ICP8 (shown in red), nuclei were visualized by DAPI staining (blue; left panel). To follow their ability to form plaques, Vero cells transfected with BAC DNA were analysed 3 days p.t. by indirect immunofluorescence using anti-ICP0 mAbs (right panel).
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 that alteration of lipid heads enzymically or enable 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 \( \alpha \)-helical transmembrane regions could facilitate lipid exchange between the leaflets (Sanyal & Menon, 2009). Thus, with an \( \alpha \)-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, 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 EcoRV. Alternatively, a stop codon was introduced at the site of interest by QuikChange 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 flanked 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),

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**Fig. 5.** Potential membrane-associated processes during nuclear egress. (a) NEC formation. (b) Recruitment of cellular and/or viral transmembrane proteins. (c) Luminal proteins physically interacting with the pUL34 TA. (d) Luminal sensor proteins for membrane bending. (e) Lipid asymmetry of membrane leaflets caused by \( \alpha \)-helical TAs and/or lipid-modifying and -flipping proteins.
Table 1. Expression vectors used in this study

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

while plaques 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 S35-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 pelleted 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, 2005) 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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