Biochemistry

 Volume 35, Number 33 August 20, 1996

Accelerated Publications

Biogenesis of Polytopic Membrane Proteins: Membrane Segments of P-glycoprotein Sequentially Translocate To Span the ER Membrane†

Angela C. Borel and Sanford M. Simon*

Laboratory of Cellular Biophysics, Box 304, Rockefeller University, 1230 York Avenue, New York, New York 10021

Received April 19, 1996; Revised Manuscript Received June 26, 1996⊗

ABSTRACT: The initial steps in the biogenesis of membrane proteins parallel those of secretory proteins. However, membrane proteins contain a signal to stop translocation across the membrane. For polytopic membrane proteins, those with multiple transmembrane segments, little is known of the temporal sequence or relationship between synthesis of the nascent proteins, translocation, folding, and integration of the membrane segments into the bilayer. Here we demonstrate that latent membrane segments translocate sequentially as they emerge from the ribosome and do not accumulate on the cytosolic side to form loops, or larger structures, prior to translocation across the membrane.

The endoplasmic reticulum (ER)[1] is the site of biogenesis of membrane proteins of the nuclear envelope, plasma membrane, Golgi, ER, and endosomes. Membrane proteins appear to use much the same machinery for translocation as secretory proteins, for which the translocation process at the ER is well characterized. This is based on the observations that both secretory and membrane proteins require signal recognition particles (SRP) for translocation (Anderson et al., 1982), that they compete with each other for translocation (Lingappa et al., 1979), and that they can be cross-linked to a common membrane protein essential for translocation across the reticulum, Sec61 (Stirling et al., 1992; Görlich et al., 1992; Deshaies & Schekman, 1987). Further, there are a number of secretory and membrane proteins that are identical in sequence, with the exception of a carboxy-terminal membrane anchoring segment (McCune et al., 1980). Since these proteins are synthesized starting at their amino terminus, and cotranslationally translocated, it is reasonable to conclude that they utilize a common translocation machinery. However, translocation of secretory and membrane proteins ultimately diverges upon recognition of a latent transmembrane segment (TMS) which is selectively retained and then integrated into the membrane. In addition, the biogenesis of polytopic membrane proteins, those with multiple TMSs, requires folding and integration of the TMSs into the lipid bilayer.

One unresolved question is how the multiple TMSs of a polytopic membrane protein traverse the membrane during translocation. Does each TMS translocate sequentially, one at a time as it emerges from the ribosome, with each TMS functioning as an independent signal sequence or stop transfer sequence or do TMSs first accumulate in the cytosol as pairs or larger aggregates of TMSs prior to translocation across the membrane? According to the sequential translocation model for polytopic membrane proteins, each TMS can function as a signal sequence: either to initiate or to terminate translocation of a protein. Alternatively, a complete signal sequence may consist of two adjacent TMSs. For proteins with an amino terminus facing the cytosol, the first two TMSs, and each sequential pair, would fold together into a

† This work was supported by an Irma T. Hirschl-Monique Weill-Caulier Career Scientist Award and the Paul Ehrlich Foundation to S.M.S.

* To whom correspondence should be addressed: 212-327-8130 (voice); 212-327-8022 (fax); simon@rockvax.rockefeller.edu (E-mail).

⊗ Abstract published in *Advance ACS Abstracts*, August 1, 1996.

[1] Abbreviations: CTABr, cetyltrimethylammonium bromide; ER, endoplasmic reticulum; Pgp, P-glycoprotein; SRP, signal recognition particle; TMS, transmembrane segment.

S0006-2960(96)00950-6 CCC: $12.00

translocation-competent conformation and jointly insert across the membrane.

Consistent with the sequential translocation model are the observations that many of the TMSs of opsin can independently function as signal sequences to initiate targeting to the ER (Audigier et al., 1987; Friedlander & Blobel, 1985), and the demonstration that a construct made of tandem copies of the asialylglycoprotein receptor (which has a single TMS) starts to translocate prior to completion of translation (Wessels & Spiess, 1988). Using a native polytopic membrane protein, we addressed this issue of whether individual TMSs translocate sequentially, in pairs or large conglomerates. We studied the biogenesis of the polytopic membrane protein P-glycoprotein (Pgp) which has been suggested to have 8, 10, or 12 TMSs (Zhang et al., 1993; Skach et al., 1993; Gerlach et al., 1986). The topology of the first two TMSs appears to be unambiguous and generally accepted: the amino terminus is on the cytoplasmic side. We have focused on the roles of the first two TMSs of Pgp in protein translocation. When does the first TMS translocate during biosynthesis of the protein? Specifically, does it translocate prior to the emergence of the second TMS from the ribosome or as a pair following emergence of both TMSs?

Our goal was to examine Pgp in the process of translocation, a generally transient occurrence. This was approached by using mRNA templates that were truncated at various points within the coding sequence, 5′ to their stop codons. Their nascent polypeptide products should remain linked, via ester bonds, between their carboxy-terminal amino acids and the final tRNAs added to their translating ribosomes (Chuck & Lingappa, 1992; Thrift et al., 1991; Kellaris et al., 1991; Perara et al., 1986). During protein synthesis, approximately 40 of the most recently translated carboxy-terminal amino acids are inside the ribosome and are protease insensitive, allowing us to estimate the amount of the protein that had left the ribosome, free to interact with either itself or the translocation machinery (Matlack & Walter, 1995; Blobel & Sabatini, 1970; Sabatini & Blobel, 1970; Malkin & Rich, 1967).

