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Biogenesis of Polytopic Membrane Proteins: Membrane Segments Assemble within Translocation Channels prior to Membrane Integration

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Summary

The initial steps in the biogenesis of membrane proteins parallel that of secretory proteins. The translocation of membrane proteins, however, must be interrupted prior to the complete traversal of the membrane. This is followed by their folding and integrating into the lipid bilayer. We have previously shown that as each latent transmembrane segment (TMS) in a polytopic membrane protein emerges from the ribosome, it sequentially translocates across the membrane. Here we demonstrate that these translocated TMSs can be extracted from the membrane with urea. This suggests that nascent TMSs do not integrate into the bilayer as they achieve a transmembrane topography. The integration is delayed until after the protein is synthesized and released from the ribosome. Prior to insertion into the bilayer, these TMSs appear to be stabilized by salt-sensitive electrostatic bonds within an aqueous-accessible compartment.

Introduction

Membrane proteins establish their transmembrane topography in the endoplasmic reticulum (ER) en route to their final destinations via the secretory pathway (Palade, 1975). It is here at the ER that these proteins must be partially translocated across the membrane bilayer, folded, and integrated into the lipid environment of the membrane. These later steps are the subject of this study.

The initial steps in the biogenesis of membrane proteins closely parallel the well-characterized biogenesis of secretory proteins in the ER. A topogenic or signal sequence in the primary amino acid structure of both secretory and membrane proteins is responsible for targeting to the ER (Blobel, 1980). Binding of a cytosolic complex, the signal recognition particle, to this signal sequence is required for the targeting of both secretory and membrane proteins (Anderson et al., 1982; Friedlander and Blobel, 1985). Competition studies have demonstrated that nascent chains of membrane proteins compete with secretory proteins for the translocation machinery, whereas cytosolic proteins do not (Lingappa et al., 1978). Certain proteins, such as μ chains of immunoglobin, exist in both secretory and membrane forms (McCune et al., 1980, 1981), differing by a stretch of amino acids located at the carboxyl terminus (McCune et al., 1980). Translocation proceeds identically for the two isoforms until the membrane-embedding signal in the carboxyl terminus is reached for the membrane form. Further evidence for common biochemical steps in the translocation of secretory and membrane proteins are the observations that nascent proteins of both kinds cross-link to the same ER-resident membrane proteins during translocation (Thrift et al., 1991) and both require the same ER membrane protein, Sec61, in order to translocate (Rothblatt et al., 1989; Green et al., 1992; Stirling et al., 1992; Kalies et al., 1994; Mothes et al., 1994).

Secretory proteins are believed to translocate through an aqueous environment, a protein-conducting channel (Blobel and Dobberstein, 1975; Gilmore and Blobel, 1985; Simon et al., 1989; Simon and Blobel, 1991, 1992; Joly and Wickner, 1993; Crowley et al., 1994). However, not much is known of the biophysical environment of a translocating membrane protein. This work examines that environment. The goal was to determine when during the biosynthesis of membrane proteins they interact with and integrate into the lipid bilayer. Nascent membrane proteins were synthesized from mRNA truncated without a termination codon at various points in the coding region for the protein. This allowed us to examine them during biogenesis, that is, while they were being translated on ribosomes, translocated across the membrane, folded, and integrated into the lipid bilayer. We recently demonstrated that, under proper conditions, these nascent polypeptides can be observed while still linked to their translating ribosomes via ester bonds between their carboxy-terminal amino acids and the last tRNA added (Borel and Simon, submitted). Thus, these truncated proteins are true "translation intermediates." Such translation intermediates are both properly targeted to the ER membrane and have domains translocated into the ER lumen, as assayed by asparagine-linked glycosylation. Therefore, the translation intermediates are also "translocation intermediates."

The environment of the translocation intermediates was explored by testing their extractability from the membrane. A protein can be biochemically defined to be integrated into the lipid bilayer if it can be extracted from the membrane only by detergent solubilization. Two different reagents have been used to test the extractability of proteins from the membrane: high concentrations of urea (Gilmore and Blobel, 1985) or a strong base, usually NaOH or Na_2CO_3, at pH 11.5, (Steck and Yu, 1973; Fujiki et al., 1982; Russel and Model, 1982). This technique, which does not solubilize the membrane, extracts only peripheral membrane and secretory proteins. Unfortunately, the ester bond between the nascent chain and its tRNA in the translocation intermediate is sensitive to treatment with strong base, and the tRNA-bound intermediates are released from their tRNAs and ribosomes. Therefore, this analysis destroys the very translation intermediates it is intended to examine. Urea is a chaotropic agent that at high concentrations denatures proteins via direct binding to their surface (Kawahara et al., 1965; Tanford, 1968, 1970; Timasheff, 1993). Since urea does not affect the stability of the peptidyl–tRNA bond, it was used as our test for membrane extractability of transmembrane segments (TMSs).

In all experiments, the extraction of translocation intermediates was compared with that of proteins that

were released from their tRNA and ribosomes (Borel and Simon, submitted). This was accomplished by treating each reaction with either of the protein synthesis inhibitors puromycin or cycloheximide. Puromycin is a tRNA analog that is used by the peptidyl-transferase to release the nascent peptide–tRNA bond (Traut and Monro, 1964; Redman and Sabatini, 1966; Redman et al., 1966; Blobel and Potter, 1967; Monro and Marcker, 1967; Skogerson and Moldave, 1968). Cycloheximide leaves this bond intact. There are substantial differences in glycosylation and, thus, in the translocation status of a protein between a nascent polypeptide that is a translation intermediate (i.e., attached to both its tRNA and its ribosome) and the same peptide released from its tRNA (Borel and Simon, submitted). For these reasons, analysis was limited either to those peptides that could be shown at the end of the experiment to be still attached to both their tRNA and their ribosome or to those peptides that had been deliberately released from their tRNA and ribosomes (by puromycin) prior to extraction.

We chose P-glycoprotein (Pgp) as our model integral membrane protein. Previously, we showed that the first TMS of Pgp moves across the membrane after exiting from the ribosome, prior to the emergence of the second TMS. Thus, the TMSs sequentially translocate across the membrane as they emerge from the ribosome. Here, we demonstrate that as translocation proceeds, before release from the ribosome, the nascent TMSs can be extracted from the membrane. However, the protein intermediates are not extracted as completely as secretory proteins, suggesting that they are partially stabilized with the membrane. Raising the salt concentration increases the extraction, while lowering the salt concentration reduces the extraction. In contrast, upon release from the ribosome, the polypeptides are resistant to extraction. Thus, the data suggest that nascent TMSs, although spanning the membrane, do not integrate into the lipid bilayer until the termination of synthesis. Until they are released from their biosynthetic ribosomes, the nascent membrane proteins remain stabilized within an aqueous-accessible compartment by electrostatic interactions.

