Co-translational Targeting and Translocation of the Amino Terminus of Opsin across the Endoplasmic Membrane Requires GTP but Not ATP*

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The tight coupling between ongoing translation and translocation across the mammalian endoplasmic reticulum has made it difficult to determine the requirements that are specific for translocation. We have developed an *in vitro* assay that faithfully mimics the co-translational targeting and translocation of the amino terminus of opsin without ongoing translation. Using this system we demonstrate that this post-translational targeting and translocation requires nucleotide triphosphates but not cytosolic proteins. The addition of GTP alone was sufficient to fully restore targeting. The addition of ATP was not specifically required, and non-hydrolyzable analogs of ATP that blocked 90% of the ATPase activity also had no inhibitory effect on translocation.

Targeting to the mammalian endoplasmic reticulum (ER)[1] membrane has been extensively studied and is a model system for other targeting events. Proteins with the appropriate signal sequence (1) bind to the signal recognition particle (SRP), which pauses translation (2–4). SRP then binds to the SRP receptor, which brings the ribosome and nascent peptide to the ER and releases the translational pause (5–7). The nascent peptide is then translocated through protein-conducting channels (8). Most studies on targeting to the ER membrane have used secreted proteins with cleaved amino-terminal signal sequences for which there is tight coupling between ongoing translation and translocation (9–13). This tight coupling between translation and translocation has complicated the analysis of the requirements for translocation in mammalian systems, as distinct from the requirements for translation.

The analysis of translocation in yeast has been more straightforward since a completely post-translational pathway has been demonstrated. Some yeast proteins can be targeted to the ER after complete synthesis and release from the ribosome (14, 15). This has allowed the *in vitro* analysis of the process in isolation of the requirements for ongoing translation, which

has provided a number of insights into mechanisms of translocation. ATP is required for post-translational translocation across the yeast ER (15), as a consequence of the involvement of both cytosolic and ER-lumenal ATPase chaperones (14, 16, 17). The lumenal chaperone BiP (also known as Kar2 and Grp78) plays a critical role in yeast translocation. Both temperature-sensitive mutations in BiP and decreased expression of BiP cause translocation defects *in vivo* (18, 19). Proteoliposomes reconstituted with purified yeast ER proteins required BiP for translocation as well (20).

The role of ATP and BiP in mammalian translocation is unresolved. ER microsomes that had been depleted of lumenal contents (including BiP) were competent to target proteins to the membrane but failed to translocate them across the microsomal membrane (21). In other experiments, proteoliposomes reconstituted with purified ER membrane components did not require BiP (22, 23). Because ongoing translation was required in these assays, the requirement for ATP could not be independently evaluated. Other *in vitro* systems have been used to partially dissociate the coupling between translation and translocation across the mammalian ER membrane. However, the targeting and translocation efficiencies were very low (24–27), suggesting that efficient translocation may only occur coupled to translation.

To investigate translocation in a mammalian system, we developed an assay using translation intermediates of opsin. Like most members of the G-protein-coupled receptor family, opsin does not have cleaved amino-terminal signal sequence, but nevertheless, the amino terminus is translocated across the ER membrane. The first transmembrane segment functions as the signal sequence, and it must emerge from the ribosome to bind SRP and target to the membrane (28). This implies that the segment of opsin that is on the amino side of the first membrane segment can only translocate uncoupled from translation. Indeed, for nascent polypeptides paused as the first transmembrane segment emerges from the ribosome, the nascent opsin can efficiently target to the ER membrane and translocate uncoupled from ongoing protein synthesis ("post-translationally") (29).

Thus, a transient step in the biogenesis of opsin can be investigated *in vitro*, isolated from the requirements for translation. Using this system, we have observed that GTP is necessary for translocation and that there is no specific requirement for ATP or cytosolic proteins.

EXPERIMENTAL PROCEDURES

General chemicals were from Sigma or Fisher unless otherwise noted. Puromycin stock was 20 mM in 500 mM Hepes-KOH (pH 7.4). Cycloheximide stock was 20 mg/ml. Emetine stock was 20 mM. ATP, GTP, and UTP stocks (100 mM) were from Amersham Biosciences. Restriction enzymes were from New England Biolabs (Beverly, MA).

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[1] The abbreviations used are: ER, endoplasmic reticulum; RBOp, ribosome-bound opsin polypeptide; tOP, truncated opsin; SRP, signal recognition particle; TB, translation/translocation buffer; AMPPNP, adenosine 5′-(β,γ-imino)triphosphate; GMPPNP, guanylyl imidodiphosphate.

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Translations were labeled with translation grade [35S]methionine from Amersham Biosciences or PerkinElmer Life Sciences.

Constructs—Opsin truncation mutants have been previously described (29). The nomenclature we use for this shortened nascent opsin construct is tOP$^{\times\times}$, for truncated opsin, with the superscript number indicating the number of codons after the end of the first transmembrane domain (which ends at amino acid 61). The first transmembrane segment is used as a point of reference because it is the first information targeting the nascent opsin to the ER membrane (28). Throughout this study we use tOP^{30}, which contains 91 amino acids total, 30 after the first transmembrane domain.

