The Amino Terminus of Opsin Translocates "Posttranslationally" as Efficiently as Cotranslationally†

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ABSTRACT: Opsin, a member of the G-protein-coupled receptor family, is a polytopic membrane protein that does not encode a cleaved amino-terminal signal sequence. The amino terminus of opsin precedes the first known targeting information, suggesting that it translocates across the endoplasmic reticulum (ER) membrane after synthesis, uncoupled from translation. However, translocation across the mammalian ER is believed to be coupled to protein synthesis. In this study we show that opsin, within a range of nascent peptide lengths, targets and translocates equally efficiently co- and posttranslationally. Longer nascent opsin peptides have a lower efficiency of cotranslational translocation but an even lower efficiency of posttranslational translocation. We also show that SRP is required for both co- and posttranslational targeting.

The endoplasmic reticulum has been extensively studied as a model for understanding protein targeting and translocation. In higher eukaryotic systems, proteins destined for the ER[1] translocate across the membrane as they are synthesized on cytosolic ribosomes (*1*−*3*). Most of the model proteins contain a signal sequence at or near the amino terminus, which is necessary and sufficient for targeting to the ER (*4*−*7*). As this portion of the nascent protein emerges from the ribosome, it is recognized by the signal recognition particle (SRP). SRP binds to the nascent signal sequence and pauses translation until the ribosome/nascent polypeptide/SRP complex interacts with the ER membrane (*8*). At the ER membrane, SRP binds to the SRP receptor (SR) (*9*), and the ribosome engages the sec61 component of the translocon (*10*). After displacement of SRP from the ribosome and signal sequence, protein translation resumes, and the nascent polypeptide translocates across the membrane through an aqueous protein-conducting channel (*11*, *12*). In vitro experiments showed that mammalian ER was required during translation, since the addition of membranes at later times did not result in targeting and translocation (*1*−*3*, *8*, *13*−*16*). These observations have led to the view that all translocation across mammalian ER is obligatorily cotranslational. This view has subsequently been supported by data indicating a tight association between the ribosome and the translocation machinery. Contact between the ribosome and the translocation machinery maintains the protein-conducting aqueous channel in an open conformation (*12*), and contacts can be observed between the ribosome and sec61 complex (*17*, *18*). It has been suggested that the junction between the ribosome and translocon is even tight enough to exclude ions (*19*), although such a tight seal is not observed in structural studies (*17*, *18*). Because of the observed linkage between translation and translocation it has been proposed that the movement of the nascent protein across the membrane may be driven by polypeptide elongation (*3*).

In contrast, some proteins in the yeast *Saccharomyces cerevisiae* can be targeted to, and translocated across, the ER after being fully synthesized and released from the ribosome (*20*−*22*). The dissociation of translocation from translation has allowed detailed analysis of translocation in yeast. The yeast posttranslational pathway is not dependent on SRP but rather requires several cytosolic chaperones (*21*−*23*). A similar SRP and ribosome-independent pathway has been demonstrated in vitro for targeting to mammalian ER membranes with short (65−70 amino acid) naturally occurring polypeptides (*24*, *25*). This has been termed "ribonucleoparticle-independent import" since neither the ribosome nor SRP is required. In addition, the targeting process did not require the SR (*24*, *25*). These proteins contain cleaved amino-terminal signal sequences, which are required for targeting to the ER (*26*). The small size of the peptide was important for the use of this pathway, however, since fusions to other proteins resulted in the loss of ribonucleoparticle-independent import (*25*). Some fusions were compatible with ribonucleoparticle-independent import but were less efficient than the regular protein alone (*27*). The addition of apyrase abolished targeting, which indicated that an energy source was required for translocation and/or targeting (*25*, *27*). The specific energy requirements are more difficult to determine both because apyrase can hydrolyze other nucleotide triphosphates and because standard cytosol preparations contain

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[1] Abbreviations: TMS, transmembrane segment; SRP, signal recognition particle; SR, SRP receptor; WG, wheat germ extract; RRL, rabbit reticulocyte lysate; tOP, truncated opsin; ER, endoplasmic reticulum.

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enzymes that can transfer high-energy phosphates between different nucleotide phosphates.

Most proteins that are targeted to the mammalian ER are larger than the ribonucleoparticle-independent import substrates and require SRP for targeting. Therefore, we investigated methods for examining intermediates of mammalian proteins in vitro. Ribosome-bound nascent polypeptides have been used to dissociate translation from translocation across mammalian ER in vitro, but the efficiency was limited, which raised the possibility that ongoing translation was in fact required for targeting and/or translocation (*28*−*30*). To extend these results, and examine the translocation across mammalian ER in detail, we wished to establish an efficient assay that uncoupled translation from translocation. To try to ensure that the in vitro results reflect the physiologic pathway, care was taken to ensure that nascent polypeptides remained ribosome bound through all experimental manipulations. Additionally, we sought to use a wild-type mammalian protein that might be expected to target to the ER uncoupled from translation. Protein synthesis was paused at various stages of synthesis and examined in detail. Since these nascent polypeptides were still bound to functional ribosomes, they mimicked transient stages for prolonged times, in order to be accessible for study.

