Molecular Cell, Vol. 2, 85–91, July, 1998, Copyright ©1998 by Cell Press

Regulation of Protein Topology by *trans*-Acting Factors at the Endoplasmic Reticulum

Ramanujan S. Hegde,* Sabine Voigt,‡ and Vishwanath R. Lingappa*†§
*Department of Physiology
†Department of Medicine
University of California, San Francisco
San Francisco, California 94143-0444
‡Department of Cell Biology
Harvard Medical School
Boston, Massachusetts 02115

Summary

In mammalian cells, the Sec61 complex and translocating chain-associated membrane protein (TRAM) are necessary and sufficient to direct the biogenesis, in the appropriate topology, of all secretory and membrane proteins examined thus far. We demonstrate here that the proper translocation of the prion protein (PrP), a substrate that can be synthesized in more than one topologic form, requires additional factors. In the absence of these additional factors, PrP is synthesized exclusively in the transmembrane topology (termed the ^{Ctm}PrP form) associated with the development of neurodegenerative disease. Thus, translocation accessory factors, acting on some but not other substrates, can function as molecular switches to redirect nascent proteins toward divergent topologic fates with different functional consequences.

Introduction

It is generally thought that in most cases, all copies of a given secretory or membrane protein exist in a single homogeneous orientation with respect to the membrane (see Schatz and Dobberstein, 1996 for a recent review of protein biogenesis). Direct examination of the synthesis and final topology of numerous proteins in vitro and in vivo confirm that this is the case for at least relatively simple substrates (for example, Blobel and Dobberstein, 1975; Katz and Lodish, 1979; Gafvelin et al., 1997). However, a few proteins have been found that can exist in two or more topological forms (Skach et al., 1993; Dunlop et al., 1995; Zhang and Ling, 1995; Levy, 1996). In most cases, the physiological significance of such observations, or the mechanisms by which this occurs, remain obscure. One example of such a protein is PrP, which is synthesized in at least three distinct topologic orientations at the ER (Yost et al., 1990; Hegde et al., 1998). The ^{sec}PrP form is fully translocated across the ER membrane, while the ^{Ctm}PrP and ^{Ntm}PrP forms each span the membrane once, in opposite orientations, at the same hydrophobic stretch (roughly residues 110–135). The ^{Ctm}PrP form has the COOH terminus of the protein in the ER lumen, while ^{Ntm}PrP has the NH_2 terminus in the lumen.

Despite this potential topological heterogeneity, only the ^{sec}PrP topology is detected at steady state in normal brain (Stahl et al., 1987; Hegde et al., 1998). The role(s) of the other topological forms observed for PrP in cellular physiology or disease had, until recently, been unclear. By analyzing transgenic mice expressing mutations in PrP that increase the relative amounts of ^{Ctm}PrP at the ER membrane, we were able to demonstrate that expression of this transmembrane form resulted in neurodegeneration (Hegde et al., 1998). Subsequently, a naturally occurring disease-causing mutation in human PrP was found to result in increased ^{Ctm}PrP synthesis in cell-free translation systems. Furthermore, elevated ^{Ctm}PrP levels were found upon analysis of samples of brain tissue taken at autopsy from clinically ill humans containing this mutation. These results together demonstrated that dysregulation of protein biogenesis and topology at the ER membrane is involved in human disease. Thus, an understanding of the events of PrP biogenesis, and protein topology in general, may be of importance to understanding the pathophysiology of neurodegeneration.

In this study, we have examined the mechanism used by PrP to achieve more than one topologic form. By manipulating the protein composition of the ER-derived microsomal membranes, we are able to demonstrate that PrP topogenesis is regulated in a manner unlike any previously examined protein. Remarkably, the default pathway of PrP biogenesis directed by the currently known translocation machinery is toward the generation of ^{Ctm}PrP. Perhaps more important is the demonstration that PrP exhibits a novel requirement for factors in addition to the currently identified translocation machinery. Thus, translocation and topology of proteins across the ER can be regulated by *trans*-acting factors.

Results

The ability to prepare functional proteoliposomes containing specific components of the ER membrane has provided substantial insight into the general mechanisms of translocation (Nicchitta and Blobel, 1990; Nicchitta et al., 1991; Görlich et al., 1992; Görlich and Rapoport, 1993). Reconstitution into liposomes of only two protein complexes, the receptor for the signal recognition particle (SRP) and Sec61 complex, is sufficient to reproduce both translocation and membrane integration of certain model proteins (Görlich and Rapoport, 1993; Oliver et al., 1995; Voigt et al., 1996). The additional inclusion of the translocating chain-associated membrane protein (TRAM) in these proteoliposomes is sufficient to reconstitute the translocation and integration of all proteins tested thus far. Many proteins are TRAM dependent, while others are only modestly stimulated by the TRAM protein (Görlich et al., 1992; Voigt et al., 1996). It appears that the TRAM protein functions to stimulate translocation at an early, signal-sequence–dependent step by facilitating proper insertion of the nascent substrate into the translocation channel (Voigt et al., 1996). To gain insight into PrP translocation and topology, these reconstitution techniques were used to

§To whom correspondence should be addressed.