Transcriptions—DNA plasmids were digested with *Bam*HI and purified with a QIAQuick PCR purification kit (Qiagen, Chatsworth, CA). The cut DNA was subsequently transcribed with a Message Machine SP6 kit (Ambion, Austin, TX) according to the manufacturer's specifications.

Translations—The nuclease-treated rabbit reticulocyte lysate extract and RNasin were from Promega (Madison, WI). Canine pancreatic membranes (ER membranes) were purchased from Promega or prepared as previously described (30). Translations were performed according to the manufacturer's specifications, except as described in the text.

Post-translational Reactions—After translation with no ER membranes present, further translation was inhibited with cycloheximide (2 mg/ml final) or emetine (2 mM final) for 10 min at 25 °C. ER membranes (0.75 μl/12.5-μl reaction) were added after treatment. The reaction was incubated at 25 °C for an additional 30 min. Some samples were explicitly released from the ribosome with puromycin (2 mM final) and incubated for 5 min at 25 °C then 5 min at 37 °C.
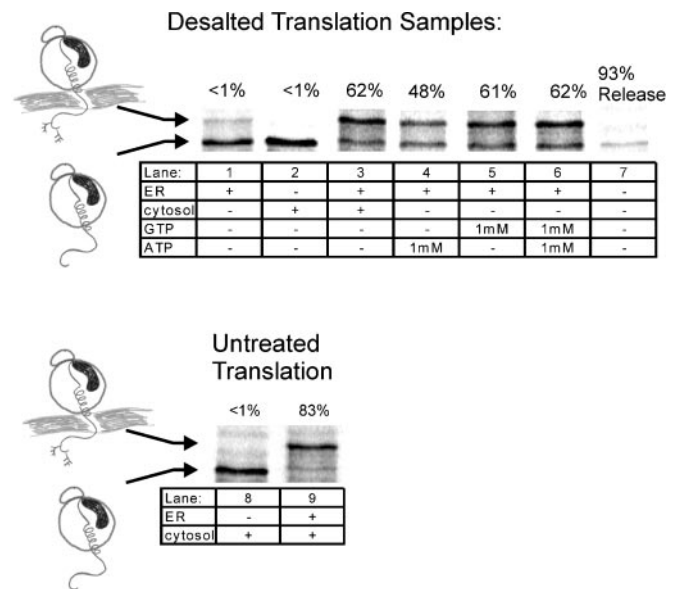
Purification of Ribosome-bound Nascent Opsin Polypeptides (RBOps) by Desalting—Translation reactions (prepared as above for post-translational reactions) were desalted using Microspin® G-25 columns (Amersham Biosciences). The column was equilibrated with translation/translocation buffer (TB:120 mM potassium acetate (pH 7.5), 50 mM Hepes-KOH (pH 7.5), 2 mM magnesium acetate) supplemented with 1 mM dithiothreitol just before use. The column was centrifuged at 735 × g_{av} for 25–30 s. The translation was diluted with buffer to a volume of 50 μl final and then added to the column. The sample was centrifuged into the column at 735 × g_{av} for 25–30 s, and the flow-through was discarded. The desalted translation mix was eluted by centrifugation for 2 min at 735 × g_{av} and diluted with TB. Supplemented reactions were typically incubated at 25 °C for 30 min.

Purification of RBOp by Centrifugation—Translations (prepared as above for post-translational reactions) were diluted in TB plus dithiothreitol, overlaid on standard harvesting buffer (1 M sucrose, 150 mM potassium acetate (pH 7.5), 50 mM HEPES-KOH (pH 7.5), 2.5 mM magnesium acetate), and centrifuged in TLA 100 rotor (Beckman Instruments) at 62,000 rpm (~148,000 × g_{av}) for 30 min at 4 °C. The resulting ribosome nascent opsin complex pellet was resuspended in TB plus dithiothreitol.

Post-translational Reactions with RBOp—For reactions, 10-μl aliquots were supplemented with 1 μl of a 10× energy stock or reticulocyte-lysate (cytosol). ER membranes (0.5 μl) were added to each 10-μl reaction.

ER Membrane Pretreatment with Inhibitors—ER membranes were diluted in TB containing 1 mM AMPPNP or GMPPNP and incubated for 10 min at 25 °C. Mock-treated samples were diluted in buffer alone under the same conditions. ER membrane aliquots were then added to purified RBOp for post-translational reactions as above. The final concentration of inhibitor was less than 200 μM in the reaction.

ATPase assay—ATPase activity was measured by determining the concentration of NADH using a Genesys 2PC spectrophotometer (Spectronic Instruments, Rochester, NY) with a coupled system (that converted 1 molecule of NADH to NAD$^+$ for each molecule of ADP generated) as previously described (31).[2] Briefly, 200-μl reactions containing 1 mM MgATP, 3 mM phosphoenolpyruvate, 0.21 mM NADH, 65 mM Hepes-KOH (pH 7.5), 1.3 mM MgCl$_2$, and 1.3 mM dithiothreitol were supplemented with 3.5 μl of protein kinase/lactate dehydrogenase enzymes (Sigma) and 15 μl of ER membranes (prepared from canine pancreas). The A_{340} was monitored for 10 min at 1-min intervals to determine the rate constant. As a control, ADP was added to some samples to directly stimulate degradation of NADH. AMPPNP did not inhibit the coupled system (data not shown).