There are two classes of mammalian full-length proteins whose structures suggest a stage that may occur uncoupled from ongoing translation. The first class includes "tail-anchored" proteins: single-pass membrane proteins with cytosolic amino termini and short carboxyl termini that reside in the membrane (*31*). The transmembrane segment, which is the uncleaved signal sequence, is not predicted to exit the ribosome until the protein is released (*31*). Accordingly, members of this class have been shown to target to the ER after release from the ribosome (*32*, *33*), without SRP (*33*−*35*), and independently of the sec61 complex (*33*). The second class includes membrane proteins with extracellular amino termini that lack a cleaved signal sequence (*14*, *36*). The first transmembrane segment (TMS) functions as the signal sequence (*5*, *37*). This implies that the portion of the protein synthesized before the TMS may translocate post-translationally. Since the topological organization of these proteins suggests that their synthesis may have a stage that may be uncoupled from translation, we chose to examine one example in greater detail in order to better understand general principles of mammalian protein translocation.

Opsin is a protein from the second class of proteins that may have a stage of translocation that does not require ongoing protein synthesis. Opsin is a member of the G-protein-coupled receptor family and has been extensively studied as a model membrane protein (*5*, *36*, *38*−*50*). In this study we examine the targeting and translocation of the amino terminus of opsin. The amino terminus of mature opsin resides in the intradiscal (extracellular) space of the photoreceptor and during biogenesis is translocated into the ER lumen. As noted above, opsin lacks a cleaved signal sequence (*36*) and is targeted to the ER membrane by the first TMS (*5*, *50*). To explicitly determine the requirements for ongoing translation, the translocation of nascent opsin of various lengths was tested in the presence of protein synthesis inhibitors.

We show that efficient targeting and translocation of nascent opsin, like the tail-anchored proteins, is not dependent upon protein synthesis. However, in contrast to the tail-anchored proteins, the targeting and translocation of the amino terminus are efficient only while the nascent opsin is still functionally attached to its biosynthetic ribosome. This targeting and translocation occur efficiently only with short nascent opsin polypeptides. The in vitro efficiency achieved with this assay is very high, suggesting that it mimics the in vivo reaction pathway. Furthermore, there is strong correlation between the efficiencies of the post- and cotranslational reactions. Unlike the translocation of the tail-anchored proteins, SRP is required for both cotranslational and post-translational targeting of nascent opsin to the ER membrane. Since translation can be uncoupled from targeting and translocation, these latter steps can be selectively studied under conditions which might not be permissive for ongoing protein synthesis.

EXPERIMENTAL PROCEDURES

All chemicals were from Sigma (St. Louis, MO) or Fisher Chemicals (Chicago, IL) except where indicated.

Transcriptions and Translations. The constructs used to generate truncated mRNA were made by subcloning the full-length bovine opsin sequence including the upstream untranslated bovine region from pSF1 (*5*) into pSP73 (Promega, Madison, WI). The truncations were designated tOP^{xx}, for ***t***runcated ***op***sin, with the superscript indicating the number of codons after the end of the first transmembrane domain (the total number of amino acids = tOP number +61). The first transmembrane segment was used as a point of reference since it contains the first signal sequence (*5*). In some constructs, designated ΔK, the lysines at amino acids 16, 66, and 67 were mutated to alanine, arginine, and arginine, respectively, in order to reduce levels of posttranslational cytosolic modifications. The cytosolic modification resulted in the trace band (in reactions with no ER membranes added) at the same molecular weight as the glycosylated tRNA-nascent polypeptide. The mutations at amino acids 66 and 67 are conservative substitutions, are near the very end of the polypeptide synthesized by the short truncations (tOP^{20} and tOP^{30}), and are therefore predicted to remain buried in the ribosome unless the polypeptide has been released. Human opsin encodes an alanine at position 16 rather than a lysine. Thus, this mutation should not affect function. tOP^{20} ΔK and tOP^{30} ΔK were generated by PCR, which contained a *Bam*H1 sequence at the 3′ end to mimic the wild-type constructs. These constructs showed no variation in targeting or translocation when compared with wild-type constructs (data not shown). These constructs were purified with a QIAQuick PCR purification kit (QIAGEN, Chatsworth, CA). The cut DNA or purified PCR product was transcribed with a Message Machine SP6 kit (Ambion, Austin, TX) according to the manufacturer's specifications. Translations were performed with rabbit reticulocyte lysate (RRL) or wheat germ (WG) extracts supplemented with ER membranes (Promega, Madison, WI) according to manufacturer's instructions except that the reaction temperature was 25 °C. All translations were done with the RRL system unless otherwise indicated. Typically, reactions were treated with cycloheximide (2 mg/mL final) for 10 min at 25 °C, emetine (2 mM final) for 10 min at 25 °C, or puromycin (2 mM final) for 5 min at 25 °C, and then 5 min at 37 °C. For posttranslational incubations with membranes, 1.5 units of

ER membranes was added per 12.5 μL reaction. Translations were labeled with translation grade [^{35}S]methionine from Pharmacia Amersham Biotech (Piscataway, NJ) or Dupont NEN (Boston, MA). To inhibit glycosylation in some reactions, 0.25 mM acetylated glycosylation consensus tripeptide (NYT) was added prior to the addition of mRNA.

Fractionation. Membranes were harvested by sedimentation or flotation harvest. Membranes to be sedimented were overlaid on standard harvesting buffer [SHB; 1 M sucrose, 150 mM KOAc (pH 7.5), 50 mM Hepes−KOH (pH 7.5), 2.5 mM $Mg(OAc)_2$] and centrifuged in a TLA 100 rotor (Beckman, Palo Alto, CA) at 70000 rpm (189000g_{av}). Flotation-harvested samples were mixed with 7 volumes of 2.34 M sucrose [flotation solutions also contained 140 mM KOAc, 20 mM Hepes (pH 7.4), 5 mM $Mg(OAc)_2$, and 1 mM DTT]. The samples were overlaid with 1.9 and 0.25 M sucrose and centrifuged at 243000g_{av} in a TLA 100 rotor (Beckman, Palo Alto, CA) at 4 °C for 1 h. Membrane-associated proteins were located between the 1.9 and 0.25 M sucrose layers.