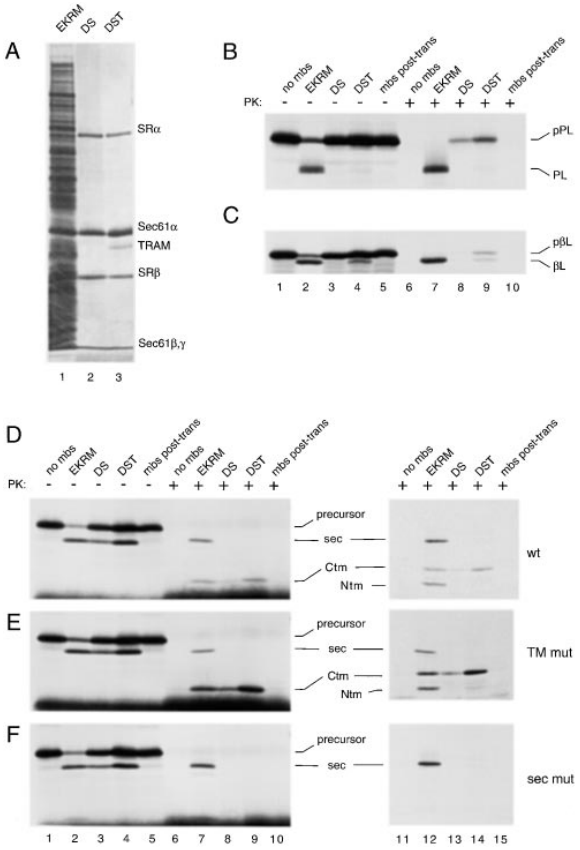

Figure 1. Analysis of PrP in Proteoliposomes Containing Purified Translocation Components

(A) EDTA-salt washed rough microsomes (EKRM) and proteoliposomes containing SRP receptor and Sec61 complex without (DS) or with TRAM (DST) were prepared and analyzed by SDS–PAGE and silver staining. These membranes were then included during translation reactions of prolactin (B), β-lactamase (C), wild-type PrP (D), a transmembrane-favoring PrP mutant (TM mut) (E), and a secretory-favoring PrP mutant (sec Mut) (F), and the samples assessed for translocation by PK digestion. Two samples in each set contained no membranes, one of which received EKRM posttranslationally (just before the proteolysis reaction). Protection of the translation product from PK digestion indicates translocation into the lumen. In the PrP substrates, the positions of the PK digestion products indicative of the different topological forms are indicated. The panels on the left (lanes 1–10) are total translation products analyzed directly, whereas the panels on the right (lanes 11–15) are immunoprecipitates of the samples in lanes 6–10 using the 3F4 anti-PrP monoclonal antibody (Rogers et al., 1991). This allows the additional visualization of the ^{Ntm}PrP fragment, which comigrates with and is obscured by the globin in the total translation products.

assess specific requirements for each of the topological forms.

For these experiments, purified components of the ER membrane were reconstituted with pure phospholipids into proteoliposomes that were then tested for translocation. Figures 1a–1c demonstrate that proteoliposomes containing only SRP receptor (also termed docking protein) and Sec61 complex (DS membranes) were able to translocate prolactin (a TRAM-independent substrate), but not β-lactamase (a TRAM-dependent substrate). As demonstrated previously, the additional inclusion of TRAM in the proteoliposomes (DST membranes) largely restored translocation of β-lactamase, while stimulating prolactin translocation modestly. Analysis of PrP translocation in these proteoliposomes revealed that only the ^{Ctm}PrP form is made (Figure 1d). Most notably, the ^{sec}PrP form was not seen regardless of whether TRAM was present or not.

Similar results were obtained with a transmembrane-favoring PrP mutant (in which three alanine-to-valine changes were made at positions 113, 115, and 118) that increases the percent of PrP made in the ^{Ctm}PrP form (Hegde et al., 1998). As expected, this mutant PrP did not generate ^{sec}PrP in the DS or DST proteoliposomes (Figure 1e). Identical results were obtained when another ^{Ctm}PrP-favoring mutant, termed KH→II (in which the lysine and histidine residues at positions 110 and 111 were changed to isoleucines), was analyzed in these proteoliposomes (data not shown).

We also analyzed the behavior of a secretory-favoring PrP mutant (in which a glycine at position 123 is changed to proline) that is incapable of making the transmembrane forms of PrP (Hegde et al., 1998). This mutant only makes ^{sec}PrP, behaving like a simple secretory protein without the unusual properties of multiple topologic forms exhibited by wild-type PrP. Remarkably, however, it was not possible to translocate this mutant into DS or DST proteoliposomes (Figure 1f). Furthermore, identical results were obtained with another secretory-favoring PrP mutant, ΔSTE (in which amino acids 104–113 are deleted), that is also incapable of being made in the transmembrane forms (data not shown). Indeed, these secretory-favoring PrP mutants are the only substrates examined thus far that completely fail to translocate in DST membranes. Because a wide variety of secretory proteins are able to translocate into the lumen of these DST proteoliposomes (Figures 1b and 1c and Voigt et al., 1996), the inability of wild-type or mutant PrP to be fully translocated into the lumen suggests that additional ER protein(s) are required to make ^{sec}PrP.