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[2] L. Greene and S. Henikoff, Kinesin Home Page, www.proweb.org/kinesin/methods/ATPase_assay.html.

FIG. 1. **Desalted translations require the addition of nucleotide triphosphate energy sources for efficient targeting and translocation.** A translation of tOP^{30} was incubated at 25 °C for 30 min and treated with emetine (2 mM final) for 10 min at 25 °C. Aliquots of the translation (*lanes 8* and *9*) were incubated in the presence (*lane 8*) or absence (*lane 9*) of ER membranes. The rest of the translation was depleted of small molecules with a G-25 column. The resulting eluted volume was diluted to 100 μl final. An aliquot was treated with puromycin (2 mM final) for 5 min at 25 °C and 5 min at 37 °C (*lane 7*). Aliquots were supplemented with ER membranes only (*lane 1*), cytosol only (*lane 2*), or both cytosol and ER membranes (*lane 3*). Other aliquots were supplemented with ER membranes and the indicated nucleotides (*lanes 4–6*). Samples were then incubated for 30 min at 25 °C (except *lane 7*). The numbers indicate the percent glycosylation (except *lane 7*).

RESULTS

We previously showed that nascent intermediates of opsin could target to the ER membrane and even translocate the amino terminus across without ongoing translation (29). In this work we examined the specific requirements for this process by removing components from the translation mix before the addition of ER membranes. As before, we generated RBOps by *in vitro* translation of mRNA that had been truncated at specific positions within the coding region without a stop codon. Then the RBOp were isolated from other components of the translation mix using a G-25 column or ultracentrifugation. These purified RBOp could be incubated with ER membranes and other supplements to determine the requirements for targeting and translocation. RBOp were purified over a G-25 column after treatment with emetine to block any further translation (Fig. 1). The indicated band represents the nascent polypeptide still covalently bound to the tRNA as previously shown (29). The nascent polypeptides were still bound to functional ribosomes, since 93% could be released from the tRNA by puromycin (*lane 7*). Release of nascent polypeptides by puromycin requires the labile aminoacyl-tRNA bond to be positioned near the functional peptidyltransferase site (33). Some components required for targeting and/or translation were removed by the G-25 column, because adding ER membranes to depleted RBOp did not result in appreciable glycosylation of the nascent peptides (*lane 1*). Without ER membranes added, supplementation of RBOp with cytosol alone did not stimulate glycosylation (*lane 2*). However, supplementation with cytosol and ER membranes together did result in efficient glycosylation (62%, *lane 3*). This was comparable with the efficiency of glycosylation of samples that were not run over the G-25 column (*Untreated Translation*, 83%, *lane 9*). Because the majority of the nascent polypeptides could be released by puromycin and the efficiency
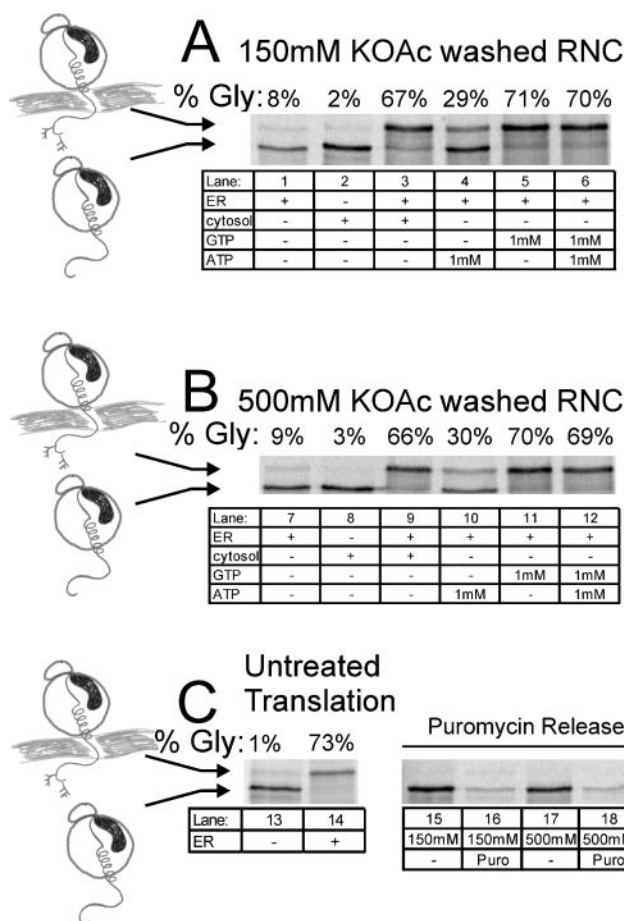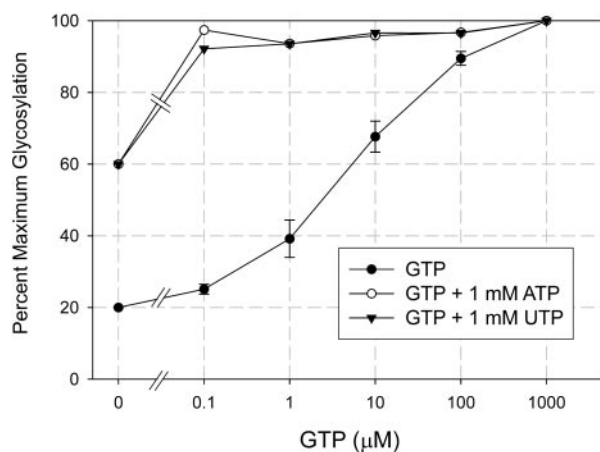
FIG. 2. **The RBOp is still competent to translocate the nascent chain after a high salt wash with only added GTP.** A translation of tOP[30] without membranes adjusted to 150 mM potassium acetate (*KOAc*) or 500 mM potassium acetate was centrifuged at 62,000 rpm for 30 min to purify the RBOps, and these were resuspended with buffer and supplemented with ER membranes (*ER*) and energy as indicated. RNC, ribosome nascent opsin complex. For comparison, a sample of translation that was not treated with potassium acetate (*Untreated Translation*) was incubated with and without membranes. Samples of both batches of RBOps that were treated with puromycin (*lanes 16 and 18*) are compared with untreated samples (*lanes 15 and 17*). The *lower arrow* indicates the tRNA-nascent chain, and the *upper arrow* indicates the glycosylated tRNA-nascent chain.