Results

Translation Products from Truncated mRNA

The pSPMDR1 DNA template which encodes Pgp was linearized at different restriction endonuclease sites (see Table 1). The DNA was transcribed, and the mRNA transcripts, truncated at different 3′ positions, were translated in in vitro translation reactions. When assaying translocation, we augmented these translations with pancreatic ER microsomes. To examine each Pgp intermediate, reactions were first tested for proper targeting to the ER microsomes. All intermediates, including the shortest construct with only one TMS of Pgp, were at least 90% targeted to the ER membrane (data not shown). These targeted intermediates were used for further analyses. Although much work has focused on elucidating the topography of this protein, there is not yet a consensus on the number of TMSs (Chen et al., 1986; Gerlach et al., 1986; Gros et al., 1986; Zhang et al., 1993). For our purposes, it is accepted that Pgp has its amino terminus on the cytoplasmic side of the ER (Georges et al., 1993; Skach et al., 1993). The true final topography or topographies of subsequent TMSs of Pgp will not affect our observations.

The translation reactions from the truncated mRNA were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and usually yielded at least two translation products (Figures 1B and 2) and often many more. Each of these has been characterized (Borel and Simon, submitted). One product (marked with an open circle) migrated at the expected molecular mass of the translated protein. A second migrated at a predicted molecular mass that was 25–30 kDa heavier (marked with an open square). This heavier product was observed in the presence and absence of ER vesicles. However, it was not seen after any treatment that dissociates the peptide–tRNA bond, such as NaOH or Na_2CO_3, raising the temperature of the translation reaction, or adding puromycin (see lanes 3 and 4 of Figures 1B or 2). When these translation reactions were treated with cefyltrimethylammonium bromide to precipitate tRNA, only the bands indicated by open squares were in the pellet. All the bands marked by open circles remained in the supernatant. Thus, although this slower-migrating band (open square) has not been reported in previous studies using truncated mRNA, the data indicate that this band represents the true tRNA-bound nascent chain. When the translation reaction was solubilized with detergent and fractionated on a sucrose gradient, it was this slower, open square tRNA-bound band that migrated with the ribosomes (Borel and Simon, submitted).

Often more than one of these slower-migrating bands marked by open squares were observed (see lanes 1

Table 1. Biosynthetic Intermediates: Pgp-Shortened Proteins

Restriction Enzyme of *MDR1* Template DNA	Length of DNA Template (bp)	Peptide Length (amino acids)	Estimated Molecular Mass (kDa)	Distance from Putative TMS
BbsI	329	110	11.9	TMS1+38
BanI	404	135	14.7	TMS2−6
PvuII	415	138	15.2	TMS2−3
ApaLI	490	163	17.9	TMS2+22 or TMS3−25
BsmAI	537	179	19.7	TMS2+39 or TMS3−8
BsaAI	625	208	22.9	TMS3−1
NsiI	921	307	33.8	TMS5−10
TaqI	1132	377	41.5	TMS6+31
EcoRI	1174	391	43	TMS6+45

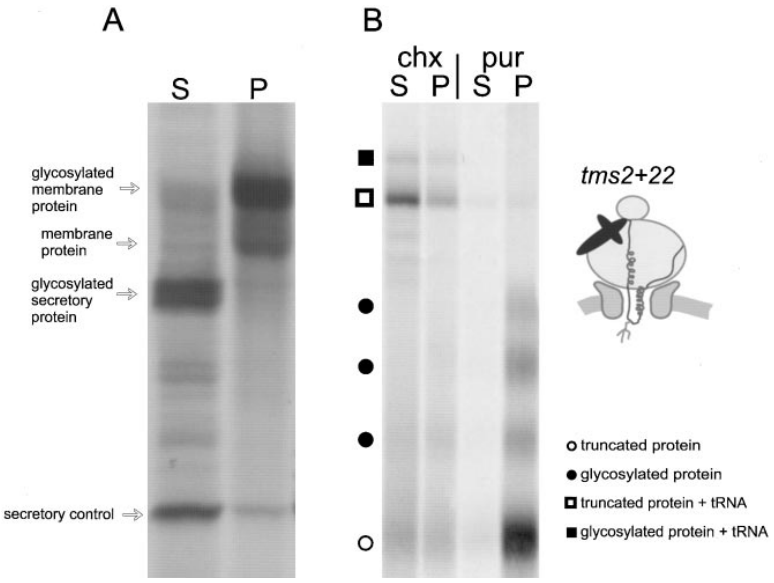

Figure 1. Urea Fractionation of Translocation Intermediates

(A) Urea treatment fractionates secretory and membrane proteins. Test secretory control proteins (pre-pro-α factor) and membrane control protein ($pSPS_LG_{ST}P$) were translated in the presence of ER vesicles. The translation products for each protein were mixed, and the ER vesicles were harvested, resuspended in 4.5 M urea, and then centrifuged to separate supernatant (S) and pellet (P) fractions, as described in Experimental Procedures. The pre-pro-α factor and its glycosylated forms were consistently in the supernatant fraction. In contrast, the $pSPS_LG_{ST}P$ and its glycosylated forms were consistently in the pellet (membrane) fraction. Similar results were observed using other control membrane proteins such as bovine opsin and vesicular stomatitis virus glycoprotein (data not shown). These control extractions were performed in parallel with all test extractions.

(B) Urea treatment fractionates tRNA-bound translocation intermediates from released-peptides. The mRNA TMS2+22 was translated in reticulocyte lysate with pancreatic ER microsomes as described in (A). The translation mix was divided in half and treated with either puromycin (pur) or cycloheximide (chx) (as described in Experimental Procedures), and then the ER membranes were harvested and treated with urea as described above for the control proteins pre-pro-α factor and $pSPS_LG_{ST}P$. The tRNA-bound nascent peptide (marked with open and closed squares) was consistently found in the supernatant, extracted from the membrane fraction. In contrast, nascent peptides that had been released from their biosynthetic tRNA and ribosomes with puromycin (marked with closed and open circles) could not be extracted from the membrane and were consistently in the pellet fraction.

and 2 in each of the autoradiograms in Figure 2). Each has been shown to be the consequence of different isoaccepting tRNAs bound to nascent polypeptide of the same size (Borel and Simon, submitted). When the tRNAs from these translation reactions were precipitated with cefyltrimethyl-ammonium bromide, only the multiple bands marked by open squares were observed in the pellet. Upon subsequent treatment with base to release the tRNA, only a single [^{35}S]methionine-radiolabeled band remained that migrated with the bands marked by open circles. Thus, the different open square bands contain only a single protein species.