In principle, the inability of DST membranes to make ^{sec}PrP could be due to a defect in targeting, proper insertion into the translocation site, or at a posttargeting step in translocation. Because all of the PrP substrates (wild-type and mutants) contain the identical signal sequence (as well as N-terminal 103 amino acids), it seemed unlikely that the total translocation defect seen with the secretory-favoring PrP mutants would be at the targeting step. However, we directly tested the ability of each of these substrates to properly target and insert into the translocation site by examining translocation intermediates of each PrP substrate. For this experiment, in vitro transcription was used to generate truncated mRNAs (and thus lacking an in-frame stop codon) coding for the first 180 amino acids of each PrP substrate. These mRNAs were used to program translation reaction containing either total rough microsomes or DST proteoliposomes. Then, the extent to which each substrate could be assembled as a translocation intermediate at the membrane was assessed. As a positive control, an early translocation intermediate of preprolactin (containing the first 86 amino acids) was analyzed in parallel.

Figure 2 demonstrates that in total microsomal membranes, each of the substrates targets very efficiently

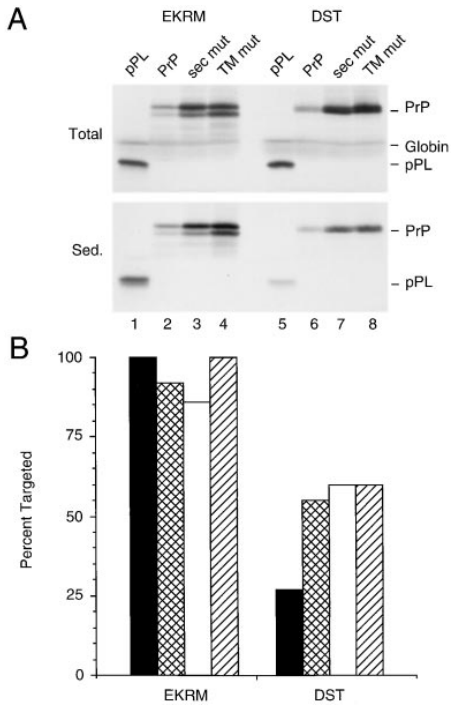

Figure 2. Targeting of PrP to the Membrane

(A) Translocation intermediates encoding the first 86 amino acids of preprolactin (pPL) or the first 180 amino acids of wild-type PrP, the secretory-favoring PrP mutant (sec Mut), or the transmembrane-favoring PrP mutant (TM mut) were translated in RRL in the presence of EKRM or DST membranes. The samples were divided and membranes were isolated by sedimentation from one of the aliquots. Equal amounts of the total (top gel) and sedimented (bottom gel) samples were analyzed by SDS–PAGE and autoradiography.

(B) The percent of total chains recovered by sedimentation is plotted as "Percent Targeted."

to the membrane and is nearly quantitatively sedimented with the microsomal membrane. Each of the PrP substrates was also found to target to DST proteoliposomes with slightly greater efficiency than was observed for the preprolactin substrate. Furthermore, the inability of high salt treatment (500 mM KOAc) to strip off the membrane-targeted PrP substrates indicates that they are tightly docked at the membrane. Yet, prolactin, and not the secretory-favoring PrP mutant, translocates into the lumen (Figure 1). These data strongly suggest that the translocation defect in PrP is at a posttargeting step. Furthermore, since PrP also targets and docks in a high salt–resistant manner to DS membranes (data not shown), it appears that the signal sequence of PrP is not TRAM dependent.

Given that the minimal translocation components were unable to support the biogenesis of the ^{sec}PrP form, we sought to reconstitute its translocation in proteoliposomes prepared with proteins extracted from total ER membranes (Nicchitta and Blobel, 1990; Nicchitta et al., 1991; Görlich et al., 1992). In principle, such proteoliposomes should contain (in varying amounts) each protein found in the starting membranes (which support ^{sec}PrP translocation), and thus should be capable of translocating ^{sec}PrP to some extent. For this experiment, total ER microsomal membranes were extracted with varying amounts of detergent, and the solubilized proteins reconstituted into proteoliposomes (Figure 3a). These different preparations of proteoliposomes were assayed for the translocation of prolactin, β-lactamase, and PrP. Prolactin translocated well into each of these membranes (Figure 3b), while β-lactamase showed optimal translocation only in membranes prepared from higher detergent extracts (Figure 3c). This optimum at higher detergent concentrations reflects dependence of β-lactamase translocation on TRAM, which is extracted better at these concentrations (data not shown). Regardless, both of these substrates were translocated into these proteoliposomes at levels comparable to the starting membranes, indicating that the basic translocation machinery had been well reconstituted.