FIG. 3. **Low GTP supplemented with ATP or UTP is as efficient as high GTP.** Centrifugation-purified ribosome-nascent chain complexes were exposed to membranes in the presence of varying concentrations of GTP (0–500 μM) alone (*closed circles*) or in the presence of 1 mM ATP (*open circles*) or 1 mM UTP (*triangles*). The percent glycosylation normalized to the maximum signal with ER membranes and cytosol is plotted against the GTP concentration.

of glycosylation was comparable with the untreated samples, we can conclude that the treatment did not damage the ribosome-bound nascent chains.

To determine the specific energetic requirements for targeting and translocation, individual nucleotide energy sources were added to RBOp, with ER membranes present (*lanes 4–6*). The addition of GTP supported glycosylation as efficiently as cytosol (compare *lanes 3* and *5*). The addition of ATP and GTP was no more efficient than GTP alone (compare *lane 5* to *lane 6*). The addition of ATP alone (*lane 4*) supported moderate glycosylation of the tRNA-nascent opsins (48%). Because the addition of GTP was as efficient as supplementation with cytosol, the only significant/relevant factors removed by the desalting treatment were small energy molecules.

In yeast, post-translational translocation depends on cytosolic proteins to maintain the nascent polypeptide in a translocation competent state (17, 34). To investigate which, if any, cytosolic proteins are required for translocation of the amino terminus of opsin, RBOp were harvested by centrifugation in physiological (150 nM) or high salt (500 mM potassium acetate) conditions (Fig. 2). A high salt wash has been used previously to remove the nascent polypeptide-associated complex from

ribosome-nascent peptide complexes (35). The translation was diluted with buffer (physiologic or high salt) and centrifuged through a similarly buffered sucrose cushion. Both the physiological as well as the salt-washed RBOp were then incubated with ER membranes and supplemented as indicated.

RBOp that had been harvested in high salt were as efficient in targeting and translocation as those harvested under physiological conditions (Fig. 2). Incubation with cytosol and ER membranes together resulted in glycosylation of 67% of the nascent opsin polypeptides in physiological (*lane 3*) and 66% in high salt (*lane 9*). The ability of nucleotide triphosphates to substitute for cytosol was tested in the presence of ER membranes (*lanes 4–6*, *10–12*). The addition of ATP stimulated glycosylation of 29% of the tRNA-nascent opsin in physiological salt (*lane 4*) and 30% in high salt (*lane 10*). Consistent with the previous observations (Fig. 1), the addition of GTP was more efficient than ATP and supported glycosylation of 71% of the tRNA-nascent opsin in physiological salt (*lane 5*) and 70% in high salt (*lane 11*). Once again the GTP was as efficient as the addition of cytosol to the RBOp purified with physiological salt (*lane 3*) or with high salt (*lane 9*) and nearly as efficient as the untreated sample (*lane 14*). The combination of ATP and GTP was no more efficient than GTP alone (*lane 6* and *12*). The addition of ER membranes alone showed only barely detectable levels of glycosylation (*lanes 1* and *7*), indicating that this RBOp purification procedure also removed the majority of cytosol. As expected, cytosol in the absence of ER membranes did not support glycosylation (*lanes 2* and *8*). The ability of puromycin to efficiently release tRNA-nascent polypeptide demonstrated that the nascent opsin was bound to a functional ribosome throughout the salt wash and purification. RBOps were incubated with (*lanes 16* and *18*) or without puromycin (*lanes 15* and *17*), and about 90% of the nascent polypeptides were released by puromycin.

Lumenal chaperones have been implicated in both co-and post-translational translocation of proteins across the ER membrane (18, 19, 21, 36–38). Because these chaperones are ATPases (39) we had expected a role for ATP in the translocation of opsin. The endoplasmic reticulum has transporters for ATP and other nucleotides (40). Our results indicated only a modest effect of ATP that was less efficient than that of GTP alone (Figs. 1 and 2). We examined whether the ATP would have a more robust effect in the presence of reduced levels of GTP. RBOps purified by centrifugation were incubated with

membranes and supplemented with a range of GTP concentrations either alone (Fig. 3, *closed circles*) or in the presence of 1 mM ATP (*open circles*).