An additional set of [^{35}S]methionine-labeled bands (closed squares) that migrated more slowly than the tRNA-bound open square bands were observed only in the presence of ER vesicles. They were not seen when the ER vesicles were pretreated with a tripeptide of Asn–Tyr–Thr, an acceptor peptide that is a competitive inhibitor of asparagine-linked glycosylation. The closed square bands also disappeared after treatment with puromycin. Thus, the closed square bands were glycosylated, tRNA-bound nascent peptides. Another set of [^{35}S]methionine-labeled bands (closed circle) were observed that migrated more slowly than the mature peptide (open circle). These closed circle bands were observed neither in the absence of ER vesicles nor in the presence of inhibitors of asparagine-linked glycosylation. Their mobility, though, was unaffected by NaOH or puromycin. Therefore, the bands marked by closed circles were glycosylated forms of the truncated tRNA-released peptides.

Urea Extraction of Control Membrane and Secretory Proteins

Urea treatment has been used to distinguish between peripheral and integral membrane proteins (Gilmore and Blobel, 1985). The assay was tested by using a 4.5 M urea treatment on known control membrane and secretory proteins (Figure 1A). Approximately 80% of pre-pro-α factor, a secretory protein, was in the supernatant fraction, demonstrating that it was almost completely extracted. After treatment with 4.5 M urea, 90% of the membrane control protein, $pSPS_LG_{ST}P$ (Rothman et al., 1988), remained in the pellet, associated with the membrane fraction (Figure 1). Indistinguishable results were observed for other transmembrane control proteins, the vesicular stomatitis virus glycoprotein and bovine opsin and the control secretory protein pre-pro-lactin. Varying the concentration of urea from 4.0 to 7.0 M had minimal effect on the results. The experimental conditions chosen gave results that maximized the amount of control membrane protein in the pellet and secretory protein in the supernatant fraction.

Urea Extraction of Translocation Intermediates

The effects of these urea treatments (4.5 M urea in 150 mM KCl) were tested on six of the translocation intermediates (Figure 2). In the first two lanes of each panel of Figure 2 are translation reactions that were treated with cycloheximide (to leave the translocation intermediates intact). The next two lanes were treated with puromycin (to release the peptides from the tRNA). For all constructs tested, >95% of the peptide released from the tRNA was found in the membrane pellet (Figure 2, lanes 3 and 4 of each autoradiogram), indicating that they could not be extracted with urea. This was true for all constructs tested, from the shortest (truncated 38 amino acids after the first TMS) to the longest (truncated 45 amino acids after the sixth TMS).

In striking contrast, a majority (50%–80%) of the translocation intermediates, those nascent peptides still

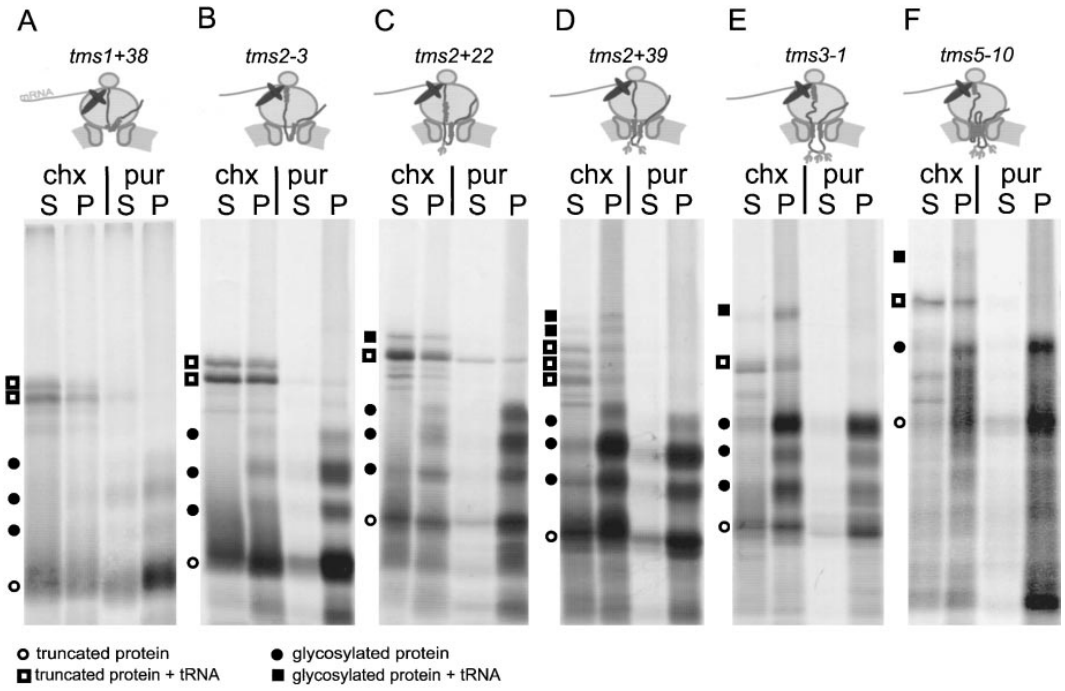

Figure 2. Urea Extractions on Translocation Intermediates in Physiological Salt

The shortened Pgp mRNAs listed in Table 1 were synthesized with pancreatic ER microsomes and treated with urea to extract nonmembrane proteins as described in Figure 1B. The results are shown for six of the constructs. Each translation was split in half and treated with cycloheximide (chx) or puromycin (pur). This allowed us to contrast the fractionation of translocation intermediates that were still attached to tRNA (cycloheximide-treated samples, lanes 1 and 2 of each gel) and nascent peptides that had been released from their tRNA (puromycin-treated samples, lanes 3 and 4 of each gel). For all constructs, greater than 90% of the tRNA-released peptides fractionated as membrane proteins (bands marked with closed or open circles in lanes 3 and 4 of each autoradiogram). In contrast, for all of the constructs, a majority (50%–70%) of the tRNA-attached peptide fractionates as a secretory protein (bands marked with open or closed squares in lanes 1 and 2 of each autoradiogram).

attached to their tRNA, were found in the supernatant fraction after urea treatment, indicating that they could be extracted from the membrane (Figure 2, lanes 1 and 2 of each autoradiogram). Once again, in these cycloheximide-treated lanes, the analysis focused on those bands (marked by open and closed squares) demonstrably bound to tRNA. It is difficult to resolve whether the peptides unintentionally released from the tRNA (open or closed circles) were released prior to fractionation, during fractionation, or during subsequent sample preparation.