Analysis of PrP in these same membrane preparations yielded interesting results (Figure 3d). As expected, the ^{Ctm}PrP form was made efficiently in all of these proteoliposomes (at levels comparable to the starting membranes), reflecting its ability to be translocated by the same minimal machinery used by prolactin or β-lactamase (e.g., see Figure 1). However, ^{sec}PrP and ^{Ntm}PrP were made efficiently in only some of the membrane preparations, showing optimal translocation at 0.5% and 0.45%, respectively. In these optimally active proteoliposomes, both ^{sec}PrP and ^{Ntm}PrP were made substantially better than in the DST membranes (compare to Figure 1d). This is in contrast to the ^{Ctm}PrP form, which was made efficiently in the DST proteoliposomes as well as in a broader range of the crude proteoliposomes in Figure 3. Thus, it appears that in the proteoliposomes made from total proteins, a translocation machinery that is competent to make all three forms of PrP can be assembled.

These results indicate that proteins in addition to the minimal machinery necessary for translocation (SRP receptor, Sec61 complex, and in some cases TRAM) are functionally present in the proteoliposomes made with the 0.5% detergent-extracted proteins. To demonstrate this directly, we sought to separate this component(s) from the minimal machinery. We took advantage of the fact that SRP receptor and Sec61 complex are not glycosylated to prepare a glycoprotein-depleted detergent extract that, when assembled into proteoliposomes, is capable of protein translocation (Görlich et al., 1992). These glycoprotein-depleted proteoliposomes (along with nondepleted and mock-depleted proteoliposomes) were assayed for prolactin, β-lactamase, and PrP translocation (Figure 4a). As expected, prolactin translocated efficiently into each of these three membrane vesicles. By contrast, the TRAM-dependent substrate β-lactamase failed to translocate into the glycoprotein-depleted vesicles, reflecting the lack of the glycoprotein TRAM. Analysis of PrP demonstrates that the the glycoprotein-depleted vesicles contain a defect only in ^{sec}PrP translocation, with ^{Ctm}PrP and ^{Ntm}PrP both being made as in the nondepleted membranes. Since PrP contains a TRAM-independent signal sequence, it was unlikely that the inability of glycoprotein-depleted proteoliposomes to make ^{sec}PrP was due only to the lack of TRAM. This is supported further by the observation that ^{sec}PrP was not made in the DST proteoliposomes (which contained TRAM; see Figure 1d).

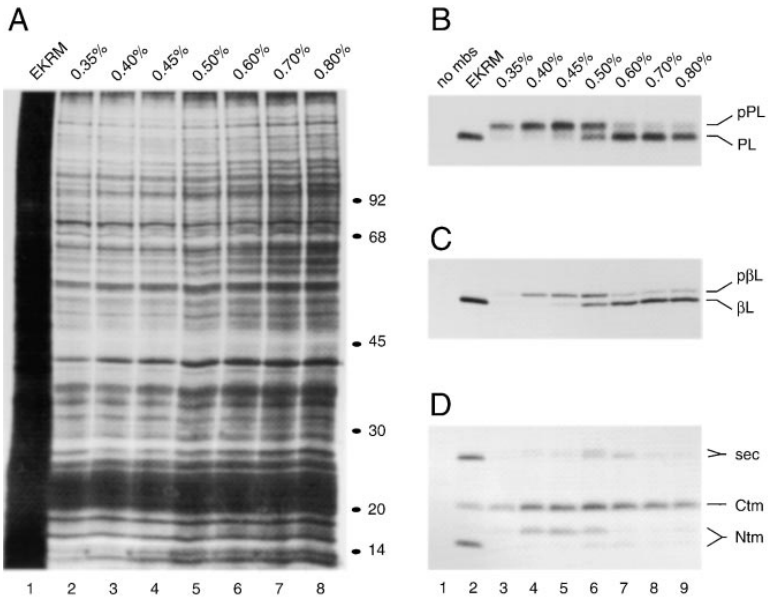

Figure 3. Analysis of PrP Translocation into Proteoliposomes Prepared from Total ER Membrane Proteins

EKRMs were extracted with cholate at various final concentrations from 0.35% to 0.80% (w/v) and the solubilized proteins reconstituted into proteoliposomes. (A) An aliquot (1 μl) of each membrane preparation was analyzed by SDS–PAGE and silver staining. Each membrane preparation was also included in translation reactions of prolactin (B), β-lactamase (C), or PrP (D). Following translation, samples were digested with PK to assess topology, and the products remaining after digestion analyzed by immunoprecipitation, SDS–PAGE, and autoradiography. The positions of various species are labeled as in Figure 1.

To demonstrate directly that the lack of TRAM alone was not responsible for the ^{sec}PrP-specific defect caused by glycoprotein depletion, these depleted proteoliposomes were replenished with either total glycoproteins or purified TRAM and assayed for translocation (Figure 4b). As expected, the defect in β-lactamase translocation was restored with total glycoproteins as well as pure TRAM, indicating that for this substrate, the defect was likely due only to the absence of TRAM. By contrast, ^{sec}PrP translocation was only restored with total glycoproteins, and not by TRAM. This indicates that a glycoprotein other than TRAM is necessary for ^{sec}PrP biogenesis, although the data do not necessarily rule out a role for TRAM in some aspect of this process.