We initially characterize the protein products from our truncated mRNA and demonstrate that, with proper handling, they can be studied while still attached to their tRNA and biosynthetic ribosomes. These translation intermediates are easily dissociated, and proteins released from ribosome-bound tRNAs behave differently from those still attached to their biosynthetic ribosomes. Thus we restricted our analysis only to those nascent intermediates preserved in a tRNA−ribosome bound state. Our results indicate that, upon emerging from the ribosome, the first TMS of Pgp moves across the membrane prior to the emergence of the second TMS from the ribosome. This can be envisioned as a growing loop entering the channel as translation progresses with the amino-terminal remaining on the cytoplasmic side of the ER. Our results also demonstrate, consistent with the conclusions from other studies using different techniques, that more than 39 of the most recently synthesized amino acids reside in the ribosome.

EXPERIMENTAL PROCEDURES

Materials. The rabbit reticulocyte lysate system was from Promega. [^{35}S]Methionine was purchased from Amersham, as well as Rainbow protein molecular weight markers. Protein synthesis inhibitors puromycin and cycloheximide were purchased from Sigma Chemical Co. Protease inhibitors were purchased from Boehringer Mannheim. The transcription reactions were done with Ambion's SP6 MEGAscript kit. Restriction enzymes were ordered from either Boehringer Mannheim or New England Biolabs. Cetyltrimethylammonium bromide (Calbiochem, San Diego, CA) was prepared as a 2% stock. All other chemicals were obtained from Fisher or Sigma Chemical Co.

Plasmids and mRNA Transcription. The plasmid pSP-MDR1, including the human gene for MDR1, was provided by Dr. M. M. Gottesman. Transcription reactions were carried out with Ambion's SP6 MEGAscript kit following restriction enzyme cleavage of the plasmid to create runoff transcripts. Transcription reactions included Ambion's Cap Analog, m7G(5′)ppp(5′)G, and were performed at 30 °C to minimize shortened RNA products. The RNA was purified with phenol and chloroform extractions, precipitated, and aliquoted for storage. Prior to use for translation reactions, the RNA was analyzed on agarose formaldehyde gels.

Protein Translations. In vitro translations were performed using Promega's rabbit reticulocyte lysate. Each 25 μL reaction contained 30 μCi of [^{35}S]methionine, 3.6 equiv of Promega's canine pancreatic ER membranes, and 40 units of RNasin ribonuclease inhibitor and was carried out at 22 °C for 50 min. Translations carried out at this temperature enhanced the amount of protein attached to tRNA molecules. Protein synthesis inhibitors cycloheximide (at a final concentration of 0.05 mg/mL) and puromycin (at 2 mM) were added at the end of translation when specified and incubated at 22 °C for 10 min. To competitively inhibit glycosylation, 30 μM acceptor tripeptide, an acylated glycosylation consensus sequence, Asn-Tyr-Thr, was added to the translation mix prior to the addition of the RNA template.

Harvesting ER Membranes. To collect proteins targeted to the ER, 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 $Mg(OAc)_2$] over a 10 μL 2 M sucrose cushion. These were centrifuged in a Beckman Optima TLX ultracentrifuge at 186000*g* for 15 min at 4 °C. The supernatant was removed, acetone precipitated, and examined by SDS−PAGE. The majority of the translation product was found in the 25 μL on top of the 2 M sucrose cushion.

Sucrose Gradient Fractionation. After translation with canine pancreatic ER membranes at 22 °C 100 μM cycloheximide or puromycin was added to inhibit further synthesis. A protease inhibitor mix (0.1 μL/mL pepstatin A, 0.1 μL/mL chymostatin, 10 μg/mL TPCK, 1 μg/mL aprotinin, and 20 μg/mL phenylmethanesulfonyl fluoride) was added, in addition to CHAPS (final concentration, 0.3%) to solubilize the membranes. Samples were layered over 5−30% sucrose gradients, including 50 mM TEA, pH 7.5, 150 mM KOAc, pH 7.5, 2.5 mM $Mg(OAc)_2$, 3 mM DTT, and 10 μg/mL bovine serum albumin. The gradients were centrifuged in a Beckman L-80 ultracentrifuge at 288000*g* for 2 h at 4 °C. Fractions were removed using a Buchler Auto Densi-flow IIc from the top of the gradient, and the absorbance at 254 nm was determined with a UV analyzer to follow the migration of ribosomes in the sucrose gradient. A monosomal peak followed by a broad polyribosomal peak was seen for each gradient. The fractions were precipitated with ice-cold acetone and run on an SDS−polyacrylamide−urea

gel. The ribosomal peak was compared to the position of the radiolabeled protein in the gradient.

Precipitation of tRNA-Bound Peptides. Cetyltrimethylammonium bromide (CTABr) was used to precipitate nucleic acids. In the precipitation procedure, 250 μL of 2% CTABr was added to a 10−20 μL translation reaction, and the mixture was vortexed. Then 250 μL of 0.5 M sodium acetate, pH 5.4, containing 200 μL/mL yeast tRNA was added, and the mixture was revortexed. Samples were incubated for 10 mins at 30 °C, followed by centrifugation for 10 min at 12000*g*. The supernatant sample was removed and precipitated with acetone. The pellet sample was also washed with acetone. Both supernatant and pellet were resuspended in 1× loading buffer and examined by SDS−PAGE.

Gel Electrophoresis and Image Analysis. Gels were prepared using 9−12% acrylamide with 6 M urea. The gels were dried and exposed either to Kodak X-Omat AR-5 film at −70 °C or to a Molecular Dynamics phosphor screen and scanned.