The TMS1+38 construct (the mRNA was terminated so that protein synthesis halts 38 amino acids after TMS1; see Table 1 for details) in Figure 2A was truncated between the first two TMSs. There was no indication of glycosylation on the translation peptide, and it was fully extracted from the membrane with urea. However, when the ER vesicles were harvested from the translation mix, over 90% of the translation intermediates were harvested as well. This indicates that this translation intermediate has been targeted to the membrane. This is further supported by the observation that after treatment with puromycin the peptide cannot be extracted with urea. Similar observations apply to the TMS2−3 construct (Figure 2B). These shortest constructs did not appear to be translocated, as assayed by glycosylation. However, they were harvested with the ER vesicles and, after puromycin treatment, could not be extracted from the membrane with urea.

It seemed possible that the attached tRNA was facilitating the urea-mediated extraction of the nascent chain from the membrane. This was tested by treating the translation mix with 20 mM EDTA prior to extraction with urea (Figure 3). The EDTA treatment disassembles the ribosomes, but leaves the peptide–tRNA bond intact. Before urea was added, the translation mix was split into three samples that were treated with puromycin (to break the tRNA bond; Figure 3, lanes 1 and 2), cycloheximide (which leaves the translation machinery intact; lanes 3 and 4), and EDTA (which breaks apart the ribosomes; lanes 5 and 6). In the presence of puromycin, the peptide was released, glycosylated on all three sites, and fractionated in the pellet. In the presence of cycloheximide, much of the tRNA-conjugated peptide was in the supernatant. After EDTA treatment to break apart the ribosome, none of the tRNA-bound peptide was extracted from the membrane pellet. This indicates that the presence of a tRNA on the peptide chain does not facilitate extraction from the membrane.

Alternatively, the ribosomes could be facilitating extraction of the translation intermediates. We consider this unlikely for the following reasons. First, following urea extraction, the ribosomes were located in the pellet, while the translocation intermediates were recovered

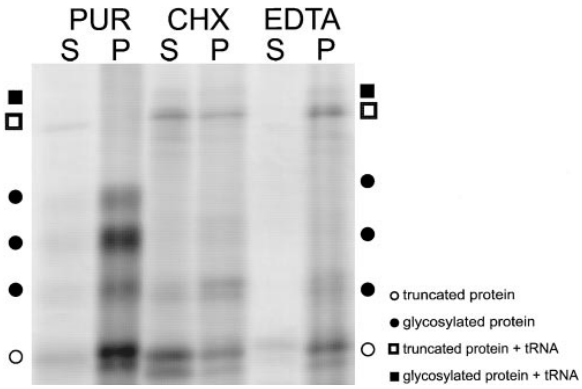

Figure 3. The tRNA Does Not Affect Fractionation of Translocation Intermediate

Translocation intermediates were synthesized as described in Figure 1. At the end of synthesis, the translation mix was divided into three and treated with puromycin (PUR, lanes 1 and 2), cycloheximide (CHX, lanes 3 and 4), or EDTA (lanes 5 and 6). Each sample was then treated with urea and fractionated as described in Figure 2. Treatment with EDTA causes the ribosome to fall apart, but leaves the nascent peptide–tRNA bond intact (see lane 6). This tRNA-bound nascent peptide (closed and open squares) fractionates with the membrane. This indicates that when the translocation intermediates are extracted from the membrane (see lanes 3 and 4) it is not because a tRNA is attached to the nascent chain and is consistent with dissociation from the ribosome being a necessary step in allowing protein integration into the bilayer.

in the supernatant. Second, after urea treatment, the samples were fractionated on a 5%–30% linear sucrose gradient. The translocation intermediates, while still bound to their tRNA, remained on top, and the ribosomes migrated down into the sucrose gradient. Thus, during the urea extraction, not only were the translocation intermediates freed from the membrane, but they were freed from the ribosome as well (Borel and Simon, submitted). This indicates that during treatment with the chaotropic agent urea, there were two competing reactions: urea was unfolding the peptide chain, allowing it to be extracted, and at the same time it was releasing the peptide chain from the ribosome, thereby allowing it to integrate into the membrane. Therefore, the ribosomes were unlikely to have facilitated extraction of the proteins from the membrane.

Effect of Salt on Urea Extraction of Translocation Intermediates

While a majority of the translocation intermediates were extracted by urea (Figure 2, compare the supernatant [S] and pellet [P] lanes for bands marked by open or closed squares), they were not as completely extracted as the test secretory control proteins (Figure 1A, lanes marked S and P for the secretory controls.) To explore the biophysical interactions affecting extraction of the translocation intermediates, we varied the concentration of KCl in the extraction buffer from 0 to 500 mM KCl. (The previous extractions in Figure 2 were done in the more physiological 150 mM KCl). Varying the salt concentration had no effect on the urea extraction of either the secretory or membrane control proteins (Figure 4A).

The effect of varying the salt concentration on the translocation intermediate TMS2−3 is shown in Figure 4B. For this construct, 60% of the translocation intermediates (open and closed squares) were extracted from the membranes with urea in physiological salt (150 mM KCl; the lanes treated with cycloheximide [chx]). However, when the KCl was eliminated from the extraction buffer, only 11% of the translocation intermediate was extracted. In sharp contrast, when the KCl concentration was raised to 500 mM, 84% of the translocation intermediates were extracted from the membrane. At all three salt concentrations, only a small amount (7%–18%) of the peptides released from their tRNA with puromycin were extracted from the membrane (open and closed circles).

The same experiment was performed with the other constructs. The results are shown for TMS2+22 (Figure 4C) and TMS5−10 (Figure 4D) individually. Results for all the constructs are summarized in Figure 5. In the absence of KCl, only small amounts of the translocation intermediates (open and closed squares) were extracted (36% of TMS2+22 and 5% of TMS5−10). At a physiological salt level, a greater percentage were extracted (72% of TMS2+22 and 41% of TMS5−10). Finally, in 500 mM KCl, most of these intermediates were extracted (94% of TMS2+22 and 61% of TMS5−10). And, again, none of the proteins released from their tRNA (open and closed circles) was extracted from the membranes.

Effect of Glycosylation on Extraction of Translocation Intermediates

In the low salt urea treatment, a substantial percentage of the translocation intermediates remained associated with the pellet fraction. The percentage of translocation intermediate that fractionated with the membrane in urea/low salt conditions was plotted (Figure 6A) as a function of peptide length for all nine intermediates. The percent in the pellet for the ribosome-attached intermediates (Figure 6A, solid stippled line with closed triangles) increased with the length of the protein and then leveled out. The first three data points, representing truncated proteins that were targeted to the ER membrane but were not glycosylated, have approximately 30% of the protein associated with the pellet. At the fourth point (TMS2+22 construct), the extraction decreased, with 59% in the pellet. Finally, the percentage of protein in the pellet reached a plateau of 70%–80% for the larger tRNA-bound intermediates. In all cases after puromycin-induced release of the translocation intermediates (broken stippled line with closed triangles), proteins that contained close to two TMSs, or more, were in the pellet (~90%) after urea/low salt treatment.