To characterize the factor involved in ^{sec}PrP biogenesis, we began fractionation of this activity. We had already determined that peripheral membrane proteins were not likely to be required by demonstrating that high salt–washed membranes were equally active as starting rough microsomal membranes (R. S. H. and V. R. L., unpublished data). To subsequently determine whether lumenal proteins are required, rough microsomal membranes were extracted with saponin, which efficiently releases lumenal contents without solubilizing membrane proteins (Panzner et al., 1995). We found that the proteoliposomes made from the saponin-extracted membranes were as active in translocating ^{sec}PrP as proteoliposomes made from the starting rough microsomal membranes (Figure 4c, compare lanes 1 and 3). As expected, the activity in these saponin-extracted membranes was depleted upon glycoprotein removal (Figure 4c, compare lanes 3 and 4), suggesting that it is the same activity observed originally.

To subsequently fractionate the activity by ConA chromatography, we took advantage of the variable rate of binding of different glycoproteins to ConA. We observed that the activity was apparently depleted rapidly by ConA, whereas many other glycoproteins are much slower to bind. Furthermore, if eluted immediately, the activity eluted efficiently, while many proteins appeared to remain bound to the resin. By combining these observations (see Experimental Procedures), we were able to prepare an enriched glycoprotein fraction that contained more than 50% of the ^{sec}PrP translocation activity while containing only 0.7% of the starting proteins (Figure 4c). Thus, an increase in specific activity of approximately 75- to 100-fold has been achieved. Further separation of this enriched fraction by other chromatographic methods should facilitate the identification of the protein(s) specifically involved in ^{sec}PrP biogenesis.

Discussion

Several independent lines of evidence in this study indicate that PrP biogenesis involves translocation accessory factors (for which we propose the general term TrAF) that are not required for the biogenesis of other secretory or membrane proteins examined thus far. First, proteoliposomes containing the known minimal translocation machinery (composed of Sec61 complex, SRP receptor, and TRAM) fail to make either ^{sec}PrP or ^{Ntm}PrP, but rather make only the ^{Ctm}PrP form; these same membranes are able to translocate all other substrates examined. Second, PrP mutants unable to make the transmembrane forms failed to translocate at all in DST proteoliposomes, while translocating efficiently into total microsomal membranes. Third, although all three forms of PrP are made in proteoliposomes prepared from certain detergent extracts of rough microsomes, the ^{sec}PrP and ^{Ntm}PrP forms are made less efficiently than the ^{Ctm}PrP form. Finally, the ability to make ^{sec}PrP in these reconstituted proteoliposomes is dependent on a glycoprotein fraction that could not be replaced with TRAM alone.

Together with the observation that the defect in PrP translocation is at a posttargeting step, these data lead to a model of PrP biogenesis involving at least one TrAF (Figure 4d). In the first step (requiring cytosolic SRP, its membrane receptor, and Sec61 complex), PrP is targeted to and docked at the translocation channel at