Consistent with the results in Figs. 1 and 2, 1 mM ATP (*open circle*, zero point on GTP axis) alone stimulated translocation but was less efficient than even 10 μM GTP alone (*closed circles*). However, when ATP was supplemented with even low concentrations of GTP, translocation was greatly improved, approximating that observed in samples supplemented with high levels of GTP.

There are three possible explanations for the observation that ATP augmented the effects of low concentrations of GTP on the efficiency of targeting and translocation. First, GTP is necessary and sufficient for targeting and translocation, and ATP is only being used to regenerate GTP from GDP (dinucleotide exchange). Second, there are distinct ATP- and GTP-dependent steps. From previous work, these steps are potentially targeting (GTP-dependent) and translocation (ATP-dependent). In this model, high GTP concentrations could allow RBOp to target multiple times to the ER membrane, eventually overcoming the inefficient ATP-dependent step. Lower GTP concentrations require a more efficient translocation step and more ATP. Third, there are distinct ATP- and GTP-dependent steps (as described above), but the ER membranes or purified RBOps contain residual adenine nucleotides (but not residual guanine nucleotides). With only GTP added, sufficient ATP can be regenerated. However, since there is no residual guanine nucleotides, GTP cannot be regenerated. Further experiments were done to differentiate these possibilities.

In cells there are abundant enzymes to generate the other nucleotide triphosphates from the large pool of ATP. One well characterized enzyme is nucleotide diphosphate kinase. Nucleotide diphosphate kinase non-specifically catalyzes the transfer of the γ-phosphate from a nucleotide triphosphate to another nucleotide diphosphate. Nucleotide diphosphate kinase is a homohexamer of 17-kDa subunits. Thus, it is expected to be too small to co-purify with ribosomes under the centrifugation conditions used. Nevertheless, it could be present with the purified RBOps or the ER membranes. To test for triphosphate exchange, UTP was used instead of ATP to supplement low levels of GTP (Fig. 3, *triangles*). UTP was as effective as ATP in increasing the efficiency of translocation at low levels of added GTP. This is still consistent with the first and third models. The specificity seen in the preceding experiments still may be due to specific requirement or differences in the levels of residual nucleotides present. To help distinguish these possibilities, more specific tests for the nucleotide requirements were used, namely the ability of analogs to inhibit the reactions.

GDP has been shown to inhibit GTP-dependent enzymes by favoring the inactive GDP-bound form. GDP addition alone had very little effect on stimulating translocation (Fig. 4). When ATP was present with lower levels of added GDP, translocation was stimulated, but at high levels of GDP there was no effect. We interpret this to mean that high levels of GDP specifically inhibit the GTP-dependent steps. At lower levels of added GDP, however, there is rapid conversion of the majority of the GDP to GTP, which can be used to support targeting and translocation. These data provide strong evidence that there is nucleotide triphosphate transfer and that GTP (or another species such as GDP that can be converted to GTP) is required. Still, a specific role for ATP may exist, since sufficient residual ATP/ADP may be present and regenerated itself in the presence of sufficient GTP.

To further explore the energetic requirements for targeting and translocation, ER membranes were treated with non-hydrolyzable analogs of GTP, 1 mM GMPPNP, and ATP, 1 mM
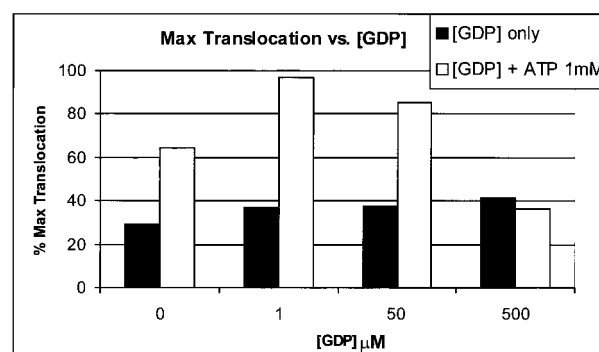