There were significant changes in the percentage of translocation intermediate extracted from the membrane with urea starting with the construct TMS2+22, the shortest intermediate to be glycosylated. With this construct, at least two events were occurring: N-linked glycosylation of the nascent peptide and translocation of the TMS (and potentially protein folding and interaction with lumenal proteins). Glycosylation has been

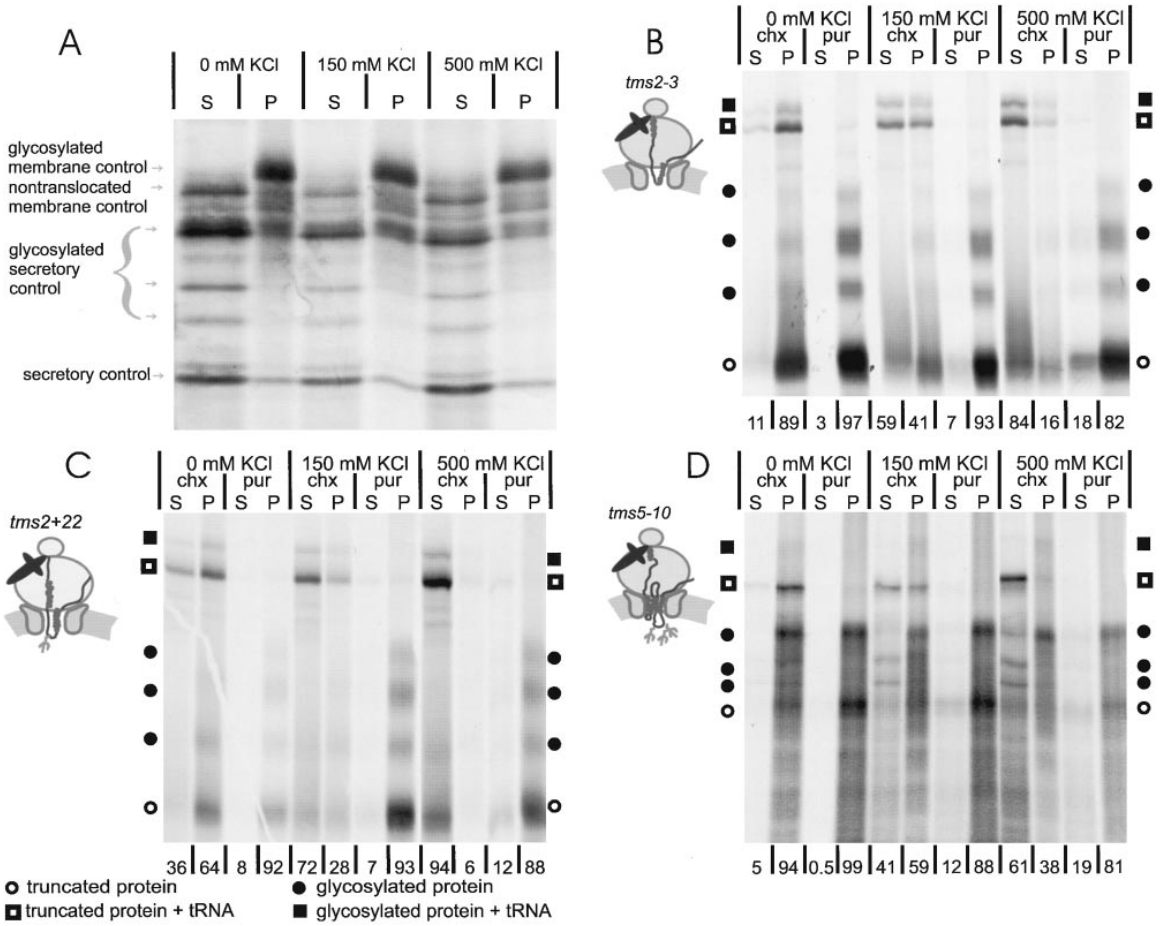

Figure 4. Effect of Varying the Salt Concentration on Extraction of Translocation Intermediates

Translocation intermediates were synthesized and urea extracted as described in Figure 2. However, the concentration of salt in the extraction media was varied from 0 to 150–500 mM.

(A) Effect of salt concentration in the urea extraction buffer on extraction of control proteins. The majority of the secretory control protein pre-pre-α factor was in the supernatant. The percentage that was extracted from the membrane was unaffected by the salt concentration. The control membrane protein, $pSPS_LG_{ST}P$, was predominantly in the pelleted membrane fraction. The percentage of the membrane-targeted glycosylated control membrane protein in the pellet was unaffected by the concentration of salt in the extraction buffer.

(B–D) Effects of varying the KCl concentration in the extraction of translocation intermediates TMS2−3 (B), TMS2+22 (C), and TMS5−10 (D). When KCl was omitted from the urea extraction buffer (first four lanes of each autoradiogram), the percentage of the translocation intermediate in the membrane fraction (lane 2) increased dramatically relative to when the buffer contained 150 mM KCl (lane 6). The opposite was observed when the KCl was increased to 500 mM. Almost none of the translocation intermediates were in the membrane fraction (lane 10): they were almost completely extracted from the membrane. In contrast, the fraction of the puromycin-released nascent chain in the membrane fraction was unaffected by the concentration of KCl (compare lanes 4, 8, and 12).

shown to be important in the biogenesis of glycoproteins (Helenius, 1994). To test whether the addition of bulky sugar groups anchored the loop between the first and second TMSs in the lumen of the ER (Simon et al., 1992), the urea extractions were repeated on each of the translocation intermediates after inhibiting glycosylation during the translation reaction with an acceptor peptide. Extractions for the translocation intermediate TMS2+39 are shown in Figure 6B. In this experiment, 26% of the protein was extracted under normal conditions (lane 1), while 25% was extracted upon inhibition of glycosylation (lane 5). Such inhibition had no effect on the extraction of peptides that had been explicitly released from their tRNA with puromycin (Figure 6B, compare lanes 3 and 4 with lanes 7 and 8). These extractions were repeated for all nine intermediates in the biosynthesis of Pgp both in the presence (closed square) and absence (closed triangle) of acceptor peptide (Figure 6A). Inhibition of glycosylation (closed square) had no effect on the urea extraction of either translocation intermediates (solid lines in Figure 6A) or peptides that had been released from their tRNA (broken lines).

Discussion

The use of translocation intermediates enabled us to analyze a protein at defined stages in its synthesis and determine which events in its biogenesis have occurred.