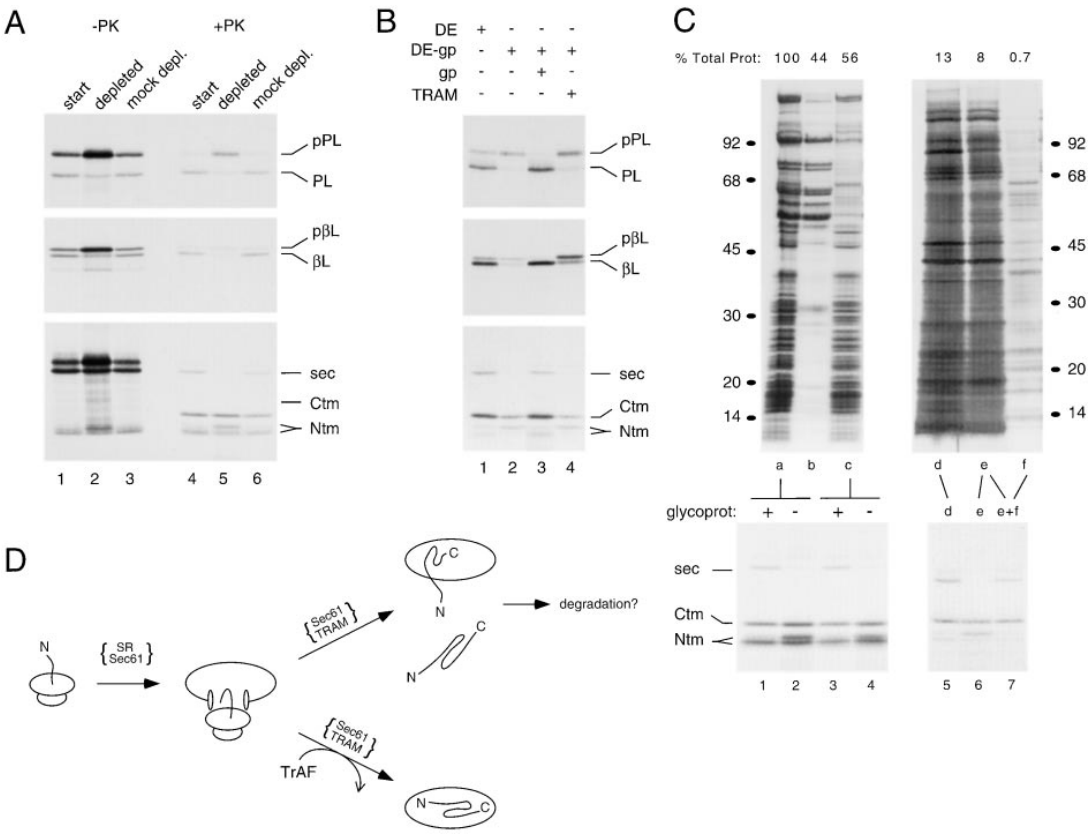

Figure 4. Role of ER Glycoproteins in PrP Translocation and Topology

(A) A 0.5% cholate extract of EKRM was prepared and either left untreated (start), depleted of glycoproteins (depleted), or mock depleted in the presence of competitive sugar (mock depl.) prior to reconstitution into proteoliposomes. These three membrane preparations were then assayed for translocation of prolactin, β-lactamase, or PrP as in Figure 1.

(B) Proteoliposomes were prepared containing various combinations of a 0.8% DBC extract of EKRMs (DE), a glycoprotein-depleted 0.8% DBC extract (DE-gp), total glycoproteins (gp), or purified TRAM as indicated above each lane. These were then assayed for translocation of prolactin, β-lactamase, and PrP as in Figure 3.

(C) Membranes were fractionated as described in the Experimental Procedures. Two eq of each fraction ([a] = EKRM, [b] = saponin extract, [c] = saponin-extracted membranes, [d] = 0.9% DBC extract of saponin-extracted membranes, [e] = ConA flowthru, and [f] = ConA eluate) were analyzed by SDS–PAGE and staining with either Coomassie blue ([a]–[c]) or silver ([d]–[f]). The relative percent of starting protein in each fraction is indicated above each lane, and molecular weight markers are adjacent to each gel. The membrane fractions (a) and (c) were used to prepare proteoliposomes containing or lacking glycoproteins and assayed for PrP translocation as in (B) (lanes 1–4). Fractions (d)–(f) were used to prepare proteoliposomes (as indicated) and also assayed for PrP translocation (lanes 5–7).

(D) Model for the mechanism of PrP biogenesis. The membrane proteins required for each step are indicated above the arrows. For simplicity, the biogenesis of the ^{Ntm}PrP form is not shown, although it would also require a TrAF for its biogenesis. See text for details.

the ER membrane. In the presence of only the minimal translocation machinery, this PrP is subsequently made in either the ^{Ctm}PrP form, or in a form that ultimately resides in the cytosol. These forms are likely to be targets of degradation in vivo under normal circumstances. If on the other hand the proper TrAF(s) are present at the translocon, PrP can be made in the ^{sec}PrP form, the topology observed in normal brain.

Although the role of ^{Ntm}PrP is currently unclear, several findings suggest that its biogenesis also requires a TrAF. First, the reconstituted membranes generated following different detergent extraction conditions (Figure 3a) show a different optimum for ^{Ntm}PrP (at 0.45%) than that observed for ^{sec}PrP or ^{Ctm}PrP (Figure 3d). Second, the ^{Ntm}PrP form is not synthesized in the DST membranes, but is made in both the starting membranes and in membranes reconstituted from total membrane proteins (Figure 1d and Figure 3d). And finally, the TrAF needed for ^{Ntm}PrP synthesis seems not to be a glycoprotein, since ^{Ntm}PrP (by contrast to ^{sec}PrP) is readily made in the glycoprotein-depleted proteoliposomes (Figure 4a). Thus, it appears that the topology of PrP is regulated by the action of more than one TrAF acting during translocation across the ER membrane.

Two implications of this work are particularly noteworthy. First, the default topology achieved by PrP in the presence of the core components of the translocation channel is the ^{Ctm}PrP form, which has been implicated in the development of neurodegeneration (Hegde et al., 1998). Thus, it appears that one potential role of the TrAF(s) involved in PrP biogenesis is to protect the cell from generating an otherwise cytotoxic form of PrP. As a corollary, it is plausible that under certain conditions (perhaps during development), TrAF activity is appropriately regulated to elicit synthesis of ^{Ctm}PrP and thus a form of programmed cell death. The second implication

is that translocation and topology are not necessarily "constitutive" features of gene expression, determined solely by the sequence of the protein being translated. Rather, it appears that for some proteins, the translocation machinery plays an important decision-making role in determining the topology and folding of the nascent chain.