RESULTS

Each of the discrete stages in the biosynthesis of membrane proteins is usually a transient event. To characterize these events, the biosynthesis of the proteins was arrested at defined points prior to completion of biosynthesis. The strategy was to cut the plasmid encoding the protein within the coding region of the DNA. Transcription should yield an mRNA truncated prior to the termination codon. Upon translation on ribosomes the nascent polypeptide should not complete synthesis and thus should be attached via its carboxy terminal amino acid and cognate tRNA to the ribosome. The human MDR1 DNA, cleaved with restriction endonuclease *Apa*LI, was used as the template for transcription and translation in Figure 1B. The resulting protein includes the first two TMSs of Pgp plus 22 amino acids (to be referred to as tms2 + 22; see Table 1), for a total size of 163 amino acids. In the first two lanes of Figure 1B, the protein was translated without ER membranes. The main product in lane 1 runs at approximately 50 kD (★) while the predicted size of the nascent peptide is 18 kDa. This 50 kDa higher molecular weight product was identified by treating the translation reaction with puromycin, an aminoacyl-tRNA analog which is incorporated into nascent peptide chains by the ribosomal peptidyl transferase, thereby dissociating the nascent peptide−tRNA bond (Figure 1B, lanes 2, 4, and 6) (Monro & Marcker, 1967; Redman & Sabatini, 1966; Traut & Monro, 1964). Upon treatment with puromycin, the major ^{35}S-labeled product shifts from 50 kDa (★) to 18 kDa species, as seen in lane 2 (●). Thus, the larger 50 kDa molecular mass band is the nascent peptide attached to a tRNA molecule. This shift from the tRNA-bound protein to the protein alone is evident upon treatment with puromycin for each intermediate (Figure 1). This is consistent with measurements of approximately 23−30 kDa molecular mass for tRNAs based on mobility on SDS−PAGE. Thus, the biosynthetic translation intermediates that are still bound to their tRNAs are readily identified by their puromycin-labile, higher molecular masses on SDS−polyacrylamide gels. The translation products were next fractionated on sucrose gradients to test if they were still attached to their biosynthetic ribosomes. Truncated Pgp mRNA (tms2 + 22) was translated in the presence of ER membranes. The reaction was split in two and treated with either puromycin or cycloheximide. Cycloheximide, like puromycin, is a protein synthesis inhibitor. However, cycloheximide does not dissociate the tRNA−peptide interaction. The ER vesicles in each reaction were then solubilized with CHAPS detergent and run on linear 5−30% sucrose gradients, and fractions were collected while monitoring the absorbance at 254 nm to assay for ribosomes. The ribosomes were detected first in a large monosomal peak followed by a broader peak, representing protein-synthesizing ribosomes complexed as polysomes on a single RNA template (Walter et al., 1981). The ribosomal peak was compared to the position of the radiolabeled translation intermediate on the gel (Figure 2A).

All of the tRNA-attached translation intermediates (★, ⩾★) in Figure 2A migrated in the same fractions of the gradient as the ribosomes. We concentrated on the tRNA-bound proteins since, at each step of sample handling and preparation, some of the nascent peptides dissociated from their ester-linked tRNA. The intermediates in this reaction had not been intentionally released from their tRNAs; thus the proteins no longer attached to tRNA were ignored.

To determine whether these tRNA-bound proteins were attached to ribosomes and not just comigrating with ribosomes in the gradient, puromycin was added to intentionally release the peptides from their tRNAs prior to the sucrose gradient. After puromycin-induced release, most of the tms2 + 22 protein ran at the expected molecular mass for the protein alone (18 kDa, ●) as well as at slightly higher molecular mass forms (⩾●, discussed below) (Figure 2B). This puromycin-released band ran at the top of the gradient (left side of gel) and no longer fractionated with the ribosomes. When the puromycin reaction did not go to completion, the residual tRNA-bound peptides still comigrated with the ribosomes.

The amino terminus of the mature Pgp faces the cytoplasm. To determine when the first TMS crosses the membrane during biosynthesis, translation intermediates were synthesized in the presence of ER membranes. Each of the truncated proteins shown was synthesized with or without ER vesicles and then separated by SDS−PAGE (Figure 1). The intermediate tms2 + 22 (163 amino acids) was the shortest construct for which we observed translocation, as assayed by glycosylation (Figure 1B). In its tRNA-bound form, the first TMS and some of the intervening sequence between the first two TMSs are potentially long enough to reach across the membrane. The products of translation reactions synthesized without ER membranes are shown in Figure 1B, lanes 1 and 2. As described above, the major ^{35}S-labeled product (★) is a tRNA-bound peptide which migrated at 50 kDa (lane 1) and after dissociation of the tRNA migrates at the expected molecular mass of a protein with 163 amino acids (18 kDa) (●). When synthesized in the presence of ER vesicles (lane 3), there is an additional slower migrating ^{35}S-labeled band (⩾★). After puromycin treatment (lane 4) this band also disappeared, and there are four faster moving bands (●, ⩾●, ⩾⩾●, ⩾⩾⩾●). To characterize these new bands, these translation reactions were repeated with acceptor peptide, a tripeptide which is a competitive inhibitor of glycosylation. This method of inhibiting glycosylation was chosen over others, such as tunicamycin or endoglycosidase F or H, to minimize the more extreme nonphysiological conditions required by these