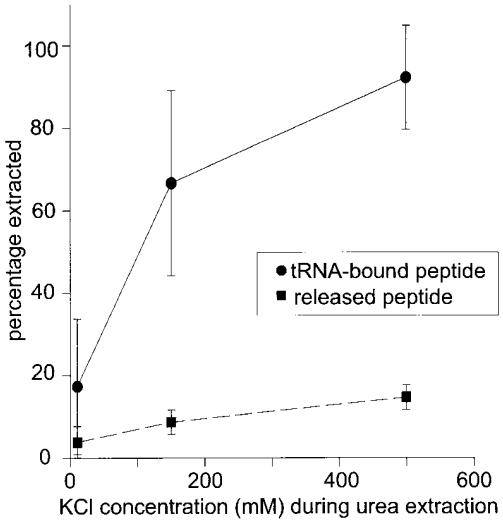

Figure 5. Effect of Salt Concentration on Extraction of Translocation Intermediates

The concentration of KCl in the urea extraction buffer was varied as described in Figure 4. The amounts of tRNA-bound translocation intermediates and tRNA-released peptides in the supernatant and pellet (membrane-associated) fractions were assayed on a phosphoimager.

Translocation intermediates are nascent polypeptides of varying lengths still bound to their tRNA and ribosomes. In this study, each nascent protein contains the amino-terminal sequence of Pgp, up to the position at which its template was cleaved, with at least the 40 carboxy-terminal amino acids between the carboxy-terminal amino acid and the membrane. Studying the nascent protein in these conformations is important for understanding its in situ interactions and characterizing the mechanism of biogenesis. Many previous studies on protein biogenesis have utilized shortened constructs of proteins with termination codons that are fully synthesized and released from the ribosome. Our results demonstrate that a biosynthetic intermediate still bound to its tRNA and ribosome behaves very differently from one that is released or synthesized with a termination codon. There are differences in both the glycosylation of the peptides and in how they interact with the membrane. The use of true translation and translocation intermediates allows us to observe at length stages that are normally only transient.

This study focuses on the relationship among three steps in the biogenesis of polytopic membrane proteins: translation, translocation, and integration. We have recently demonstrated that the first TMS of Pgp translocates across the membrane sequentially, before subsequent TMSs emerge from the ribosome (Borel and Simon, submitted). The results in this paper demonstrate that, upon achieving a transmembrane topography, this first TMS can still be extracted from the membrane with urea. This suggests it has not integrated into the hydrocarbon core of the lipid bilayer. Longer translation intermediates, including up to five of the latent TMSs, were also extracted from the membrane with urea. If, however, any of these were released from their biosynthetic ribosomes, they could not be extracted. Thus, the TMSs do not appear to integrate into the lipid bilayer either cotranslationally or cotranslocationally, but wait until the remainder of the protein is synthesized and released from the ribosome.

Nature of the Urea-Sensitive State

These observations suggest that latent TMSs that have not been released from their biosynthetic ribosomes are maintained in a yet to be defined state. They are not yet membrane proteins, since they can be extracted from the membrane without detergents and thus are not yet integrated into the lipid bilayer. Nor are they secretory proteins, since they span the membrane and do not slip without perturbation.

This paper explores the nature of this intermediate state. The concentration of salt in the urea buffer was varied to probe the nature of the bonds stabilizing the translocation intermediates with the membrane. This intermediate state was disrupted by raising the salt concentration from low (20 mM buffer) to physiological (150 mM KCl) and above (500 mM KCl). The strongest conclusion that can be drawn from this observation is that the translocated polypeptide is in an aqueous compartment: salt ions do not have access to the hydrocarbon core of the lipid bilayer (Parsegian, 1969). Because we see changes in extraction based on salt concentration, the intermediates must be in a region that is accessible to salt.

The effects of varying salt concentration (see Figures 4 and 5) are consistent with an electrostatic interaction between the translocating chain and another protein, but are inconsistent with a hydrophobic interaction (with either another protein or a lipid) as the primary stabilizing force. Electrostatic interactions are destabilized by raising salt concentrations and stabilized by lowering them. In contrast, the vast majority of hydrophobic interactions are stabilized in higher concentrations of the salts NaCl and KCl and destabilized in lower ionic conditions (Timasheff, 1993). Thus, the biosynthetic intermediates appear to be stabilized in their transmembrane topography in an aqueous-accessible compartment, most likely via ionic rather than hydrophobic interactions. Varying the chemical nature of the ion had little effect on the extraction. We tested for a potential role for an ATP-dependent chaperone in stabilizing the translocation intermediates. However, urea extractability of the translocation intermediates was unaffected by varying the concentration of ATP, GTP, or their nonhydrolyzable analogs (A. C. B. and S. M. S., unpublished data).

Glycosylation of the translocation intermediates may also stabilize them with the membrane. Their strongest association with the membrane was observed in the absence of salt. However, under these conditions, inhibiting glycosylation did not facilitate their extraction from the membrane. These results have a few possible explanations. First, glycosylation may not serve a crucial role in the biogenesis of the Pgp intermediates. The extent to which different proteins need their glycosylated groups has been shown to be variable (Helenius, 1994). Second, the glycosylation may not have much effect at the temperatures used in our work. Other studies have shown