The demonstration that *trans*-acting factors can manipulate a single substrate to achieve multiple topological forms provides a system in which the molecular basis for this regulation can be dissected. Further work is necessary to identify other substrates responsive to such regulation by either these or other TrAFs.

Experimental Procedures

Transcription, Translation, and Translocation

cDNAs encoding each substrate, engineered behind the SP6 promoter in the SP64 plasmid, have been described previously (Simon et al., 1987; Hegde et al., 1998). The transmembrane-favoring PrP mutant used in the figures had three alanine-to-valine changes at positions 113, 115, and 118. Similar results were also obtained with a second transmembrane-favoring mutant in which the lysine and histidine residues at positions 110 and 111 were changed to isoleucines (data now shown). The secretory-favoring PrP mutant used in the figures had a glycine-to-proline change at position 123. Similar results were also obtained with a second secretory-favoring mutant in which residues 104–113 were deleted (data now shown). In vitro transcription with SP6 polymerase, translation of this message in RRL, and translocation into microsomal membranes has been described (Hegde et al., 1998 and references therein). To prepare truncated message for assessment of targeting (Figure 2), the plasmids were digested with restriction enzymes (HincII at codon 180 in PrP or PvuII at codon 86 in preprolactin) and subsequently transcribed in vitro. Following translation of these truncated messages in RRL in the presence of the microsomal membranes, a 5 μl aliquot was diluted to 50 μl, adjusted to 500 mM KOAc, 5 mM $MgOAc_2$, 50 mM HEPES (pH 7.4), and layered onto 100 μl of 0.5 M sucrose in the same buffer. Membranes were sedimented by centrifugation for 4 min at 50,000 rpm in TLA100 rotor (Beckman), and the pellet dissolved in 1% SDS, 0.1 M Tris (pH 8), prior to analysis by SDS–PAGE.

Preparation of Proteoliposomes

Purification of SRP receptor, Sec61 complex, and TRAM, and their incorporation into DS or DST proteoliposomes were performed exactly as described previously (Görlich and Rapoport, 1993). The proteoliposomes in Figure 3 were prepared from EDTA and high salt–washed rough microsomes (EKRMs, prepared as described by Nicchitta and Blobel, 1990) and resuspended in extraction buffer (15% v/v glycerol, 350 mM KOAc, 12 mM $MgOAc_2$, 50 mM HEPES [pH 7.4], 5 mM 2-mercaptoethanol) at 1 equivalent (eq, as defined by Walter and Blobel, 1983) per μl. To 80 μl aliquots, 20 μl of cholate (in extraction buffer) was added to the final concentrations indicated in Figure 3, mixed well, and incubated at 4°C for 15 min, and particles larger than 20 S were removed by centrifugation. Soluble proteins were reconstituted into proteoliposomes by incubation with ~35 mg of Biobeads SM2 (BioRad) at 4°C for 12 hr, and the liquid phase was removed and diluted 5-fold with ice-cold water. The membranes were then sedimented by centrifugation (10 min at 70,000 rpm in TL100.3 rotor) and resuspended in 20 μl of 100 mM KAc, 0.25 M sucrose, 1.5 mM $MgAc_2$, 50 mM HEPES [pH 7.4], 1 mM DTT. These were used at a concentration of 1 μl per 10 μl translation reaction. For depletion of glycoproteins, detergent-extracted proteins were incubated with 0.2 vol of packed conconavalin A–coupled Sepharose beads (Pharmacia) with constant mixing for 12 hr at 4°C. Mock depletions contained 0.5 M α-D-methylmannopyannoside during the incubation with conconavalin A. The unbound proteins were removed and used for reconstitutions as described above. Depletion and replenishment of glycoproteins in Figure 4b were performed with detergent extracts prepared with 0.8% deoxyBigCHAP (DBC). Total glycoproteins were prepared by elution from glycoprotein-bound ConA beads with 1 M α-D-methylmannopyrannoside in extraction buffer containing 0.8% DBC (room temperature for 12 hr). The eluted proteins were precipitated by addition of polyethylene glycol 6000 to 15% w/v, and the precipitate (collected by centrifugation) was dissolved in the glycoprotein depleted extract. Alternatively, the equivalent amount of purified TRAM (prepared as previously described by Görlich and Rapoport, 1993) corresponding to the amount in the total glycoprotein fraction was added to the glycoprotein-depleted extract. Reconstitutions were performed by incubation with 100 mg of Biobeads per 100 μl sample, and the membrane was collected as above.

Fractionation of Membranes

EKRMs were resuspended at 0.5 eq per μl in 50 mM triethanolamine (pH 7.4), 250 mM sucrose, 1 mM DTT, and saponin was added to 1% from a 20% w/v stock (Sigma, prepared as described by Panzner et al., 1995). After 15 min on ice, the membranes were sedimented, rinsed once, and resuspended at 1 eq per μl in extraction buffer. A 0.9% detergent extract was prepared as above using DBC and warmed to room temperature. 200 μl was applied by gravity to a 100 μl column of ConA, previously equilibrated in extraction buffer containing 0.25% DBC. The column was washed with 400 μl of the equilibration buffer and eluted with extraction buffer containing 0.5 M α-D-methylmannopyannoside and 0.9% DBC. The flowthru fraction from above as well as the first 200 μl of the eluate were saved and used in reconstitutions. 100 μl of the flowthru fraction was mixed with either 100 μl of eluate or elution buffer, and reconstituted into proteoliposomes as above with 200 mg of biobeads. In parallel, 100 μl of the starting detergent extract was mixed with 100 μl of elution buffer and reconstituted.