Salt-Washed ER Membranes. This was done essentially as described in the published SRP purification protocol (*51*). Briefly, an aliquot of 10 μL of ER membranes was mixed with 10 μL of ice-cold high salt buffer [1 M KOAc (pH 7.5), 10 mM $Mg(OAc)_2$] and incubated on ice for 10 min. The stripped ER membranes were then overlaid on a cushion of SHB and centrifuged at 139000g_{av} for 15 min at 4 °C. The membranes were resuspended in 10 μL of 100 mM KOAc (pH 7.5), 50 mM HEPES−KOH (pH 7.5), 2 mM $Mg(OAc)_2$, and 1 mM DTT.

SDS−PAGE and Analysis. Samples were precipitated with 2.5 volumes of 3 M ammonium sulfate and run on 15% gels in Mini-PROTEAN II cells from Bio-Rad (Hercules, CA) according to the manufacturer's specifications. Gels were dried on model 583 (Bio-Rad, Hercules, CA) gel dryers, exposed to a phosphor screen, scanned with a STORM 860 phosphorimager, and analyzed with ImageQuant Software (Molecular Dynamics, Sunnyvale, CA). Individual bands were background subtracted using an equivalent area in the same lane. Percentage glycosylation was calculated as the fraction of the glycosylated band of the sum of the glycosylated and nonglycosylated band intensities.

RESULTS

Since the biogenesis of a membrane protein is a dynamic process, the experimental strategy was to study intermediates in protein synthesis by arresting protein translation at different points. This has been previously done using two different experimental strategies. In one method a stop codon was inserted in the coding sequence of the protein to be studied. This generated a shortened protein molecule which was released from its biosynthetic ribosome (*45*, *52*−*54*). A second method used truncated mRNA molecules that ended in the coding region without a stop codon, which stalled translation at the end of the mRNA (*5*, *28*−*30*, *55*−*57*). Nascent polypeptides, still bound to the biosynthetic ribosome, were then used to study intermediates in biogenesis. The continued presence of the ribosome has been shown to affect the status of a nascent polypeptide (*54*, *58*−*60*).

Several criteria were used to distinguish intermediates in translation from released polypeptides (*58*, *61*). The criteria used to identify these intermediates were as follows: (1) the molecular weight of peptides generated should be increased by an amount consistent with continued covalent attachment to the tRNA; (2) this increase in molecular weight should be reversed by treatment with puromycin (*62*, *63*), an aminoacyl-tRNA analogue which will displace the tRNA from a peptidyl-tRNA, but only with an active ribosome (*64*); (3) the nascent polypeptides should migrate in a sucrose gradient with the ribosome fraction unless released with puromycin. For all of our experiments these criteria were used to distinguish intermediates in translation from peptides that had been released from the ribosome.

Our first goal was to establish in vitro translation conditions that allowed nascent opsin to remain functionally bound to the ribosome. Under standard translation conditions, truncated opsin mRNA did not translate as robustly as full-length mRNA. Therefore, translation temperature and magnesium concentration were varied to optimize the translational yield. The stability of translation intermediates was assessed using the above criteria. Since translations in vitro are typically incubated between 22 and 37 °C, with increasing translational yield at higher temperatures (*65*, *66*), we examined the effect of temperature on the yield and stability of tOP^{80} intermediates at 25 and 30 °C (Figure 1A). At the end of translation, half of each sample was treated with puromycin to release nascent peptides from the ribosome. The amount of nascent peptide still bound to its tRNA was quantified after separating the translation products on SDS−PAGE.

Translations incubated at 30 °C for 10 min showed a large yield of tRNA-bound nascent polypeptides, of which 50% could be released by puromycin (Figure 1A). The yield of these translation intermediates was maximal at 20 min and then decreased steadily at later time points. At all of the time points shown, only about 50% of the nascent polypeptide bands translated at 30 °C were released from the tRNA by puromycin treatment. Samples translated at 25 °C accumulated tRNA-nascent polypeptides more slowly and reached a peak level by 60 min. During the first 10−30 min of translation at 25 °C, the fraction released with puromycin ranged from 60% to 75%, which was greater than those translated at 30 °C. Since translations at 25 °C yielded more stable, ribosome-bound tRNA-nascent polypeptides, subsequent translations were performed at this lower temperature.

Lowering the magnesium concentration in translations has also been used in vitro to improve yields of synthesized protein. Standard magnesium (0.5 mM) and reduced magnesium (0.16 mM) are compared in Figure 1B. The extent of association of the nascent polypeptide with ribosomes was quantified by fractionation on a sucrose gradient and by sensitivity to puromycin. At the lower magnesium concentration, most of the tRNA-nascent polypeptides were observed on the top of the sucrose gradient, indicating that they were not bound to ribosomes. In contrast, under standard magnesium conditions, the majority of the tRNA-nascent polypeptides did enter the gradient, indicating that they were bound to ribosomes. Furthermore, only 43% of the tRNA-nascent polypeptides were released by puromycin treatment of the low magnesium translation, while translations at standard magnesium over 80% were released by puromycin treatment (data not shown). This indicated that, at reduced magnesium concentrations, a significant portion of the tRNA-bound