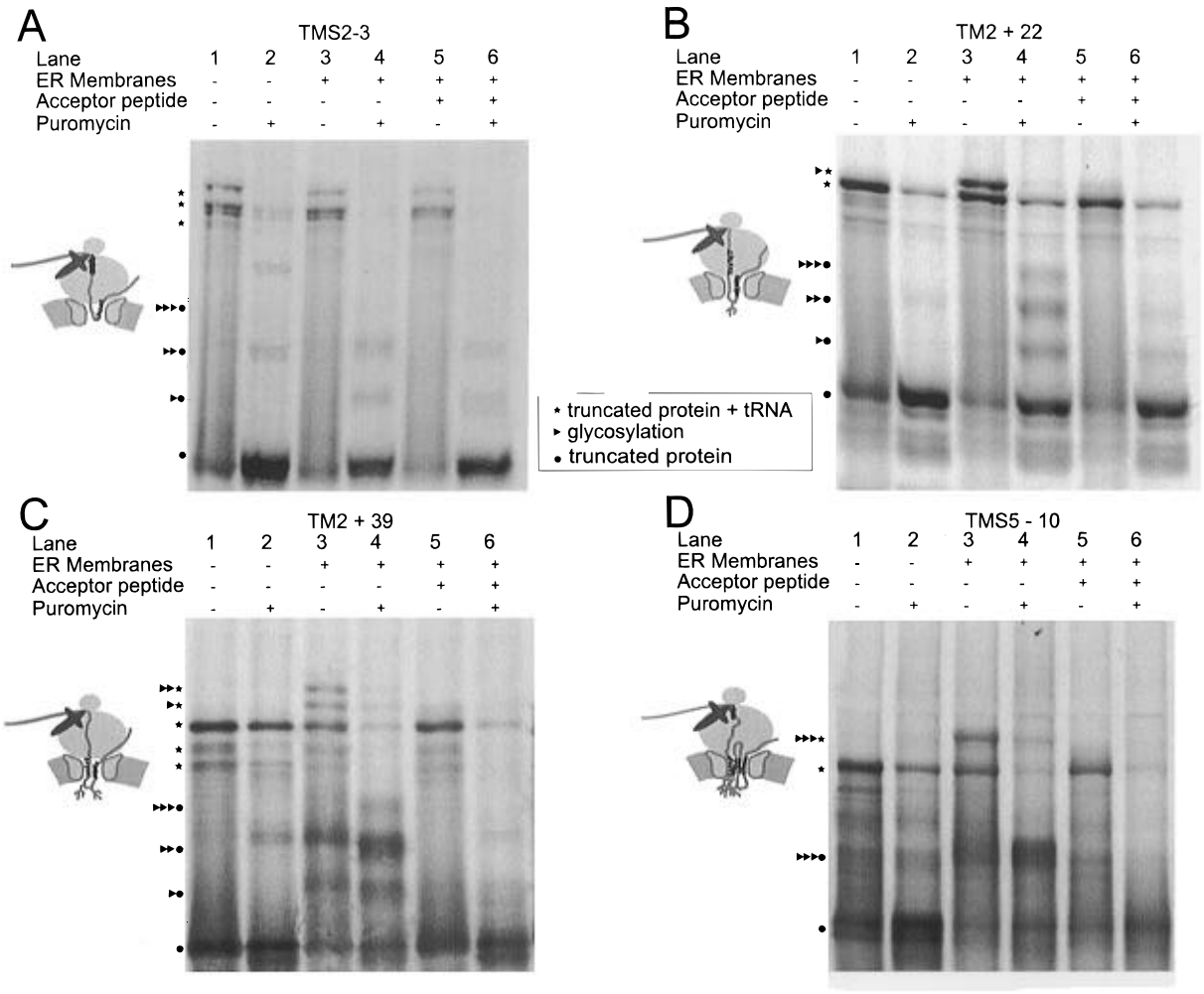

FIGURE 1: Translation products from truncated mRNA. mRNAs terminated at various points in the coding region of Pgp were translated into protein in reticulocyte lysate with (lanes 3−6) or without (lanes 1, 2) ER membranes. This yields a translation product on SDS−PAGE of the nascent peptide bound to tRNA (★). Or, when ER membranes are included and enough of the peptide has crossed the membrane, glycosylated nascent peptide bound to tRNA (➢★). The higher molecular weight bands (★) disappear when puromycin, which releases nascent chains from their tRNA, is added at the end of the translation mix and bands that migrate at the appropriate molecular weight for the nascent peptide (●) or glycosylated nascent peptides (➢●) get stronger. In the presence of acceptor peptide, a competitive inhibitor of glycosylation, the slower migrating bands (marked with ➢) are not seen (lanes 5 and 6). (A) tms2 − 3. A number of tRNA-bound bands were seen with this construct which were also present in the absence of ER membranes (lane 1) or the presence of a competitive inhibitor of glycosylation (lane 5). When puromycin is used to release the nascent chain from the ribosome, there are three bands (lane 4) which are not seen in the presence of an inhibitor of glycosylation (lane 6). (B) tms2 + 22. In the presence of membranes, a discrete band of larger apparent molecular weight is observed (lane 3, ➢★). This band is not observed when acceptor peptide, a competitive inhibitor of glycosylation, is present during the protein translation. (C) tms2 + 39. This tRNA-attached peptide is glycosylated on two of the three potential glycosylation sites (lane 3). (D) tms5 − 10. All three potential sites are fully glycosylated both on the tRNA-translation intermediates (lane 3) and on the tRNA-released forms (lane 4).

Table 1: Biosynthetic Intermediates: P-glycoprotein-Shortened Proteins

restriction enzyme of MDR1 template DNA	length of DNA template (bp)	peptide length (amino acids)	estimated molecular mass (kDa)	distance from putative membrane segment
*Bbs*I	329	110	11.9	tms1 + 38 aa
*Ban*I	404	135	14.7	tms2 − 6 aa
*Pvu*II	415	138	15.2	tms2 − 3aa
*Apa*LI	490	163	17.9	tms2 + 22 aa
*Bsm*AI	537	179	19.7	tms2 + 39 aa
*Nsi*I	921	307	33.8	tms5 − 10 aa

agents and thus preserve the tRNA−peptide bond. When tms2 + 22 was translated with ER membranes and acceptor peptide, the higher molecular mass forms did not exist for either the translation intermediate or the tRNA-released protein (lane 5). Therefore, the higher molecular mass forms of the tRNA-bound peptide product of tms2 + 22 were due to glycosylation.