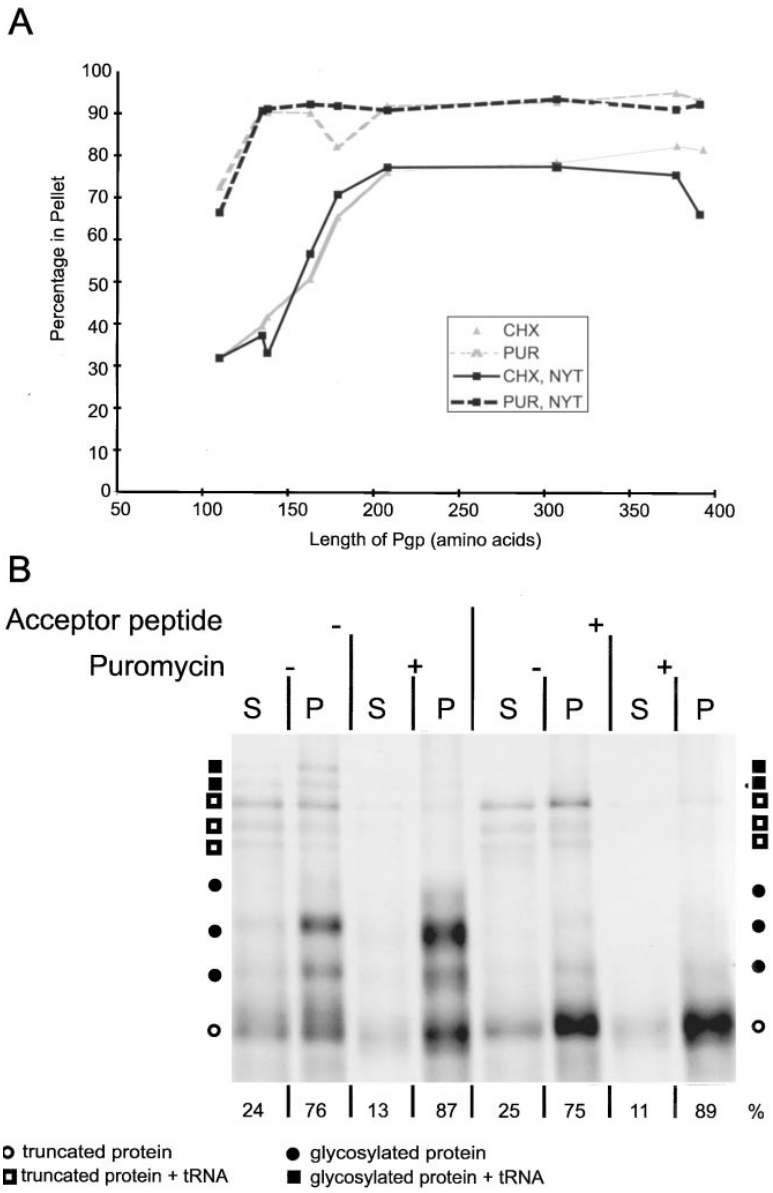

Figure 6. Glycosylation and the Extraction of Translocation Intermediates

(A) Translocation intermediates were translated and extracted as described in Figure 1 with KCl omitted from the urea extraction buffer. The percentage of protein fractionating with the membrane was plotted for the translocation intermediates (tRNA-bound bands, solid stippled line with closed triangles) and tRNA-released peptides (puromycin treatment, broken stippled line with closed triangles). The experiment was repeated with acceptor peptide in the translation mix to inhibit glycosylation. The percentage of proteins fractionating with the membrane was unaffected for either the translocation intermediates (solid line with closed squares) or the tRNA-released peptides (puromycin treatment, broken line with closed squares).

(B) The translocation intermediate TMS2+39 was translated and urea extracted as described in (A) with KCl omitted from the extraction buffer. The presence of acceptor peptide in the translation mix to inhibit glycosylation had no effect on the percentage of translocation intermediate fractionating with the membrane (compare lane 2 with lane 6). Likewise, inhibiting glycosylation had no effect on the percentage of puromycin-released peptides that fractionated with the membrane.

that the processing of newly synthesized proteins can be temperature sensitive (Machamer and Rose, 1988; Ljunggren et al., 1990; Denning et al., 1992). Loo and Clarke showed that reducing the temperature at which cell lines were grown resulted in processing and targeting of mutant Pgp proteins to the plasma membrane that were not processed at normal temperatures (Loo and Clarke, 1994). Because the translations were done at 22°C, followed by the extractions at 4°C, it is possible that removing the glycosylation groups on the intermediates had minimal effect at the low temperatures. Raising the temperature to more physiological levels may show that N-linked oligosaccharides are necessary for processing the Pgp intermediates.

A third plausible explanation for these results is that the N-linked oligosaccharides are a redundant system for Pgp biogenesis. It may be a useful stabilizing force under normal conditions, but it may not be the only one. Our experiments were done at low concentrations of salt, where there is the greatest association of translocation intermediates with the membranes. Our goal was to test whether glycosylation was responsible for this association. However, it may be that other interactions holding the translocation intermediates in place were so strong in low salt that inhibiting glycosylation had minimal effect. In fact, there is evidence that in physiological salt, glycosylation did affect the ease of extraction. All of these constructs were extracted with urea in physiological salt (Figure 2). With constructs TMS2+39 and TMS3−1, more of the unglycosylated tRNA-bound peptide was extracted into the supernatant (bands marked by open squares) than the glycosylated tRNA-bound peptide (bands marked by closed squares). Glycosylation of the tRNA-bound peptide was correlated

with reduced extraction from the membrane. However, this effect was not consistently observed with all constructs during urea extraction in 150 mM salt, nor was it observed during urea extraction in 500 mM salt (Figure 4).

Advantages of Posttranslocational Integration

The shielding of translocating chains from lipid has some advantages for the cell. Many proteins that are translocated across membranes have stretches of hydrophobic amino acids that fully cross to the lumenal side of the ER. This is particularly striking for some of the viral fusion proteins, such as influenza hemagglutinin. Energetics favor partitioning these hydrophobic stretches into lipid. If any portion of a hydrophobic domain of a protein even transiently interacted with lipid during translocation, the protein would become trapped. Subsequent translocation would be blocked until sufficient energy was expended to extract these hydrophobic domains from the lipid. These hydrophobic stretches are initially synthesized in the hydrophilic environment of the ribosome. To move them further along in an aqueous environment, from the interior of the ribosome to the interior of a water-filled protein-conducting channel and out to the lumen of the ER, does not present an energetic barrier.

There are specific advantages to shielding latent TMSs from interactions with lipids until translation is complete. This is the stage at which polytopic membrane proteins have to fold and form their proper intramolecular bonds. Many TMSs have charged groups which, by themselves, should not be stable within the lipid bilayer. These groups are often stabilized by salt bridges to charges in other TMSs. Thus, many TMSs cannot be properly integrated into the bilayer until all interacting TMSs are synthesized, translocated, and folded.

Stabilizing the translocation intermediates across the membrane, but not through their TMSs, may reflect a specific maturation process. If a TMS were bound to either lipids or the TMS of other chaperone-like proteins, it would be shielded from proper intramolecular interactions required for folding. Charged residues on either side of the TMS may stabilize the translocation intermediate. It has long been noted that there is a statistically higher density of positive and negative charges, respectively, on the cytoplasmic and lumenal/extracellular regions adjacent to the TMS (Von Heijne and Gavel, 1988). These charges may facilitate interaction with chaperones that stabilize and promote folding of polytopic membrane proteins prior to integration into the lipid bilayer.

One way of ensuring that all TMSs are properly folded prior to their integration is to postpone their embedding in the lipid until synthesis of the protein is complete. The signal for this could be the release of the ribosome from the newly synthesized protein. A number of experimental observations are consistent with a role for the ribosome in regulation of the membrane-bound protein-conducting channels. First, if translocating chains are released from their membrane-bound ribosomes under conditions that stabilize the ribosomes on the membrane, the protein-conducting channels remain open. Conditions that subsequently dissociate the ribosomes from the membrane close the channels (Simon and Blobel, 1991). Second, nascent transmembrane proteins can be cross-linked to a similar set of resident ER proteins as secretory proteins (High et al., 1991, 1993; Thrift et al., 1991; Oliver et al., 1995). They continue to be cross-linked to the same subset of ER proteins even after their TMSs are synthesized. Only upon completion of synthesis can they no longer be cross-linked to these proteins (Thrift et al., 1991). This suggests that they remain associated with the translocation machinery and do not integrate into the lipid bilayer until they are released from the ribosome.