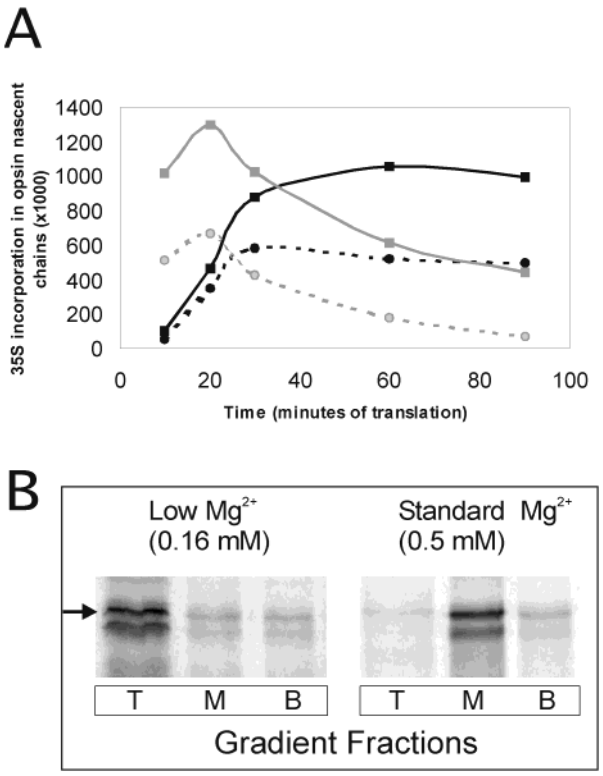

FIGURE 1: Optimizing formation of a ribosome-bound tRNA nascent polypeptide. (A) tRNA-bound nascent opsin accumulated more quickly when translated at higher temperatures but was not associated with the ribosome. Translations of tOP^{80} were incubated at 25 °C (black lines) or 30 °C (gray lines). Aliquots were removed at the indicated times and treated with puromycin or left on ice. Samples were then analyzed by SDS−PAGE and the intensities of the bands quantified. The solid lines indicate the number of counts in the tRNA band in untreated samples, and the dashed lines indicate the number of counts that were released by puromycin treatment [(untreated tRNA counts) − (puromycin-treated tRNA counts)]. (B) High magnesium is required to maintain nascent opsin molecules bound to functional ribosomes. Translations of tOP^{40} with 0.16 or 0.5 mM magnesium were incubated for 30 min at 25 °C. The translations were sedimented as described in Experimental Procedures. The top fractions (T), middle fractions (M), and bottom fractions (B) were analyzed by SDS−PAGE. The arrow indicates the nascent opsin polypeptide bound to the tRNA.

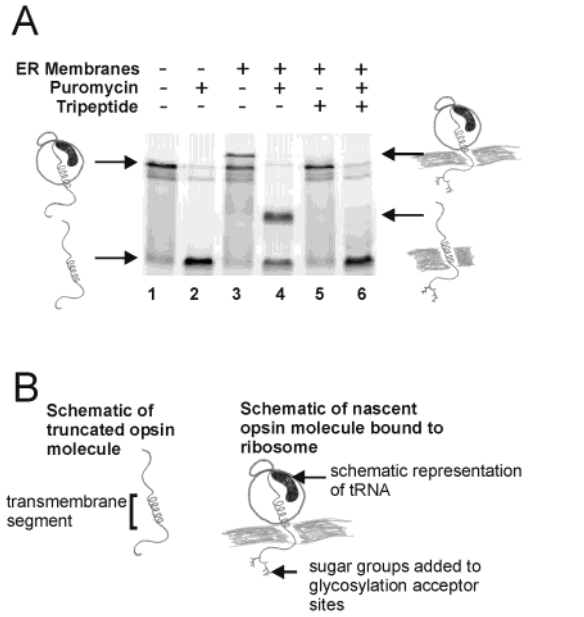

FIGURE 2: Translation of truncated mRNA with membranes generates intermediates in translation and translocation across the membrane. (A) tOP^{30} ΔK was translated for 30 min at 25 °C in the absence (lanes 1 and 2) or presence of ER membranes (lanes 3−6). Glycosylation was competitively inhibited with 0.25 mM acetylated tripeptide (N-Y-T) (lanes 5 and 6). Half of each sample was treated with puromycin to explicitly release peptides from the tRNA. (B) Schematic representation of the truncated opsin molecule indicating the transmembrane segment region. Not shown is the translocation machinery in the membrane.

nascent opsins were released from the ribosome. Each truncated mRNA can only be translated once to generate a stalled product. Thus the increased translational efficiency observed with lower magnesium may be the consequence of this decreased ribosome stability, since each released mRNA can now be reused for new rounds of translation. Since these translation conditions produce stable intermediates in translation, all subsequent experiments were done in 0.5 mM magnesium and incubated at 25 °C to maximize the yield of stable intermediates in translation.

The ribosome-nascent opsin complexes, obtained under the conditions described above, were tested for the ability to interact with ER membranes. Translation of tOP^{30} ΔK (93 amino acids total) without membranes present (Figure 2, lane 1) generated two prominent ^{35}S-labeled products when analyzed on SDS−PAGE. The lower band had an estimated molecular mass of 9 kDa, which is consistent with the predicted size of the tOP^{30} peptide. The upper band had an estimated molecular mass of 30−35 kDa. This band corresponds to the nascent peptide still attached to the last peptidyl-tRNA. The [^{35}S]methionine tRNA alone runs at approximately 25 kDa on SDS−PAGE (*66*). Treatment of the translation with puromycin (lane 2) resulted in the loss of the upper band. This showed that the tRNA-polypeptides were still bound to functional ribosomes, since puromycin can only release peptidyl-tRNA bonds in the presence of a functional ribosomal peptidyltransferase (*62*, *64*). When the translation mix contained ER membranes (lane 3), the band corresponding to the tRNA-nascent polypeptide was reduced in intensity, and an additional band of higher molecular weight was observed. This represents the tRNA-nascent polypeptide that has been glycosylated at the two glycosylation acceptor sites in the amino terminus. When this translation was treated with puromycin (lane 4), the tRNA-nascent polypeptide bands are reduced in intensity, and a band of 15 kDa increased in intensity, which is the predicted size of the tOP^{30} peptide (released from the tRNA) with two glycosylations. The identity of this band was confirmed by the addition of a competitive inhibitor of glycosylation (lanes 5 and 6), which prevented the molecular mass shifts attributable to glycosylation.