The translation mix was solubilized and fractionated on a sucrose gradient to test if the glycosylated tRNA-bound peptides were still attached to their biosynthetic ribosomes. The glycosylated tRNA-bound ^{35}S-labeled bands migrated with the ribosomes (Figure 2A, ➢★). To confirm that this ^{35}S-labeled band was the glycosylated tRNA-bound nascent peptide, the sucrose gradients were repeated on translation reactions which included acceptor peptide. The higher molecular mass form of the tRNA-bound intermediate was missing in the presence of acceptor peptide (compare Figures 2A and 2C). However, the majority of the tRNA-attached translation intermediates still migrated in the ribosome-containing fractions (Figure 2C, ★). To test if migration of the nascent peptides into the gradient was the consequence of attachment to the ribosome, the sucrose gradients were

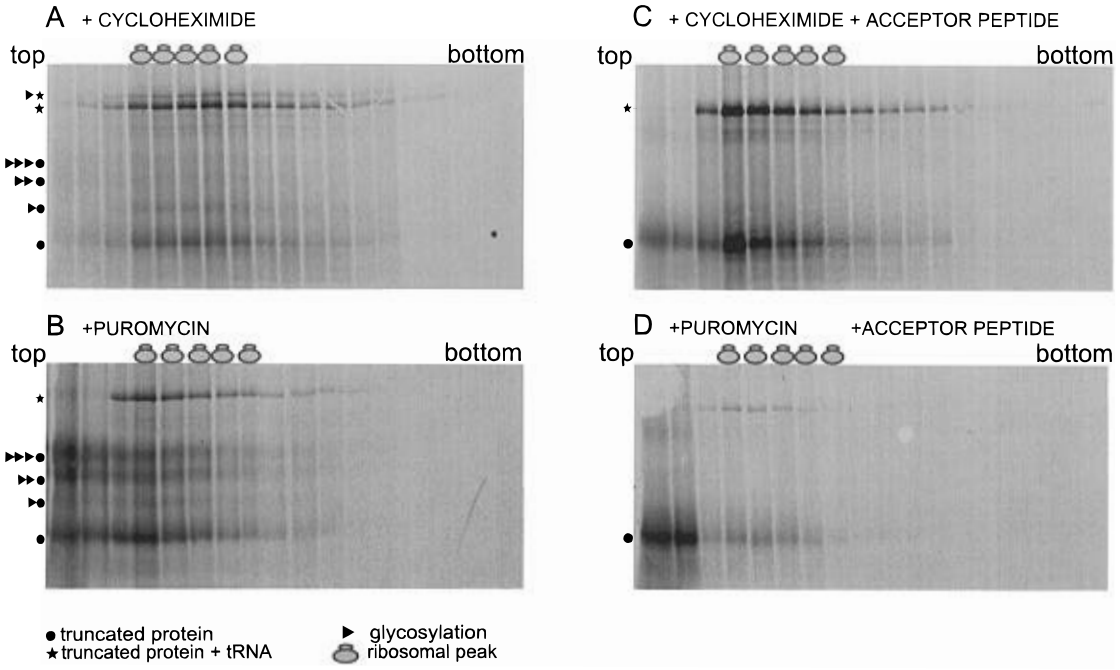

FIGURE 2: tRNA-bound nascent peptides are still attached to ribosomes. mRNA for tms2 + 22 was translated in the presence of ER microsomes, treated with either puromycin (to cleave the tRNA−peptide bond) or cycloheximide (which leaves the bond intact), and then solubilized and fractionated on a sucrose gradient. Location of the ribosomes in the gradient was detected by absorbance at an OD_{254}. The sucrose gradient was fractionated and samples were prepared for SDS−PAGE. (A) Most of the translation product, the higher molecular weight tRNA-bound nascent chains (★), migrates into the gradient along with the ribosomes. (B) When puromycin is used to break the tRNA−peptide ester bond, the released peptides (●) remain at the top of the gradient and do not comigrate with the ribosomes. (C) When the translation reaction includes acceptor peptide, a competitive inhibitor of glycosylation, the slower migrating band which comigrated with the ribosomes in panel A (➢★) disappears, confirming its identity as a glycosylated form of the tRNA-bound peptide. (D) Upon inhibition of glycosylation with acceptor peptide, and release of the nascent peptides from their tRNAs by puromycin, all of the peptide runs as a single band at the top of the gradient.

repeated on translation reactions which had been terminated with puromycin to release the nascent peptides from their tRNA and ribosomes. The majority of the proteins stayed at the top of the gradient (Figure 2D, ●). Thus the nascent peptides only comigrate in the sucrose gradient with ribosomes while attached to their tRNA.

A series of constructs shorter than the tms2 + 22 were tested in a similar manner (see Table 1 and Figure 1A). The SDS−PAGE gel in Figure 1A shows the same series of translation reactions for the shorter construct tms2 − 3. This translation reaction was repeated in the presence of ER vesicles (Figure 1A, lane 3). The major products migrated at approximately 45 kDa (★) and disappeared after puromycin treatment (lane 4), indicating that they are tRNA-bound peptides. These tRNA-bound peptides (★) were observed in the presence of acceptor peptide (lane 5) and in the absence of membranes (lane 1) indicating they were not the consequence of glycosylation. When these translation products were centrifuged through sucrose cushions to harvest and collect ER vesicles, the vast majority of the synthesized proteins are found associated with the membranes. Therefore, enough of the first TMS had emerged from the ribosome to allow proper targeting. Further, upon addition of puromycin these constructs are translocated across the ER as assayed by glycosylation on as many as three of the potential sites on the nascent peptide (lane 4, Figure 1A). This confirms that these short translation intermediates are targeted to the ER. It also demonstrates that the same peptide will assume different characteristics depending upon whether it is a translation intermediate (still attached to its biosynthetic ribosome) or a fully released peptide.

There are lower molecular mass bands visible in the gel below the indicated tRNA-bound protein (band marked with a star in Figure 1A). More than one band of tRNA-conjugated protein was seen with many of the truncated constructs we tested. These additional bands were seen in the absence of ER membranes (Figure 1A, lane 1), and their intensity was unaffected by acceptor peptide (Figure 1A, lane 5), indicating that they were not a consequence of glycosylation. The multiple bands might be the consequence of stacking of ribosomes behind the original "caught" ribosome on the mRNA template (Figure 1A, below the starred product) (Wolin & Walter, 1988), and the different molecular masses might be the consequence of different length peptides. Alternatively, these different bands could be the result of different isoaccepting tRNAs with different mobility on SDS−PAGE, all conjugated to the same truncated polypeptide.