It has been recently reported that the TMS of one type II membrane protein (having only a single TMS and its amino terminus located on the cytoplasmic side of the membrane) could be cross-linked to lipids during protein biogenesis (Martoglio et al., 1995). This observation, which differs from ours, may be due to the use of different model proteins. A type II membrane protein with a single TMS may have an explicit signal to trigger integration into the membrane prior to release of the peptide chain from the ribosome. In contrast, as mentioned above, there may be distinct advantages to not integrating the TMS of polytopic membrane proteins until the entire protein is in a transmembrane conformation. We have confirmed our observations using other model polytopic membrane proteins such as opsin and the sodium–calcium exchanger (data not shown).

There may be novel topogenic sequences in the polypeptide that, in addition to previously reported signals to start transfer or stop transfer across the membrane, signal to integrate into the membrane. We have analyzed only translocation intermediates of Pgp up to the large cytoplasmic loop between the sixth and seventh TMSs. This is partially the result of a technical limitation: it is very difficult to discern the tRNA-bound bands for larger peptides. However, one potentially interesting observation is that many polytopic membrane proteins have multiples of six TMSs separated by large cytoplasmic loops. The ATP-binding cassette proteins such as Pgp, cystic fibrosis transmembrane conductance regulator, and yeast Ste6p all have two groups of six TMSs separated by a large cytoplasmic loop. The sodium channel has four such groups of six TMSs, each separated by a large cytoplasmic loop. It is possible that these proteins are each integrated into the membrane six TMSs at a time. The large cytoplasmic loops may have a novel kind of signal "integration" sequence, which triggers the protein-conducting channel (either directly or indirectly through the ribosome) to open laterally into the plane of the membrane to allow protein integration into the bilayer.

Experimental Procedures

Materials

Protein molecular mass markers and [^{35}S]methionine were obtained from Amersham. Protease inhibitors were purchased from Boehringer Mannheim. Restriction enzymes were either from Boehringer Mannheim or New England Biolabs. Except when indicated, all other chemicals were obtained from Fisher or Sigma Chemical Company.

Plasmids and mRNA Transcription

The plasmid pSPMDR1, which includes the human gene for MDR1, was provided by Dr. M. M. Gottesman. The secretory control protein,

pre-pro-α factor was supplied by Promega. The membrane control protein ($pSPS_LG_{ST}P$) (Rothman et al., 1988) was supplied by Dr. V. Lingappa, and vesicular stomatitis virus glycoprotein was provided by Dr. A. Helenius. Transcription reactions were carried out following the instructions of the manufacturer (SP6 MEGAscript kit, Ambion) following restriction enzyme cleavage of the plasmid. Transcription reactions were done at 30°C to minimize shortened mRNA products and included Cap Analog (Ambion) m7G(5′)ppp(5′)G. All mRNA was purified with phenol and chloroform extractions, precipitated, and aliquoted for storage.

Protein Translations

In vitro translations were performed using the Promega rabbit reticulocyte lysate system, except one-half volume of the suggested reticulocyte lysate was used and the reaction was supplemented with a buffer containing 120 mM KOAc (pH 7.5), 50 mM HEPES (pH 7.5), and 2 mM $Mg(OAc)_2$. Pancreatic microsomal membranes were either from Promega or were prepared as described by Walter and Blobel (1983). Each 25 μl reaction contained 30 μCi of [^{35}S]methionine, 3.6 equivalents of microsomal membranes, and 40 U of RNasin ribonuclease inhibitor (Promega) and were carried out at 22°C for 50 min. This temperature enhanced the amount of nascent protein that remained attached to tRNA molecules. Protein synthesis inhibitors cycloheximide (at a final concentration of 0.2 mg/ml) or puromycin (at 2 mM) were added when specified and incubated at 22°C for 10 min. To inhibit glycosylation competitively, we added 0.03 mM acceptor tripeptide, an acylated glycosylation consensus sequence, Asn–Tyr–Thr, to the translation mix prior to the addition of RNA template.

Maintenance of the tRNA bond was essential for this analysis. Many commonly used experimental manipulations destroy the intermediate; they do not survive precipitation with trichloroacetic acid or membrane extraction protocol utilizing NaOH or Na_2CO_3. Lowering the temperature of translation reactions optimizes the amount of protein that remains suspended by its ribosome. Finally, the translation intermediates were kept on ice when possible to prevent reactions that may hydrolyze the peptide–tRNA bond.

Harvesting Microsomal Membranes

Translation products were layered on top of 100 μl of 1.0 M sucrose (with 20 mM $KHPO_4$ [pH 7], 1 mM β-mercaptoethanol, and 2 mM $Mg[OAc]_2$) and 10 μl of 2 M sucrose to harvest proteins that were targeted to the ER microsomes. After centrifugation in a Beckman Optima TLX Ultracentrifuge at 186,000 × g for 15 min at 4°C, the top of the cushion was removed. The supernatant, containing protein not associated with the membrane vesicles, was acetone precipitated and examined by SDS–PAGE. The majority of the translation product was generally found with the ER microsomes in the 25 μl portion of the sucrose left behind, layered on top of the 2 M sucrose cushion. This fraction was either examined by SDS–PAGE or used for subsequent experiments.

Urea Extraction

The targeted proteins were extracted with a solution of 4.5 M urea, 150 mM KCl, 1 mM β-mercaptoethanol, and 5 mM $Mg(OAc)_2$ with a buffer of either 20 mM K–HEPES (pH 7.2) or 20 mM $KHPO_4$ (175 μl of a 5.15 M urea stock was added to the 25 μl harvested sample). After thorough mixing, the sample was kept on ice for 30 min. The samples were centrifuged in a Beckman Optima TLX Ultracentrifuge at 279,174 × g_{av} for 30 min at 4°C to pellet the membranes. Variations on the procedure included the varying the KCl concentration (from 0 to 500 mM) and varying the urea concentration (from 3 to 7 M). The supernatant was removed and precipitated with 5 vol of ice-cold acetone. Precipitation with trichloroacetic acid was found to hydrolyze the tRNA–peptide bond and release the peptide from its ribosome. Both the supernatant and pellet samples were resuspended in 1× loading buffer (with 4% SDS, 0.125 M Tris [pH 6.8], 8 M urea, 10% β-mercaptoethanol, and 0.025% bromophenol blue) and were run on SDS–polyacrylamide gels.

Gel Electrophoresis and Image Analysis

SDS–PAGE used 9%–12% polyacrylamide with 6 M urea. After drying, the gels were exposed either to Kodak X-OMAT AR-5 film at −70°C or to a Molecular Dynamics phosphor screen. The scanned images were analyzed with Molecular Dynamics software. The volume integration of each object on the image was defined as the sum of the values for all pixels minus the background of the object.

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