To test if translocation across the membrane required ongoing translation, protein synthesis was inhibited (with cycloheximide or emetine) prior to the addition of membranes. When tOP^{20} was translated in reticulocyte lysate in the absence of ER membranes, over 98% of the tRNA-polypeptides were unglycosylated (Figure 3, lane 1). [The trace band that comigrated with the glycosylated tRNA-bound peptide in some gels in the absence of ER membranes was not due to glycosylation. When several lysines were removed, this band was not seen, suggesting that the modification was due to ubiquitination (see Figure 2).] In

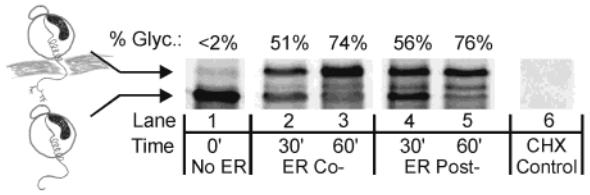

FIGURE 3: Posttranslational targeting and translocation of tOP^{20}. Translations of tOP^{20} were incubated for 30 min at 25 °C without ER membranes (lane 1) or with ER membranes present cotranslationally (ER Co-, lanes 2 and 3). A portion of the translation without membranes was treated with cycloheximide, and then ER membranes were added (ER Post-, lanes 4 and 5). Cycloheximide was added to a sample prior to incubation at 25 °C (CHX, lane 6).

contrast, when tOP^{20} was translated in the presence of ER membranes, at 30 min 51% of the tRNA-bound nascent opsin was glycosylated (lane 2), and at 60 min 74% was glycosylated (lane 3). When ER membranes were added after cycloheximide, at 30 min 56% of the nascent tRNA-bound opsin was glycosylated (lane 4), and at 60 min 76% was glycosylated (lane 5). There was no detectable translation when cycloheximide was added at the beginning of a translation reaction (lane 6). Thus, in the absence of ongoing translation the amino terminus of opsin was able to target and translocate as efficiently as when membranes were present during chain elongation. Moreover, the kinetics of translocation were equivalent for both cotranslational and posttranslational translocation reactions and thus independent of ongoing translation.

The observation that the amino terminus of tOP^{20} translocates across the ER membrane posttranslationally could be either an indication that opsin was paused at approximately the right length for the targeting reaction to occur or a reflection of a property of the amino terminus (unrelated to the timing of targeting). To clarify the requirement for targeting and translocation of the amino terminus with respect to length, nascent opsin polypeptides paused at different lengths were examined (shown schematically in Figure 4B). Nascent opsin of increasing length was translated with ER membranes present cotranslationally or posttranslationally. The efficiency of cotranslational translocation decreased with increasing lengths of nascent opsin to a level of 30% (Figure 4A). This is comparable to the level of glycosylation seen with full-length opsin translated in the presence of ER membranes (data not shown). The efficiency of posttranslational translocation was similar to that of cotranslational translocation for opsin constructs shorter than tOP^{80}. However, for constructs longer than tOP^{80} (which encodes the first three transmembrane segments), posttranslational translocation was significantly less efficient.

Fractionation by flotation harvest was used as an independent measure of targeting to the ER membrane. When $tOP^{20}\Delta K$ was translated without membranes, the entire translation remained in the cytosolic fraction (Figure 5, lanes 1 and 2). When membranes were present either co- or posttranslationally, almost all of the glycosylated protein fractionated with the membranes, while the unglycosylated tRNA-bound protein remained in the cytosolic fraction. Puromycin was added to some samples to explicitly release the nascent polypeptide from the ribosome prior to the addition of membranes (lanes 7 and 8). Only trace amounts of released peptides were in the membrane fraction. This demonstrated that although ongoing translation is not required for efficient targeting to the ER membrane, continued association with the ribosome is required. Furthermore, the glycosylated tRNA-polypeptides were stably associated with the ER membrane, which provides further evidence that the truncated mRNA generated intermediates in the biogenesis process.

Constructs as short as tOP^{20} and tOP^{30} targeted to the ER very efficiently despite only encoding only 20 and 30 amino acids after the first functional signal sequence. It has been previously shown that proteins with a signal sequence at the amino terminus cannot engage SRP or target to the ER until 40−50 amino acids have been synthesized after the signal sequence (*53*, *67*−*69*). This spacer is thought to be required in order for the signal sequence to span the internal protein tunnel of the ribosome and have access to the cytosol. This raised the possibility that the targeting of these short intermediates was not SRP-dependent. To be certain that the posttranslational reaction was similar to the cotranslational reaction, the requirement for SRP was tested.

