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An ATP-binding membrane protein is required for protein translocation across the endoplasmic reticulum membrane

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The role of nucleotides in providing energy for polypeptide transfer across the endoplasmic reticulum (ER) membrane is still unknown. To address this question, we treated ER-derived mammalian microsomal vesicles with a photoactivatable analogue of ATP, 8-N_3ATP. This treatment resulted in a progressive inhibition of translocation activity. Approximately 20 microsomal membrane proteins were labeled by [α^{32}P]8-N_3ATP. Two of these were identified as proteins with putative roles in translocation, α signal sequence receptor (SSR), the 35-kDa subunit of the signal sequence receptor complex, and ER-p180, a putative ribosome receptor. We found that there was a positive correlation between inactivation of translocation activity and photolabeling of αSSR. In contrast, our data demonstrate that the ATP-binding domain of ER-p180 is dispensable for translocation activity and does not contribute to the observed 8-N_3ATP sensitivity of the microsomal vesicles.

Introduction

Protein transport across cellular membranes is fundamental for organelle biogenesis and cell growth. The transfer of large hydrophilic proteins across the lipid bilayer is thermodynamically unfavorable, and therefore energy must be expended in the process. In many cases, part of the energy appears to be provided by the hydrolysis of ATP. For example, ATP is required for protein import into chloroplasts (Grossman *et al.,* 1980; Flugge and Hinz, 1986) and mitochondria (Pfanner and Neupert, 1986; Eilers *et al.,* 1987) and for translocation across bacterial membranes and the endoplasmic reticulum (ER)[1] membrane (Hansen *et al.,* 1986; Rothblatt and Meyer, 1986; Waters and Blobel, 1986; Chen and Tai, 1987; Lill *et al.,* 1989).

Part of the requirement for ATP can be attributed to the need to keep substrate proteins in a "translocation competent" or "unfolded" state, as has been demonstrated for mitochondrial import (Pfanner *et al.,* 1987) and post-translational translocation across the ER membrane in the yeast *S. cerevisiae* (Chirico et al., 1988; Deshaies *et al.,* 1988). It is likely that there is a further requirement for nucleotide hydrolysis to provide the energy for polypeptide chain transfer across the membrane. However, it has been difficult to address this problem experimentally.

Translocation across mammalian ER occurs in at least four discrete steps: signal sequence recognition by signal recognition particle (SRP), targeting to the ER via the SRP receptor, nascent chain insertion into the membrane, and subsequent translocation of the polypeptide chain (Rapoport, 1990). Both SRP and SRP receptor bind GTP (Connolly and Gilmore, 1989; Miller and Walter, unpublished data), and GTP binding is required to complete the first three steps of translocation (Connolly and Gilmore, 1986; Connolly *et al.,* 1991). Thus, multiple rounds of GTP binding and hydrolysis may ensure the proper vectorial delivery of the nascent chain to the site of translocation. However, GTP hydrolysis by SRP and SRP receptor probably does not contribute to the vectorial movement of the remainder of the nascent chain across the membrane (Connolly *et al.,* 1991).

In studies that further elucidate the nucleotide requirements for protein translocation, Garcia and Walter (1988) found that there is a requirement for ATP to translocate pre-elongated nascent chains across the ER membrane. Similarly, Mueckler and Lodish (1986) found that

[1] Abbreviations used: BIP, Ig heavy-chain binding protein; DTT, dithiothreitol; ER, endoplasmic reticulum; pKRMs, puromycin-treated, salt-washed microsomal membranes; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SRα, the α subunit of the SRP receptor; SRP, signal recognition particle; SSR, signal sequence receptor; TEA, triethanolamine; TpKRMs, trypsinized pKRMs; UV, ultraviolet.

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ATP hydrolysis is required to translocate and insert an integral membrane protein. These studies do not distinguish whether ATP is required by a cytosolic protein that unfolds the pre-elongated nascent chains or whether ATP is used by an ER membrane protein that acts during translocation. If the second case is true, then there should be at least one ATP-binding protein in the ER membrane that is required for translocation. We have tested this directly by using a photoactivatable analogue of ATP, 8-N_3ATP, to cross-link the ATP binding proteins in the membrane and to assess their role in translocation.

Results

Microsomes photolabeled with 8-N_3ATP are inhibited for translocation activity

8-N_3ATP is an ATP analogue that can be used to photocross-link ATP-binding proteins. On exposure to ultraviolet (UV) light, the azide group on the probe becomes activated to a nitrene, and the nucleotide analogue becomes covalently attached to the protein to which it is bound (Potter and Haley, 1983). Thus, ATP-binding proteins that require nucleotide hydrolysis for activity might be inactivated by this procedure. To determine if an ATP-binding protein in the ER membrane is required for protein translocation, we asked whether microsomes photocross-linked with 8-N_3ATP are impaired for translocation activity (Figure 1A).