FIG. 4. **Low GDP enhances but high GDP inhibits targeting and translocation in the presence of ATP.** Centrifugation-purified ribosome-nascent chain complexes of tOP[30] were prepared as before and were supplemented with membranes and nucleotides as indicated. The glycosylation was normalized to the level seen with cytosol present with membranes.
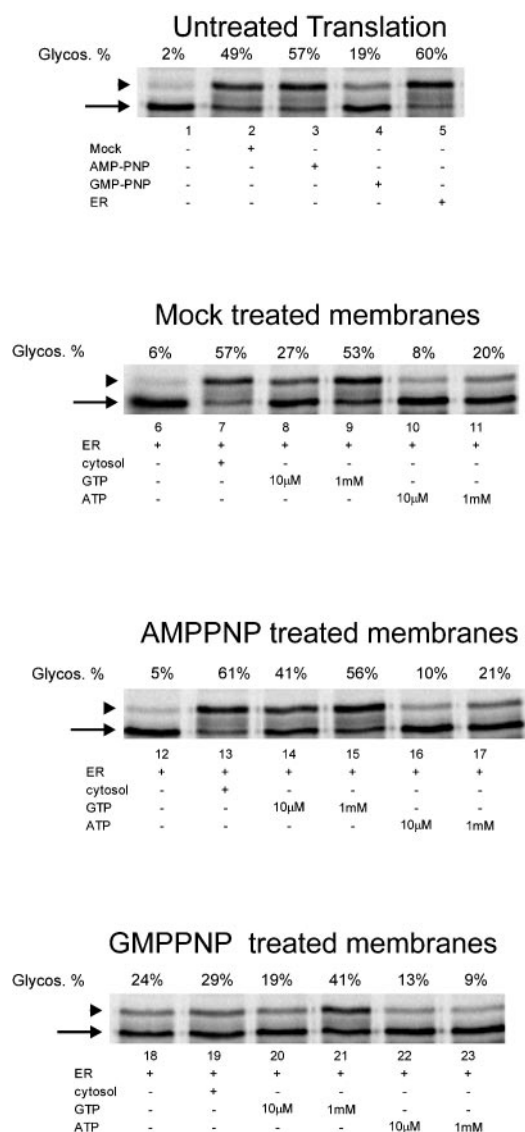
AMPPNP (Fig. 5). The *top panel* shows ER membranes treated with inhibitors or mock-treated used with unpurified translations. There was efficient glycosylation of nascent opsin (60%) with the addition of untreated control ER membranes (*lane 5*), which was comparable with mock-treated membranes (*lane 2*, 49%) and AMPPNP-treated membranes (*lane 3*, 57%). In contrast, the glycosylation of nascent opsin was reduced to 19% in the presence of ER membranes pretreated with GMPPNP (*lane 4*). With no membranes added, background glycosylation was not detectable (*lane 1*). Similar results were observed with the inhibitors used at 10 mM (data not shown).

The treated ER membranes were then tested with purified RBOp and specific nucleotide supplementation. Mock-treated ER membranes (*lanes 6–11*) gave results very similar to those seen earlier with untreated ER membranes. Glycosylation was efficient with the addition of membranes and cytosol (*lane 7*). The addition of 10 μM GTP stimulated translocation above background (*lane 8*), whereas 1 mM GTP was nearly as efficient as cytosol (*lane 9*). The addition of ATP, even at 1 mM, was less efficient than 10 μM GTP (compare *lanes 10* and *11* to *8* and *9*). Without any added energy source (*lane 6*), translocation was barely detectable.

When AMPPNP pretreated membranes were supplemented with the same energy sources, the results were very similar to the mock-treated membranes (compare the *bottom panel* to the *middle panel*). Cytosol stimulated translocation efficiently (*lane 13*), low GTP was less efficient (*lane 14*), and high GTP was as efficient as cytosol (*lane 15*). Low and high ATP were still inefficient (*lanes 16* and *17*). With no added energy there is only barely detectable glycosylation (*lane 12*).

With the GMPPNP pretreated membranes (*bottom panel*) there were two differences from the mock- and AMPPNP-treated membranes. First there was a higher background level of glycosylation with only membranes added (*lane 18*), which is consistent with previous work showing that GMPPNP can have a positive effect on targeting (25–27, 41) but still less efficiently than a similar concentration of GTP (data not shown). Second, GMPPNP inhibited stimulation of translocation by other energy sources. The addition of cytosol (*lane 19*) did not stimulate translocation detectably above background (compare with *lane 18*). Low GTP (*lane 20*) had no stimulatory effect, but high GTP (*lane 21*) moderately increased the targeting and glycosylation. The addition of ATP had no significant effect (*lanes 22* and *23*).

Because AMPPNP did not inhibit translocation it was important to independently confirm that ATPase activity in the treated ER membranes had indeed been inhibited by the compound. The ER has been shown to be able to transport ATP and other nucleotides (40). However, we tested for effects of GMP-

Untreated Translation

Mock treated membranes

AMPPNP treated membranes

GMPPNP treated membranes

FIG. 5. **GMPPNP pretreatment of membranes inhibits targeting and translocation of RBOp but AMPPNP pretreatment does not.** Ribosome-nascent chain complexes of tOP[30] were harvested by centrifugation as described in Fig. 2. ER membranes were incubated with either 1 mM AMPPNP, 1 mM GMPPNP, or with buffer alone (*mock-treated*) for 10 min at 25 °C. Aliquots of membranes were added to RBOp samples and supplements as shown. The final residual concentration of AMPPNP or GMPPNP was 200 μM. Complete mixes were incubated at 25 °C for 30 min before analysis by SDS-PAGE. *Arrows* indicate the tRNA-nascent chains, and the *arrowheads* indicate the glycosylated (*Glycos.*) tRNA-nascent chains.

PNP and AMPPNP on the lumenal ATPase activity of ER membranes (Fig. 6). Membranes were pretreated with the non-hydrolyzable nucleotide triphosphate analogues as before, and ATP hydrolysis was assayed by coupling release of ADP to the oxidation of NADH, which was monitored by A_{340}. Membranes pretreated with AMPPNP showed an inhibition of 89% of the ATPase activity. There was only a slight loss of activity with mock treatment and a moderate loss of activity with 1 mM GMPPNP treatment.