Most preparations of pancreatic ER contain sufficient SRP to support targeting and translocation in vitro (*70*). This residual SRP can be removed with a high-salt wash. An SRP-

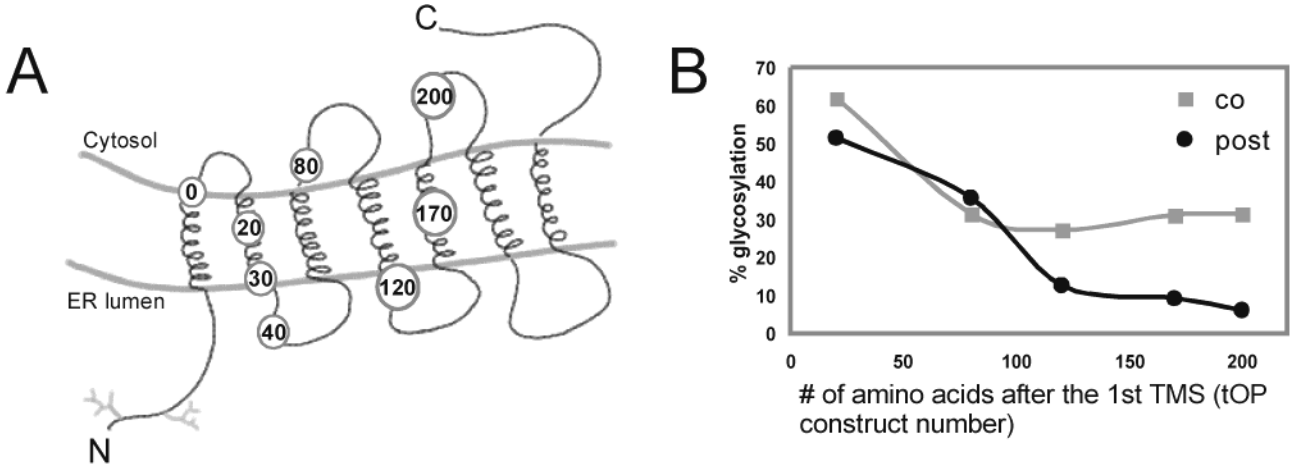

FIGURE 4: The efficiency of co- and posttranslational translocation depends on the length of the nascent chain. (A) Opsin nascent peptides of increasing lengths were translated either in the presence of ER membranes (co) or only exposed to ER membranes after treatment with cycloheximide (post). The averaged efficiency of glycosylation is plotted against the construct number. (B) Schematic of the sites of truncation used in part A.

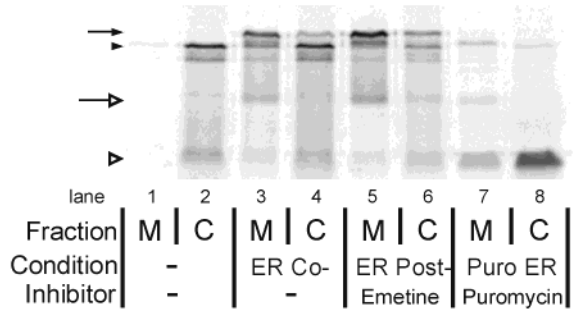

FIGURE 5: Nascent opsin molecules must remain attached to ribosomes to efficiently target to the ER membrane. tOP^{20} ΔK was translated without ER membranes (lanes 1 and 2), with ER membranes present cotranslationally (lanes 3 and 4), with ER membranes present only posttranslationally (lanes 5 and 6), or with ER membranes added after puromycin treatment (lanes 7 and 8). Posttranslational reactions were treated with cycloheximide prior to membrane addition. To release nascent chains from the ribosome, translations were treated with puromycin. Samples were separated into membrane (M) and cytosol (C) fractions by flotation harvest as described in Experimental Procedures. Fractions were analyzed by SDS−PAGE. Key: filled arrow, glycosylated tRNA-nascent opsin; filled arrowhead, tRNA-nascent opsin; outline arrow, glycosylated released opsin; outline arrowhead, released opsin.

dependent protein translated in reticulocyte lysate will translocate across salt-washed membranes but one translated in wheat germ extract will not since reticulocyte lysate contains functional SRP while wheat germ does not (*8*). Preprolactin was used as a control since it is known to require SRP for targeting. Preprolactin translated in reticulocyte lysate supplemented with salt-washed membranes (K-RM) was targeted to the ER, and the signal sequence was cleaved (Figure 6A, lane 2). In contrast, there was no signal sequence cleavage of preprolactin synthesized in wheat germ supplemented with salt-washed membranes (lane 4).

When translated in vitro, both full-length opsin and an opsin truncated at 93 amino acids have been shown to require SRP for cotranslational targeting to the ER membrane (*5*). We tested if the short arrested opsin also required SRP for targeting to the ER. The translation intermediate tOP^{30} was synthesized in reticulocyte lysate (a source of SRP) or wheat germ (lacking functional SRP) in the presence of untreated membranes (RM) or salt-washed membranes (K-RM) (Figure 6B). In the absence of ER membranes there was no detectable glycosylation in either reticulocyte lysate (Figure 6B, lane 5) or wheat germ translations (lane 8). Translation of tOP^{30} in reticulocyte lysate with untreated membranes present cotranslationally showed efficient glycosylation of the nascent polypeptides (lane 6). Glycosylation in the presence of salt-washed membranes was nearly as efficient (lane 7, 16% reduced relative to the unwashed membranes). The presence of SRP in the reticulocyte lysate largely compensated for the loss of SRP in the salt-washed membranes. Thus, the salt-washed membranes showed only a small reduction in the efficiency of targeting and translocation, as assayed by glycosylation, and were not significantly damaged by the treatment.