As shown in Figure 1A, full length preprolactin synthesized in a reticulocyte lysate translation extract was efficiently processed to prolactin when untreated microsomes were added to the extract (Figure 1A, compare lanes 1 and 2). However, after photocross-linking with 8-N_3ATP, microsomes had a reduced capacity for translocation; thus, they were 68% active compared with untreated membranes (Figure 1A, compare lanes 2 and 3). After continued photocross-linking, their activity compared with untreated membranes was reduced to ~25% and finally 3%, as assessed by a decrease in processed prolactin and an increase in full-length preprolactin (Figure 1A, lanes 4 and 5). These results suggest that there are microsomal components involved in translocation that are sensitive to photocross-linking with 8-N_3ATP. Mock-treated microsomes exposed to UV in the absence of 8-N_3ATP were almost fully active for translocation compared with untreated membranes (Figure 1A, lane 6), indicating that neither UV irradiation alone nor subsequent handling of the microsomes resulted in a significant reduction in translocation activity.

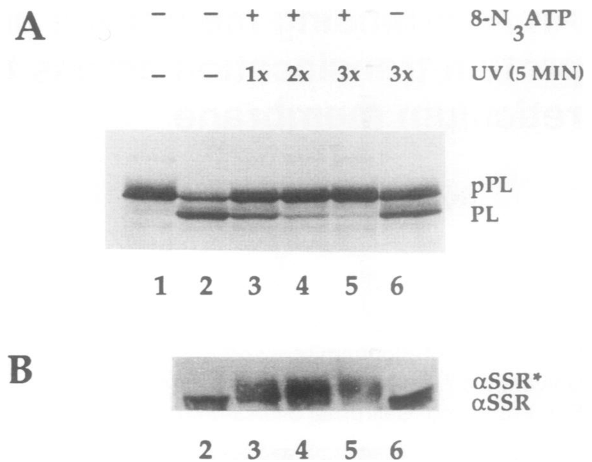

Figure 1. **Inhibition of protein translocation activity by 8-N_3ATP correlates with photolabeling of αSSR.** (A) Translation/translocation reactions were carried out in the absence of EKRMs (lane 1) or presence of EKRMs that were either untreated (lane 2), treated with 5 mM 8-N_3ATP (lanes 3–5), or mock treated by UV irradiation (lane 6). EKRMs were UV irradiated for 1×, 2×, and 3× 5 min as indicated. Samples were analyzed by SDS-PAGE. The precursor pPL and processed form of preprolactin (PL) are indicated. (B) The microsomal membranes used in (A) were analyzed by Western blotting with antiserum against αSSR. αSSR and photolabeled αSSR (αSSR*) are indicated. When activated and quenched 8-N_3ATP was added separately to a translocation assay, no effect on translation or translocation was observed (data not shown). Thus, the effect observed is not a primary effect on translation or a nonspecific inhibition due to the presence of the activated 8-N_3ATP.

If 8-N_3ATP is binding to bonafide ATP-binding sites, then the binding should be competed for by ATP or other ATP analogues. Using a threefold excess of ATPγS, we found that microsomes photolabeled with 8-N_3ATP were >50% protected from inhibition compared with microsomes treated in the absence of ATPγS (Figure 2A, compare lanes 3 and 4). In this experiment some degree of inhibition of translocation activity is expected because binding of 8-N_3ATP is irreversible during the time of irradition, whereas binding of ATPγS is reversible. When microsomes are irradiated with UV in the presence of 5 mM ATP alone, no affect on translocation activity was observed (data not shown), thus the inactivation caused by treatment with 8-N_3ATP requires the presence of the photoactivatable azido group.

α signal sequence receptor (SSR) and ERp-180 cross-link to 8-N_3ATP

Our results indicate that at least one ATP-binding protein in the membrane causes an inhibition