Acknowledgments

We would like to thank Tom Rapoport for useful discussions, encouragement, and comments on this manuscript.

Received April 1, 1998; revised May 12, 1998.

References

Blobel, G., and Dobberstein, B. (1975). Transfer of proteins across membranes. I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. J. Cell Biol. *67*, 835–851.

Dunlop, J., Jones, P.C., and Finbow, M.E. (1995). Membrane insertion and assembly of ductin: a polytopic channel with dual orientations. EMBO J. *14*, 3609–3616.

Görlich, D., Hartmann, E., Prehn, S., and Rapoport, T.A. (1992). A protein of the endoplasmic reticulum involved early in polypeptide translocation. Nature *357*, 47–52.

Görlich, D., and Rapoport, T.A. (1993). Protein translocation into proteoliposomes reconstituted from purified components of the endoplasmic reticulum membrane. Cell *75*, 615–630.

Gafvelin, G., Sakaguchi, M., Andersson, H., and von Heijne, G. (1997). Topological rules for membrane protein assembly in eukaryotic cells. J. Biol. Chem. *272*, 6119–6127.

Hegde, R.S., Mastrianni, J.A., Scott, M.R., DeFea, K.A., Tremblay, P., Torchia, M., DeArmond, S.J., Prusiner, S.B., and Lingappa, V.R. (1998). A transmembrane form of the prion protein in neurodegenerative disease. Science *279*, 827–834.

Katz, F.N., and Lodish, H.F. (1979). Transmembrane biogenesis of the vesicular stomatitis virus glycoprotein. J. Cell Biol. *80*, 416–426.

Levy, D. (1996). Membrane proteins which exhibit multiple topological orientations. Essays Biochem. *31*, 49–60.

Nicchitta, C.V., and Blobel, G. (1990). Assembly of translocation-competent proteoliposomes from detergent-solubilized rough microsomes. Cell *60*, 259–269.

Nicchitta, C.V., Migliaccio, G., and Blobel, G. (1991). Biochemical fractionation and assembly of the membrane components that mediate nascent chain targeting and translocation. Cell *65*, 587–598.

Oliver, J., Jungnickel, B., Görlich, D., Rapoport, T., and High, S. (1995). The Sec61 complex is essential for the insertion of proteins into the membrane of the endoplasmic reticulum. FEBS Lett. *362*, 126–130.

Panzner, S., Dreier, L., Hartman, E., Kostka, S., and Rapoport, T.A. (1995). Posttranslational protein transport in yeast reconstituted with a purified complex of Sec proteins and Kar2p. Cell *81*, 561–570.

Rogers, M., Serban, D., Gyuris, T., Scott, M., Torchia, T., and Prusiner, S.B. (1991). Epitope mapping of the Syrian hamster prion protein utilizing chimeric and mutant genes in a vaccinia virus expression system. J. Immunol. *147*, 3568–3574.

Schatz, G., and Dobberstein, B. (1996). Common principles of protein translocation across membranes. Science *271*, 1519–1526.

Simon, K., Perara, E., and Lingappa, V.R. (1987). Translocation of globin fusion proteins across the endoplasmic reticulum membrane in Xenopus laevis oocytes. J. Cell Biol. *104*, 1165–1172.

Skach, W.R., Calayag, M.C., and Lingappa, V.R. (1993). Evidence for an alternate model of human P-glycoprotein structure and biogenesis. J. Biol. Chem. *268*, 6903–6908.

Stahl, N., Borchelt, D.R., Hsiao, K., and Prusiner, S.B. (1987). Scrapie prion protein contains a phosphatidylinositol glycolipid. Cell *51*, 229–240.

Voigt, S., Jungnickel, B., Hartmann, E., and Rapoport, T.A. (1996). Signal sequence-dependent function of the TRAM protein during early phases of protein transport across the endoplasmic reticulum membrane. J. Cell Biol. *134*, 25–35.

Walter, P., and Blobel G. (1983). Preparation of microsomal membranes for cotranslational protein translocation. Methods Enzymol. *96*, 84–93.

Yost, C.S., Lopez, C.D., Prusiner, S.B., Myers, R.M., and Lingappa, V.R. (1990). Non-hydrophobic extracytoplasmic determinant of stop transfer in the prion protein. Nature *343*, 669–672.

Zhang, J.T., and Ling, V. (1995). Involvement of cytoplasmic factors regulating the membrane orientation of P-glycoprotein sequences. Biochemistry *34*, 9159–9165.