Translation of tOP^{30} in wheat germ with untreated membranes present cotranslationally showed glycosylation which was less efficient than for the reticulocyte translations (lane 9). Glycosylation in the presence of salt-washed membranes (lane 10) was significantly less efficient (68% reduction). This reduction in efficiency can be attributed to

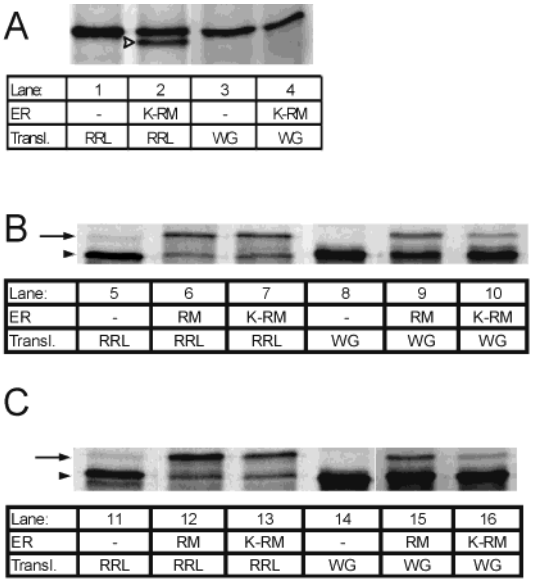

FIGURE 6: SRP is required for cotranslational and posttranslational targeting and translocation of nascent opsin. (A) Salt-stripped ER membranes failed to translocate preprolactin. Translations of preprolactin were incubated for 30 min at 25 °C without ER membranes (lanes 1 and 3) or with salt-stripped ER membranes (K-RM, lanes 2 and 4). The translations were performed with either rabbit reticulocyte lysate (RRL) or wheat germ extract (WG). The arrowhead indicates the signal sequence cleaved prolactin. (B) SRP was required for cotranslational targeting of short nascent opsin. tOP^{30} was translated in either RRL or WG without ER membranes (lanes 5 and 8) or in the presence of untreated ER membranes (RM, lanes 6 and 9) or salt-stripped ER membranes (K-RM, lanes 7 and 10). The RRL translation mix also provides SRP, while the WG translation mix does not. Key: arrow, glycosylated tRNA-nascent opsin; arrowhead, tRNA-nascent opsin. (C) SRP was required for posttranslational targeting of short nascent opsin. Lanes are as in (B) except that translations of tOP^{30} in either RRL or WG were arrested with cycloheximide and then incubated with no additions (lanes 11 and 14), with ER membranes (RM, lanes 12 and 15), or with salt-stripped ER membranes (K-RM, lanes 13 and 16).

the lack of SRP. Therefore, SRP is required to target these short opsin nascent polypeptides cotranslationally.

We next tested for the potential involvement of SRP in the posttranslational targeting and translocation of opsin (Figure 6C). After translation of tOP^{30}, cycloheximide was added prior to the addition of ER membranes. Without membranes, there was no detectable glycosylation (lanes 11 and 14). Translation of tOP^{30} in reticulocyte lysate supplemented with untreated membranes posttranslationally showed efficient glycosylation of the nascent polypeptides (lane 12). Glycosylation with salt-washed membranes added posttranslationally was nearly as efficient (lane 13, 12% reduction). Translation of tOP^{30} in wheat germ with untreated membranes added posttranslationally showed less efficient glycosylation compared to the reticulocyte lysate samples (but was comparable to wheat germ cotranslational samples). Glycosylation was significantly reduced with the posttranslational addition of salt-washed membranes (lane 16, 60% reduction) compared to untreated membranes.

Since both cotranslational and posttranslational targeting and translocation are reduced in efficiency with limiting SRP (but not with SRP supplementation from RRL), we concluded that SRP is also required for the posttranslational translocation of nascent ribosome-attached opsin.

DISCUSSION

The biogenesis of membrane proteins involves many steps, some occurring in parallel and others occurring sequentially. These steps include synthesis, interactions with cytosolic proteins (e.g., SRP, NAC, ribosomal proteins), targeting of different domains to the ER, selective translocation of different domains through the protein-conducting channel, interactions with lumenal proteins of the ER, folding of the nascent protein, posttranslational modifications (glycosylation, disulfide bonds), and integration into the hydrocarbon core of the lipid bilayer. The translocation of most membrane proteins in mammalian systems is tightly coupled to translation. Our goal was to identify and characterize a system in which a mammalian membrane protein, or at least a sizable domain of a protein, is translocated in a physiological context after synthesis. This would allow us to differentiate between requirements for translation and targeting from translocation.

The model system we used was opsin, a seven transmembrane domain, G-coupled receptor. Many members of this family lack an amino-terminal signal sequence but, nevertheless, translocate their amino terminus into the lumen of the ER (*5*, *36*, *50*). Thus, it has been suspected that translocation might be independent of ongoing translation. Our strategy was to arrest synthesis of opsin at various steps in synthesis and then, in the absence of ongoing translation, add ER membranes and assay for translocation.

To ensure that our in vitro assay faithfully and efficiently mimicked in vivo synthesis, we established the following criteria to ensure we were examining true intermediates in the biogenesis of opsin: (1) the nascent opsin had to still be attached to its tRNA; (2) the nascent opsin-tRNA still had to be attached to its ribosome (as assayed by fractionation in a sucrose gradient); (3) the nascent opsin-tRNA had to be attached to the ribosome at the peptidyl transferase (as assayed by sensitivity to puromycin); and (4) the nascent opsin-tRNA-ribosome had to be competent to target to the endoplasmic reticulum (as assayed by harvesting with the ER membranes or glycosylation by an ER lumenal oligosaccharyl transferase). Unfortunately, many of the conditions that optimize protein translation in vitro destabilize the interaction of nascent chains with the ribosome and do not lead to the synthesis of stable intermediates. As a result, we chose to optimize conditions (25 °C, 0.5 mM magnesium) that maximized the synthesis of true intermediates in biosynthesis, recognizing that this could result in lower amounts of total translation product.