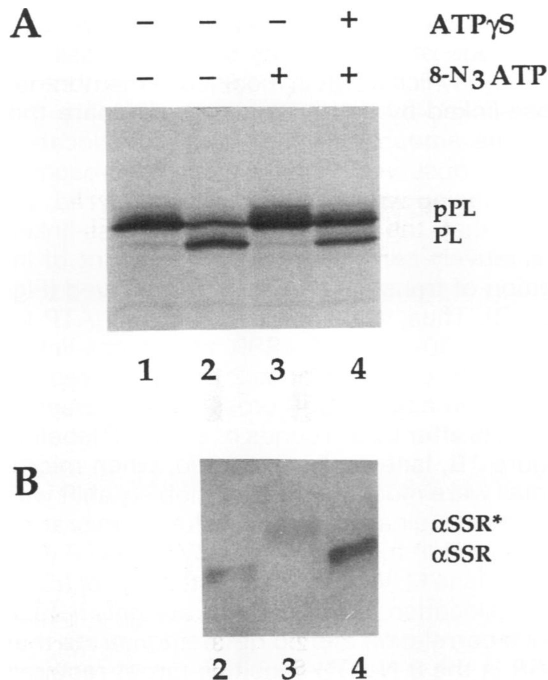

Figure 2. **ATPγS inhibits 8-N_3ATP cross-linking to membrane proteins.** (A) Translation/translocation assays were carried out in the absence (lane 1) or presence of EKRMs. EKRMs were mock treated by UV irradiation for 15 min (lane 2) or photolabeled with 5 mM 8-N_3ATP in the absence (lane 3) or presence of 10 mM ATPγS (lane 4). The precursor pPL and processed PL are indicated. (B) The microsomal membranes indicated in (A) were analyzed by Western blotting with antiserum against αSSR (lanes 2–4). αSSR and photolabeled αSSR (αSSR*) are indicated.

of translocation activity when it is cross-linked by 8-N_3ATP. To identify the 8-N_3ATP-binding proteins in the membrane that are the potential targets for the inhibition, microsomal membranes were photolabeled with [α^{32}P]8-N_3ATP, and the profile of labeled proteins was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Approximately 20 membrane proteins were cross-linked with the ATP analogue (Figure 3, lane 1). All the photolabeling observed can be competed for by excess unlabeled 8-N_3ATP (Figure 3, lane 2), indicating that the binding sites for [α^{32}P]8-N_3ATP are saturable. Moreover, ATPγS competed out nearly all photolabeling by [α^{32}P]8-N_3ATP (Figure 3, lane 3), indicating that the binding of 8-N_3ATP to these proteins was specific.

Two of the major [α^{32}P]8-N_3ATP-labeled proteins approximately comigrate with proteins that are thought to be involved in protein translocation: αSSR, a 35-kDa subunit of the SSR complex (Wiedmann *et al.,* 1987) and the 180-kDa protein, which we term ER-p180, identified as a putative ribosome receptor by Savitz and Meyer (1990). We tested the identity of the [α^{32}P]8-N_3ATP-labeled products by immunoprecipitation with antibodies raised against these proteins.

SSR is an integral membrane glycoprotein comprised of a 35-kDa α subunit (αSSR) and a 22-kDa β subunit (βSSR) (Wiedmann *et al.,* 1989; Görlich *et al.,* 1990). αSSR was identified by photoaffinity labeling to be in close proximity to