To distinguish between these possibilities, the tRNA-bound proteins were precipitated with cetyltrimethylammonium bromide (CTABr) (Kellaris et al., 1991) and resuspended in either neutral buffer or NaOH to subsequently hydrolyze all of the tRNA−peptide bonds. If the multiple tRNA-bound bands were due to stacked ribosomes, then upon hydrolysis of the tRNA−peptide bond, we should observe a ladder of decreasing size peptides. Alternatively, if the multiple tRNA-bound bands were the consequence of different isoaccepting tRNAs, then upon hydrolysis with NaOH we should observe only a single polypeptide band.

A translation reaction of tms2 − 6 is shown in Figure 3, lane 1. Neither of these bands is due to glycosylation since their intensity is unaffected by inhibition of glycosylation

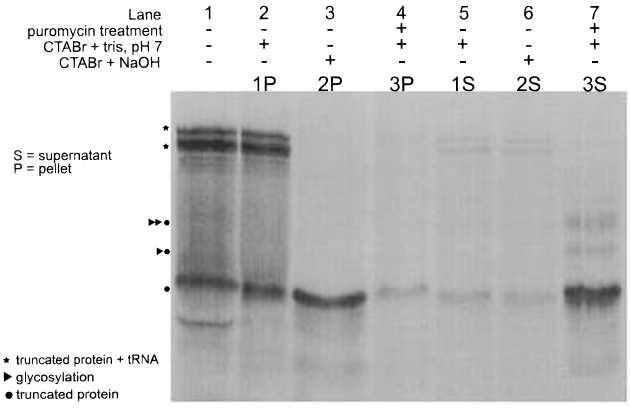

FIGURE 3: Multiple tRNA-bound bands are from different isoaccepting tRNAs. On most of our truncated constructs we observed multiple tRNA-bound bands. Some of these bands were not inhibited by acceptor peptide and were seen in the absence of ER membranes. The mRNA for tms2 − 6 was translated, the tRNA-bound conjugates were precipitated with CTABr, and the tRNA−peptide bond was hydrolyzed with NaOH. The presence of a single band upon release of the tRNA in lane 3 indicates that the different tRNA-bound bands are from peptides of indistinguishable mobility in SDS−PAGE.

in the translation mix and the mobility of the tRNA-bound bands is not shifted when translated in the absence of membranes (data not shown). Aliquots of this same reaction were treated with CTABr to precipitate all of the tRNA-bound peptides. Then both the supernatants and pellets were treated with either neutral Tris-HCl at pH 7 (sample 1, lanes 2 and 5) or 100 mM NaOH (sample 2, lanes 3 and 6). The CTABr precipitated pellet (lane 2, sample 1P) is almost indistinguishable from the complete translation reaction in lane 1, and no radiolabeled nascent protein is left in the supernatant (lane 5, sample 1S). This indicates that (a) the vast majority of the nascent peptide is bound to the tRNA, (b) CTABr is extremely efficient at precipitating the tRNA-bound peptides, and (c) neutral Tris does not adversely affect the stability of the tRNA−peptide bond.

Treatment of the CTABr-precipitated sample with NaOH yielded only a single radiolabeled band (●), running at the predicted mobility of the tms2 − 6 polypeptide chain (see lanes 3 and 6). This demonstrates that each of the higher molecular mass bands was the consequence of tRNA bound to the same tms2 − 6 peptide and thus the multiple higher molecular mass bands were the consequence of different isoaccepting tRNAs bound to the same polypeptide.

When the translation reaction was treated with puromycin prior to solubilization with CTABr, no radiolabeled protein was seen in the pellet (lane 4, sample 3P), and the entire sample was seen in the supernatant (lane 7, sample 3S) migrating as the tRNA-released tms2 − 6 protein (●). This confirms that CTABr only precipitates proteins that are still bound to tRNA. Since most of the label was in a single band (circle), it was an independent test that there was only a single polypeptide bound to the tRNA. The puromycin-treated samples often show bands of slightly higher molecular mass (⩾●) than those of the tRNA-released protein (●). This demonstrates that, upon puromycin release, the nascent tms2 − 6, like tms2 − 3 (Figure 1A), was translocated and glycosylated. Occasionally, we also observed a small ladder of peptides or decreasing molecular mass which could be the consequence of stacked ribosomes.

The gel in Figure 1C shows translation reactions of tms2 + 39 (180 amino acids). When synthesized in the presence of ER membranes, there were two higher molecular mass bands (lane 3, marked with a star and one or two triangles). These bands disappeared upon treatment with puromycin in lane 4, indicating that they were tRNA-bound bands. These bands also disappeared when acceptor peptide was included in the translation mix in lane 5, indicating that they were the result of glycosylation. Thus, two of the three potential sites on the tRNA-conjugated protein tms2 + 39 were glycosylated. Once the protein was released from the ribosome with puromycin, all three potential sites were glycosylated (see lane 4, marked with a circle and one, two, or three triangles). The glycosylation of only two sites indicates that the loop between the first two TMSs had not fully translocated across the membrane. Thus, the 39 amino acids after the second TMS are not sufficient for TMS2 of this tRNA−ribosome-attached peptide to have fully emerged from the ribosome to span the membrane.

