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

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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 CmPrP 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; Gaßel et al., 1997). However, a few proteins have been found that can exist in two or more topological forms (Schach 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 CmPrP form is fully translocated across the ER membrane, while the CmPrP and CmPrP forms each span the membrane once, in opposite orientations, at the same hydrophobic stretch (roughly residues 110-135). The CmPrP form has the COOH terminus of the protein in the ER lumen, while NmPrP has the NH2 terminus in the lumen.

Despite this potential topological heterogeneity, only the CmPrP 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 CmPrP 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 CmPrP synthesis in cell-free translation systems. Furthermore, elevated CmPrP 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 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 topology is regulated by the currently known translocation machinery toward the generation of CmPrP. 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...
restored translocation of β-lactamase, while stimulating prolactin translocation modestly. Analysis of PrP translocation in these proteoliposomes revealed that only the CtmPrP form is made (Figure 1d). Most notably, the secPrP 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 CtmPrP form (Hegde et al., 1998). As expected, this mutant PrP did not generate secPrP in the DS or DST proteoliposomes (Figure 1e). Identical results were obtained when another CtmPrP-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 secPrP, 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 translocated into the lumen suggests that additional translation components are required to make secPrP.

In principle, the inability of DST membranes to make secPrP 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
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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, \(\beta\)-lactamase, and PrP. Prolactin translocated well into each of these membranes (Figure 3b), while \(\beta\)-lactamase showed optimal translocation only in membranes prepared from higher detergent extracts (Figure 3c). This optimum at higher detergent concentrations reflects dependence of \(\beta\)-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 \(\text{ConPrP}\) 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 \(\beta\)-lactamase (e.g., see Figure 1). However, \(\text{secPrP}\) and \(\text{NmpPrP}\) 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 \(\text{secPrP}\) and \(\text{NmpPrP}\) were made substantially better than in the DST membranes (compare to Figure 1d). This is in contrast to the \(\text{ConPrP}\) 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, \(\beta\)-lactamase, and PrP translocation (Figure 4a). As expected, prolactin translocated efficiently into each of these three membrane vesicles. By contrast, the TRAM-dependent substrate \(\beta\)-lactamase failed to translocate into the glycoprotein-depleted vesicles, reflecting the lack of the glycoprotein TRAM. Analysis of PrP demonstrates that the glycoprotein-depleted vesicles contain a defect only in \(\text{secPrP}\) translocation, with \(\text{ConPrP}\) and \(\text{NmpPrP}\) 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 \(\text{secPrP}\) was due only to the lack of TRAM. This is supported further by the observation that \(\text{secPrP}\) was not made in the DST proteoliposomes (which contained TRAM; see Figure 1d).
To demonstrate directly that the lack of TRAM alone was not responsible for the \(^{35}S\)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 \(\beta\)-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, \(^{35}S\)PrP translocation was only restored with total glycoproteins, and not by TRAM. This indicates that a glycoprotein other than TRAM is necessary for \(^{35}S\)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 \(^{35}S\)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 luminal proteins are required, rough microsomal membranes were extracted with saponin, which efficiently releases luminal 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 \(^{35}S\)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 \(^{35}S\)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 \(^{35}S\)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 \(^{35}S\)PrP or \(^{35}S\)NtmPrP, but rather make only the \(^{35}S\)CtmPrP 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 \(^{35}S\)PrP and \(^{35}S\)NtmPrP forms are made less efficiently than the \(^{35}S\)CtmPrP form. Finally, the ability to make \(^{35}S\)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
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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 nPrP 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 CmPrP 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 hPrP form, the topology observed in normal brain.

Although the role of nPrP 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 nPrP (at 0.45%) than that observed for cPrP or cmPrP (Figure 3d). Second, the nPrP 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 nPrP synthesis seems not to be a glycoprotein, since nPrP (by contrast to cPrP) 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 cPrP 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 cPrP 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 not 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 not 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 RRL, and translocation into microsomal membranes has been described exactly as described previously (Go è rlich and Rapoport, 1993). The transmembrane-favoring PrP 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-methylmannopyranoside and 0.9% DBC. The flowthrough fraction from above as well as the first 200 μl of the eluate were saved and used in reconstitutions. 100 μl of the flowthrough fraction was mixed with either 100 μl of eluate or elution buffer, and reconstituted into proteoliposomes as above with 200 μg of biobeads. In parallel, 100 μl of the starting detergent extract was mixed with 100 μl of elution buffer and reconstituted.

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**References**


Fractionation of Membranes

ERMs 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 warned 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-methylmannopyranoside and 0.9% DBC. The flowthrough fraction from above as well as the first 200 μl of the eluate were saved and used in reconstitutions. 100 μl of the flowthrough fraction was mixed with either 100 μl of eluate or elution buffer, and reconstituted into proteoliposomes as above with 200 μg of biobeads. In parallel, 100 μl of the starting detergent extract was mixed with 100 μl of elution buffer and reconstituted.