Intermediates in the synthesis of opsin were able to efficiently target and translocate in the absence of all protein synthesis and elongation of the nascent polypeptide. The translocation efficiency was significantly greater than previously described for elongation-independent translocation of a longer opsin construct in vitro (*29*), other constructs with an amino-terminal signal sequence (*28*, *30*, *56*), and even the cotranslational translocation exhibited by full-length opsin expressed in vitro (data not shown). Furthermore, no previous publication demonstrated posttranslational translocation of an intermediate in protein translation: a nascent polypeptide still covalently attached to a tRNA. The weak efficiency previously observed for posttranslational translocation has been used to support the hypothesis that the movement of the nascent chain across the membrane is driven by the elongation of the nascent polypepetide. The efficiency of the translocation of the amino terminus of opsin in our experiments is inconsistent with that model.

The efficiency of posttranslational translocation dropped as the length of the arrested fragment of opsin exceeded 80 amino acids after the first membrane segment. In fact, the efficiency was highest at the nascent chain lengths approximating the point where the first topogenic signal (TMS 1) is predicted to be emerging from the ribosome. This suggests that the nascent opsin molecules were arrested at the point in synthesis that they would normally bind to SRP. This is consistent with the hypothesis that a function of translational arrest by SRP is to keep the nascent polypeptide at the length needed for targeting, thus extending the time during synthesis that nascent chains may productively interact with ER membranes. This would prevent both folding of proteins prior to translocation and cytosolic exposure of secretory and lysosomal enzymes (*8*). Although arrest is not absolutely required for targeting and translocation (*71*), the existence of an "SRP window" for interaction of a nascent chain was proposed on the basis of a mathematical model of the SRP−ribosome−membrane interactions (*72*). A specific range of chain lengths that bound SRP was found by comparing the ability of SRP to bind to nascent chains of various lengths, with the affinity decreasing as the size increased (*67*).

There were a few surprising observations in these experiments. First, in previous studies the posttranslational targeting of shortened opsin was inefficient. In contrast, in this study the posttranslational targeting and translocation were very efficient (and equal to cotranslational) for nascent chains as long as 143 amino acids (two TMS predicted out of the ribosome). This indicates that this large amino acid segment did not inhibit targeting, despite any potential folding or secondary structure. Furthermore, the high efficiency suggests that it is representative of a physiological step in the biogenesis of opsin. We performed the same assay with another membrane protein P-glycoprotein, whose first transmembrane segment also provides the first targeting information. However, in the case of P-glycoprotein, the amino terminus remains in the cytosol. Thus it would not require a translocation step that is independent of protein synthesis. In agreement with this prediction, P-glycoprotein showed no posttranslational translocation (data not shown).

A second surprising observation was that efficient posttranslational targeting of opsin required continued association with the ribosome and SRP. This is in contrast to the two other mammalian model systems for posttranslational translocation: membrane proteins whose bulk is in the cytosol with a transmembrane segment at their carboxyl terminus and short (65−70 amino acid) peptides, both of which target to the ER after release from the ribosome, independently of SRP (*24*, *25*, *27*, *32*). Our results are consistent with other studies of posttranslational translocation of other classes of proteins, which did require SRP and continued association with the ribosome (*28*, *30*, *56*, *73*). However, we have not resolved whether the ribosome simply serves a passive role in maintaining an opsin structure that is competent for translocation or if it serves a more active role in engaging the translocation machinery.

A third surprising observation was the efficiency with which opsin targeted to the ER when as few as 20 amino

acids were synthesized after the first transmembrane segment. Previously published data suggested that opsin would not target until at least 40 amino acids had been synthesized after the transmembrane segment. This was based on both the observation that, during synthesis of a protein, the 35−50 most recently added amino acids are still buried with the tunnel of the ribosome (*61*, *74*−*77*) and the previously published studies suggesting that a signal sequence cannot engage SRP and mediate targeting to the ER until at least 30−50 additional amino acids have been synthesized (*53*, *67*−*69*).

There are several possible explanations for these differences. The previous studies of translocation of shortened opsin examined nascent peptides that were released from their biosynthetic ribosomes. The greater efficiency of targeting that we observed with shorter chains may be the consequence of studying a nascent polypeptide that is still attached to its biosynthetic ribosome. Other estimates of the nascent chain length required for the interaction with SRP were based on studies of proteins with a signal sequence at the extreme amino terminus. The assays used for targeting in many studies were cleavage of the signal sequence or tight binding to the ER membrane (measured by fractionation). For the signal sequence to be cleaved requires exposure of the cleavage site (at the carboxyl end of the signal sequence) to a lumenal enzyme. Tight binding to the membrane may require a large portion of the nascent polypeptide to interact with the translocation machinery in the membrane. Each of these assays may in fact require a longer nascent chain than is required for targeting alone by an amino-terminal signal sequence. In the case of opsin, there is an extension preceding the signal sequence, the translocation of which can be assayed by glycosylation.

The ability to efficiently dissociate the steps of translation from targeting and translocation provides an experimental system in which it is possible to test the requirements for proteins, small molecules, and nucleotides that are specifically required for the latter steps of protein biosynthesis.

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