Previous estimates of 40 amino acids in the ribosome have been based on the accessibility of protease to the nascent polypeptide (Matlack & Walter, 1995; Blobel & Sabatini, 1970; Sabatini & Blobel, 1970; Malkin & Rich, 1967). Our results, using a different approach, confirm that more than 39 amino acids are needed after a latent transmembrane for it to extend far enough out of the ribosome to span the membrane. Further, these results allow us to benchmark the relative position of the translocation intermediates relative to the ribosome, the membrane, and the lumen of the ER. The translation of the construct tms5 − 10 is similar to that of the others (Figure 1D). There is a ^{35}S-labeled band at the predicted size of the peptide (●) and additional slower migrating bands (★, lane 1, 3) that disappear when the of the translation mix is treated with puromycin (lanes 2, 4). When tms5 − 10 is translated in the presence of ER vesicles, there are additional bands (⩾★) that migrate more slowly and which are not seen in the presence of acceptor peptide (lane 5). These appear to be glycosylated on all three potential sites in the loops between the first two TMSs.

These translation reactions were repeated with a number of constructs (Table 1). The shortest three constructs [108, 135, and 148 amino acids (including tms2 − 3 shown in Figure 1A)] were not long enough to be glycosylated. However, these nascent peptides harvested with the ER membranes, and upon release with puromycin, they were glycosylated. Thus the first TMS was sufficiently exposed from the ribosome to be properly targeted to the ER membrane, but not enough of the loop after this TMS was translocated to allow glycosylation. The construct tms2 + 22 in Figure 1B was the first intermediate to show signs of glycosylation of its tRNA ribosome-bound form. Because the second TMS of tms2 + 22 is located within the ribosome, we concluded that the first TMS can move across the membrane independently of TMS2. As the truncated proteins become longer and are able to further translocate, the second and third glycosylation sites of the tRNA-bound proteins are modified as seen in Figure 3c,d.

DISCUSSION

These studies utilizing the translation products from truncated mRNA demonstrate the following points about the biogenesis of polytopic membrane proteins. First, truncated

mRNAs produce nascent peptides that are still attached to their tRNA and biosynthetic ribosomes, and thus they are true "translation intermediates". Second, these translation intermediates can also be "translocation intermediates" bound to their ribosome on the cytoplasmic side and modified by the oligosaccharidyltransferase in the lumen of the ER. Third, during biosynthesis, there appears to be a number of different isoaccepting tRNAs that can all function at the same codon. Fourth, there are at least 39 amino acids between the sites of assembly at the peptidyltransferase and the membrane-bound translocation apparatus. Fifth, in polytopic membrane proteins with multiple TMSs, the transmembrane signal sequence that "initiates" translocation moves across the membrane prior to interaction with the "terminating" signal sequence which, in turn, pauses translocation. Therefore, TMSs do not appear to translocate across as "loops" or preassemble as larger aggregates in the cytosol prior to translocation.

The use of truncated mRNA to generate translation intermediates captured what would otherwise be a transient event in the biogenesis of membrane proteins. The translation products fall into two groups: peptides that are translation intermediates that can be demonstrated to be still attached to both their biosynthetic tRNA and ribosomes and those peptides that have broken this bond. This study focuses on these translation intermediates. When synthesized in the presence of ER membranes, these intermediates bound to the tRNA, and its biosynthetic ribosome on the cytoplasmic side is N-glycosylated, an enzymatic process that only occurs within the lumen of the ER. Thus, they are both translation intermediates and translocation intermediates. Peptides that have been released from their tRNA are not equivalent to the translocation intermediates. They are glycosylated on more sites (Figure 1A,B; compare lanes 1 and 2); translation intermediates can be extracted from the membrane, and released peptides behave like integral membrane proteins and cannot be extracted (Borel & Simon, 1996), and after solubilization the translocation intermediates fractionate with ribosomes and released tRNA-released peptides do not. Thus, our analysis will focuse on these tRNA-conjugated peptides.

The issue of whether a protein translocates cotranslationally or immediately thereafter was first clearly addressed by Redman and Sabatini (1967), who showed that nascent chains of the secretory protein amylase were found in membrane-bound compartments upon release from the ribosome with puromycin. This indicated that the nascent chains were either crossing the membrane cotranslocationally or immediately crossed the membrane upon release from the ribosome. Our results clearly demonstrate that a chain that is still attached to its biosynthetic tRNA and ribosome is simultaneously modified by the ER-lumenal enzyme oligosaccharidyltransferase and therefore must be translocating cotranslationally.

We frequently observed multiple puromycin-labile tRNA-bound bands when we were examining translation intermediates on SDS−PAGE. These multiple bands were not the consequence of glycosylation: they were observed both in the presence of competitive inhibitors of glycosylation and in the absence of ER membranes. These are apparently not the consequence of different size peptides but of different size tRNAs bound to the same peptide. When these tRNA-bound bands were harvested and then the tRNA−peptide bond was broken, there was only one peptide species. Thus, these bands are likely the consequence of different isoaccepting tRNAs and not protein products synthesized from ribosomes stacked behind an original "caught" ribosome on an mRNA template. There have been numerous studies aimed at determining the number of most recently assembled amino acids that potentially reside within the large aqueous tunnel (Matlack & Walter, 1995; Connolly et al., 1989; Sabatini & Blobel, 1970; Blobel & Sabatini, 1970; Malkin & Rich, 1967) observed in the large ribosomal subunit (Yonath et al., 1987). The extent of the polypeptide buried in the ribosome has been typically assayed by accessibility to protease. Most of these studies conclude that approximately 35−55 amino acids are inside the ribosome, and as many as 70 amino acids are protected by the combined structure of the ribosome and membrane-bound translocation machinery. Alternatively, it has been proposed that nascent chains immediately exit the ribosome (Ryabova et al., 1988). It has been argued that the protease protection referred to above could be the consequence of limited access to the nascent chain in the area around the ribosome by proteases or that protease resistance is the result of a cytosolic nascent chain associated complex (NAC) that is bound to the polypeptide (Wang et al., 1995).