DISCUSSION

Here we examine the requirements for targeting and translocation across the mammalian ER. This has proven difficult due to the tight coupling between targeting/translocation and ongoing translation with the commonly studied model proteins (9, 42). We therefore sought other proteins with a stage of
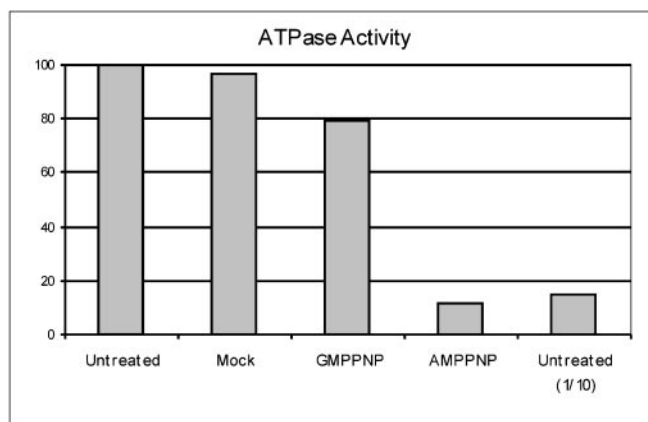
FIG. 6. **AMPPNP inhibits ER membrane ATPase *in vitro*.** 15-μl aliquots of ER membranes were treated with 1 mM AMPPNP, GMP-PNP, or mock-treated with buffer alone for 10 min at 25 °C. Membrane samples were added to an ATPase detection mix containing NADH and the enzymatic coupling system, and the decrease in the A_{340} was measured at 1-min intervals. The rate of degradation of NADH, which is directly related to the rate of ADP production, is shown for each sample.

biogenesis that could potentially be uncoupled; that is, "tail-anchored" proteins (43) and membrane proteins with translocated amino-terminal extensions but no cleaved signal sequence (44). The tail-anchored proteins target and translocate after release from the ribosome (45), independently of SRP (46) and the Sec61 complex (47). A representative protein from the second group is opsin, whose first transmembrane segment is the signal sequence (28). The amino-terminal domain of opsin has 36 amino acids that are synthesized before the beginning of the first transmembrane segment, which is not sufficient for targeting (48). In addition, there are two *N*-linked glycosylation sites in this domain that can be used as a "reporter" to follow the translocation of this segment of the protein.

We have established an assay for efficient targeting and translocation of nascent opsin polypeptides without ongoing translation (29). The efficiency of post-translational translocation in this *in vitro* assay was nearly identical to the efficiency of co-translational translocation. SRP is required, and the nascent polypeptide must remain bound to an active ribosome (29). Thus, the *in vitro* translation-independent assay likely mimics a physiologic step in the *in vivo* targeting and translocation. Because ongoing translation is not required, the system can be manipulated in ways that were previously not possible. In the present study we examined the requirements for energy sources and cytosolic factors by first removing small molecules from the translation and then by purifying RBOp from the bulk translation by centrifugation. Glycosylation of the amino terminus of opsin was used to as a marker of translocation due to the ease of the assay and the relative efficiency of the reaction with optimized ER membranes. This assay then has an end point that reflects only those nascent chains that have both targeted to and translocated the amino terminus across the ER membrane. The intermediate step of targeting before translocation is much more difficult to examine experimentally, since detection requires physical separation of the targeted and untargeted material. Using a quick centrifugation method, this was done previously, and the majority of targeted nascent chains chased into a glycosylated form within 5–10 min of targeting.[3] In addition, in all experiments that assayed targeting directly (using physical separation methods) glycosylated nascent polypeptides were not present in the untargeted material (29).

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[3] E. M. Kanner and S. M. Simon, unpublished results.

The removal of small molecules from translation reactions by desalting resulted in ribosome-bound nascent opsin complexes that were functional intermediates in biogenesis. This was assessed in two ways. First, nascent opsin polypeptides could still be released from the tRNA by puromycin. This requires that nascent opsin be bound to the ribosome (33, 49, 50). Second, targeting and translocation of the amino terminus, as assayed by glycosylation, could be reconstituted with the addition of the appropriate nucleotide energy source. This indicated that only small molecules were removed by the treatment. Reconstitution was as efficient as the addition of cytosol and 75% as efficient as the reaction with ribosome nascent opsin complexes that had not been desalted.

The efficiency of both the regular and the reconstituted post-translational translocation reaction was somewhat variable. When comparisons were made between experiments, the results were normalized to the maximum translocation efficiency of the unpurified samples (as in Figs. 3 and 4). When normalized this way, the error bars were very small. This suggests the variability as in the isolation of the labile translation intermediate (29).[3]

With pure nucleotides added back, GTP was more efficient (62%) in supporting targeting and translocation than ATP, which in the same experiment was 48%. However, this is quite different from the 1% observed in the absence of any energy source. Because cytosolic enzymes might not be completely removed by desalting, it is possible that there was some enzyme-mediated transfer of triphosphates between the nucleotide pools. Previously published work has shown that there is an energy requirement for targeting and translocation (24). Specifically, GTP has been found to be required for targeting nascent opsin to the ER membrane (25–27, 41, 51). Examination of the role of ATP for mammalian translocation has not been possible due to the requirements for ongoing translation. To more specifically examine the role of ATP in translocation, centrifugation was used to further purify RBOp from cytosolic components. These more highly purified RBOps could efficiently target and translocate opsin to the ER when supplemented only with nucleotide triphosphate energy sources. The efficiency of targeting and translocation was unaffected by a high salt wash of the RBOp. Only SRP has ever been shown to stay bound to ribosomes under these conditions (52). This indicates that cytosolic proteins were not required for the efficient targeting and translocation of ribosome nascent opsin complexes. The lack of a cytosolic protein requirement distinguishes this mammalian post-translational reaction from the yeast post-translational reaction, which requires two ATP-dependent chaperones, one in the cytosol (14–17) and one in the lumen of the ER (18, 19, 39, 53, 54).