We have taken an alternative approach to measuring the distance between the site of the most recently synthesized carboxy amino acid and the membrane. This was measured by increasing the number of amino acids on the carboxy side of a latent TMS until it was far enough from its site of synthesis to allow groups on its lumenal side to be glycosylated. With intermediate tms2 + 39, consisting of two TMSs of Pgp plus 39 amino acids, the loop between the first and second TMSs is not fully glycosylated on all three sites while the peptide was still tRNA- and ribosome-held. This suggests that the 39 amino acid spacer on the carboxy-terminal side of the second TMS is not long enough to allow it to reach from its site of assembly at the peptidyltransferase and stretch fully across the membrane. Our findings confirm the previous estimates of the distance between the sites of translocation at the membrane and of biosynthesis inside the ribosome. However, they do not address the issue of whether this distance is maintained inside or on the surface of the ribosome.

These results demonstrate that the first TMS of Pgp translocates prior to emergence of the second TMS from the ribosome. The translation intermediary tms2 + 22 is glycosylated on one of three possible glycosylation sites between the first and second TMS (Figure 1B). However, this translation intermediary is still attached to the ribosome (Figure 2), with TMS2 still inside the ribosome. Even with an additional 17 amino acids after this construct (tms2 + 39), the second TMS still has not fully emerged from the ribosome, and only two of the three potential sites are glycosylated (Figure 1C). Because the protein intermediate is glycosylated, it can be inferred that the first TMS has positioned itself across the membrane in its predicted orientation. This also suggests that TMS1 can translocate independently of TMS2. Additionally, because the amino terminus of Pgp remains on the cytoplasmic side of the ER, it is likely that the first TMS of the Pgp peptide chain moves across the membrane without associating with a second or multiple TMSs.

This system can be used to study other translocation events, such as folding of the nascent chain and integration of TMSs into the lipid bilayer. Our results indicate that

nascent translocation intermediates do not behave the same as the identical peptides that have been released from their tRNA and ribosomes: there are significant differences in glycosylation, and thus transmembrane topology (Figure 1A−C; compare lanes 3 and 4), and significant differences of membrane integration as well (Borel & Simon, 1996). While this technique is a powerful method for analyzing protein biogenesis, care should be taken to ensure that analysis is limited to those products that are true translation/translocation intermediates.

ACKNOWLEDGMENT

We thank Marjorie Russel, Peter Model, and members of the Simon laboratory for many stimulating discussions and comments on the manuscript and Michele Baez and Shoshona Hort for technical assistance.

REFERENCES

Anderson, D. J., Walter, P., & Blobel, G. (1982) *J. Cell Biol. 93*, 501.
Audigier, Y., Friedlander, M., & Blobel, G. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5783.
Blobel, G., & Sabatini, D. D. (1970) *J. Cell Biol. 45*, 130.
Borel, A. C., & Simon, S. M. (1996) *Cell 85*, 379.
Chuck, S. L., & Lingappa, V. R. (1992) *Cell 68*, 9.
Connolly, T., Collins, P., & Gilmore, R. (1989) *J. Cell Biol. 108*, 299.
Deshaies, R. J., & Schekman, R. (1987) *J. Cell Biol. 105*, 633.
Friedlander, M., & Blobel, G. (1985) *Nature (London) 318*, 338.
Gerlach, J. H., Endicott, J. A., Juranka, P. F., Henderson, G., Sarangi, F., Deuchars, K. L., & Ling, V. (1986) *Nature (London) 324*, 485.
Görlich, D., Prehn, S., Hartmann, E., Kalies, K.-U., & Rapoport, T. A. (1992) *Cell 71*, 489.
Kellaris, K. V., Bowen, S., & Gilmore, R. (1991) *J. Cell Biol. 114*, 21.
Lingappa, V. R., Cunningham, B. A., Jazwinski, S. M., Hopp, T. P., Blobel, G., & Edelman, G. M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3651.
Malkin, L. I., & Rich, A. (1967) *J. Mol. Biol. 26*, 329.
Matlack, K. E., & Walter, P. (1995) *J. Biol. Chem. 270*, 6170.
McCune, J. M., Lingappa, V. R., Fu, S. M., Blobel, G., & Kunkel, H. G. (1980) *J. Exp. Med. 152*, 463.
Monro, R. E., & Marcker, K. A. (1967) *J. Mol. Biol. 25*, 347.
Perara, E., Rothman, R. E., & Lingappa, V. R. (1986) *Science 232*, 348.
Redman, C. M., & Sabatini, D. D. (1966) *Proc. Natl. Acad. Sci. U.S.A. 56*, 608.
Ryabova, L. A., Selivanova, O. M., Baranov, V. I., Vasiliev, V. D., & Spirin, A. S. (1988) *FEBS Lett. 226*, 255.
Sabatini, D. D., & Blobel, G. (1970) *J. Cell Biol. 45*, 146.
Skach, W. R., Calayag, M. C., & Lingappa, V. R. (1993) *J. Biol. Chem. 268*, 6903.
Stirling, C. J., Rothblatt, J., Hosobuchi, M., Deshaies, R., & Schekman, R. (1992) *Mol. Biol. Cell 3*, 129.
Thrift, R. N., Andrews, D. W., Walter, P., & Johnson, A. E. (1991) *J. Cell Biol. 112*, 809.
Traut, R. R., & Monro, R. E. (1964) *J. Mol. Biol. 10*, 63.
Walter, P., Ibrahimi, I., & Blobel, G. (1981) *J. Cell Biol. 91*, 545.
Wang, S., Sakai, H., & Wiedmann, M. (1995) *J. Cell Biol. 130*, 519.
Wessels, H. P., & Spiess, M. (1988) *Cell 55*, 61.
Wolin, S. L., & Walter, P. (1988) *EMBO J. 7*, 3559.
Yonath, A., Leonard, K. R., & Wittmann, H. G. (1987) *Science 236*, 813.
Zhang, J.-T., Duthie, M., & Ling, V. (1993) *J. Biol. Chem. 268*, 15101.

BI960950Q