Based on several criteria, we conclude that GTP is specifically required for the targeting and translocation of ribosome nascent opsin complexes. First, the addition of only GTP to RBOp was as efficient as the addition of complete cytosol and nearly as efficient as the unfractionated translation reaction. Second, the level of targeting and translocation correlated in a dose-dependent fashion with the amount of GTP added. Third, although low levels of GDP (with ATP present) are stimulatory, high levels of GDP inhibited the targeting and translocation reaction. Fourth, pretreatment with non-hydrolyzable GTP analogs inhibited the targeting and translocation reaction, whereas pretreatment with ATP analogs did not.

Several steps of targeting are known to require GTP. It is required for SRP binding to the signal sequence of nascent proteins (3, 55, 56) and for the SRP receptor to bind SRP (57–59). GTP hydrolysis is required in each case for proper recycling. GMPPNP has been shown to cause SRP to remain associated with the ER membrane, presumably bound to the SRP receptor, and to block further targeting to those membranes (60). Although pretreatment of the ER membranes with GMPPNP was inhibitory, the addition of this non-hydrolyzable analog to a targeting and translocation reaction had a mildly stimulatory effect (data not shown). One explanation may be that GMPPNP can support a single SRP cycle by displacing GDP. In previously published work, GMPPNP was fully or partially effective at replacing GTP (25–27, 41, 51).

ATP is thought to be important for translocation (61–63). In yeast systems, there are two ATP requirements. First there are cytosolic chaperones that presumably keep the proteins in a translocation-competent state (17, 34). The second ATP requirement is for the lumenal chaperone BiP, which is an ATPase and required for translocation (14, 39). We have examined the requirements for ATP in this mammalian-dissociated system. When the level of added GTP was reduced, ATP could stimulate translocation but only to the level seen with high GTP alone. This effect could be entirely explained by triphosphate energy transfer. First, the same effect of ATP was observed with low levels of added GDP, and second, UTP could substitute for ATP in similar assays. Because it is clear there is triphosphate exchange, this did not eliminate the possibility that there was residual ATP/ADP in the system, which would account for our observed lack of an ATP requirement (since it could be continuously regenerated).

To eliminate the possibility that there was an ATP-dependent step that we failed to uncover due to residual ATP/ADP, ER membranes were pretreated with non-hydrolyzable inhibitors before the addition to purified RBOp. Non-hydrolyzable inhibitors have been shown to prevent BiP from releasing bound peptide even in the presence of ATP (64). AMPPNP has also been used at high concentrations to compete tightly bound ADP from purified mammalian BiP (65). Pretreatment of ER membranes with 1 mM AMPPNP inhibited 89% of the ATPase activity (Fig. 6). However, ER membranes pretreated with AMPPNP (1 mM) showed no targeting or translocation defect (Fig. 5). Membranes that were pretreated with even higher concentrations of AMPPNP (10 mM) were also fully functional for targeting and translocation (data not shown).

We can, therefore, conclude that there is no specific requirement for ATP in this *in vitro* targeting and translocation system based on several criteria. First, ATP alone had only a modest effect on targeting and translocation, which was much lower than the effect of cytosol or of GTP alone (Figs. 1, 2, and 3). Second, the effect of ATP was much more pronounced when even very small amounts of GTP or GDP were present (Figs. 3 and 4), suggesting that the ATP is only acting by regenerating usable GTP. Third, the effect of ATP could be completely mimicked by other nucleotide triphosphates (Fig. 3) or by a nucleotide-regenerating system (data not shown), which further suggests that ATP only serves as a source of high energy triphosphates. Fourth, non-hydrolyzable ATP analogs do not affect the targeting or translocation reactions, which suggests that there is no specific ATPase that is required.

The post-translational targeting and translocation assay that we have developed for short nascent opsin polypeptides permits the uncoupling of these steps from ongoing translation. Thus, it is now possible to investigate the energetic requirements for targeting and translocation as distinct from the requirements for the translation process itself. Although GTP was required for targeting and translocation, ATP was dispensable. Because GTP is known to be important for targeting to the ER membrane, we can assign the GTP requirement to the targeting step. We detected no further energy requirements for the translocation step. The ATPase BiP is thought to have a

role in the translocation of proteins across the ER. We were unable to detect any specific requirement for ATP hydrolysis. The amino terminus of opsin is only 36 amino acids and may not be large enough to require any additional proteins to cross the membrane. There are other members of the G-protein-coupled receptor family with considerably longer amino-terminal extensions. It is possible that such proteins have additional requirements. Because G-protein-coupled receptor family members with amino termini longer than 200 amino acids encode cleaved signal sequences, it is possible that there are upper limits on the size of the peptides that can be translocated post-translationally. This can now be studied using *in vitro* systems similar to the one described here in which targeting/translocation steps can be dissociated from translation itself.

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