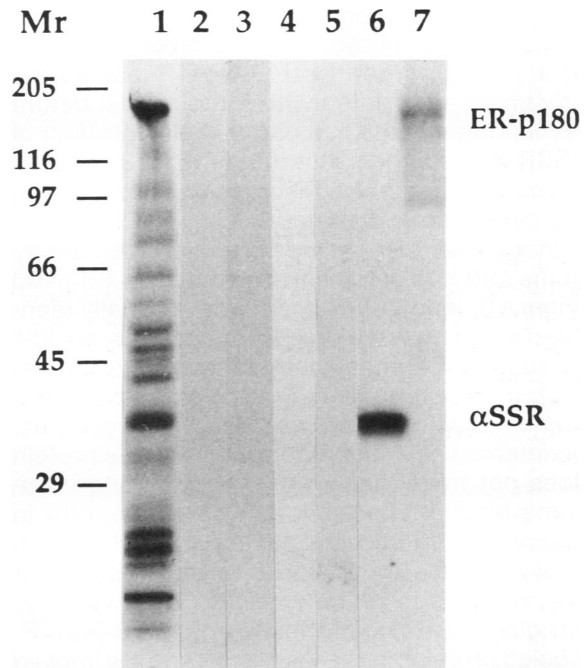

Figure 3. **Analysis of [α^{32}P]8-N_3ATP-labeled microsomal membrane proteins.** EKRMs were photolabeled for 5 min with 25 μM [α^{32}P]8-N_3ATP in the absence (lane 1) or presence of either 15 mM unlabeled 8-N_3ATP (lane 2) or 1 mM ATPγS (lane 3). EKRMs photolabeled with 25 μM [α^{32}P]8-N_3ATP were treated with proteinase K and were analyzed by SDS-PAGE either directly (lane 4) or after immunoprecipitation with antiserum raised against αSSR (lane 5). EKRMs were photolabeled with 25 μM [α^{32}P]8-N_3ATP and prepared for immunoprecipitation with antibodies against αSSR (lane 6) or ER-p180 (lane 7). Note that the degree of labeling was equivalent for all lanes and the exposure times are comparable between lanes 1–4 and lanes 5–7. αSSR, ER-p180, and protein standards (Mr) $\times 10^{-3}$ are indicated. No proteins were labeled when the samples were incubated with [α^{32}P]8-N_3ATP but not exposed to UV light or when the [α^{32}P]8-N_3ATP was activated and quenched before being incubated with the membranes (data not shown). Most proteins became cross-linked when the exposure time to activating light was between 1 and 5 min (data not shown), the time scale that is indicative of specific binding (Potter and Haley, 1983). No additional proteins were labeled when the time of UV exposure was increased to 15 min (data not shown). Thus, none of the labeling seen is due to the presence of a long-lived reactive group or a secondary reactive group created by extended exposure to UV light.

the nascent chain as it is being translocated across the membrane (Krieg *et al.,* 1989; Wiedmann *et al.,* 1987). Although its function is still unknown, it is thought that SSR is actively involved in translocation and may comprise part of a protein translocation channel (Simon and Blobel, 1991). Antibodies raised against αSSR (Görlich *et al.,* 1990) immunoprecipitate the 35-kDa [α^{32}P]8-N_3ATP-labeled product, suggesting that αSSR itself is an ATP-binding protein (Figure 3, lane 6). αSSR is predicted to have a single transmembrane spanning domain and a carboxy-terminal cytoplasmic tail of ~5 kDa that is sensitive to degradation by proteolysis (Prehn *et al.,* 1990). When [α^{32}P]8-N_3ATP-labeled microsomes are treated with proteinase K before immunoprecipitation, a photolabeled product of αSSR is no longer detected (Figure 3, lane 5), indicating that 8-N_3ATP cross-links to αSSR in the cytoplasmic domain.

In a similar manner we confirmed the identity of the 180-kDa cross-linked product as ER-p180 (Figure 3, lane 7). ER-p180 was originally identified as a ribosome receptor because a soluble proteolytic fragment derived from this protein inhibits ribosome binding to microsomal membranes (Savitz and Meyer, 1990). However, experiments done in our lab show that this protein does not fractionate with the majority of ribosome binding sites that can be assayed for in microsomal membranes (Nunnari *et al.,* 1991). Thus, the role for ER-p180 in translocation, if any, remains to be determined. However, it is intriguing that two of the major [α^{32}P]8-N_3ATP-labeled proteins in the ER membrane are implied to function during translocation and thus are potential targets for the inhibition of translocation activity observed.

No photolabeled products were immunoprecipitated by antibodies that recognize βSSR, the α subunit of SRP receptor (SRα), signal peptidase, or Ig heavy-chain binding protein (BIP) (data not shown). BIP is a soluble protein residing in the ER lumen that is known to bind to ATP (Kassenbrock and Kelly, 1989) and thus might be expected to cross-link 8-N_3ATP. However, all the cross-linked sites are sensitive to degradation by exogenously added protease (Figure 2, lane 4), indicating that they are all cytoplasmically exposed. Thus, under the conditions used, [α^{32}P]8-N_3ATP labels only ATP-binding sites exposed to the cytoplasm.

Inactivation of translocation activity by 8-N_3ATP correlates with photolabeling of αSSR

We observed that when cross-linked to 8-N_3ATP, αSSR undergoes a mobility shift when analyzed by SDS-PAGE (Figure 1B). We took advantage of this mobility shift to assess the extent to which αSSR is modified in membranes cross-linked by 8-N_3ATP and to compare this with the amount of inhibition of translocation activity observed. Thus, when microsomes photolabeled with 8-N_3ATP were analyzed, we found that the extent of αSSR cross-linked qualitatively correlates with the amount of inhibition of translocation activity observed (Figure 1B). Thus, after one round of 8-N_3ATP labeling, ~30–40% of αSSR was cross-linked (Figure 1B, compare lanes 2 and 3). Moreover, the percentage of αSSR cross-linked increases to >90% after three rounds of 8-N_3ATP labeling (Figure 1B, lane 5). As expected, when microsomes were mock treated, no mobility shift was detected, indicating that the altered migration is indeed due to cross-linking by 8-N_3ATP (Figure 1B, lane 6). With respect to the role of αSSR in translocation, these findings are only a qualitative correlation and do not demonstrate that αSSR is the 8-N_3ATP-sensitive target required for translocation.

We have already demonstrated that the presence of ATPγS during photocross-linking protects the membranes from the inhibition of translocation activity caused by 8-N_3ATP cross-linking. Thus, we compared the extent of αSSR cross-linked in membranes photolabeled in the presence and absence of 8-N_3ATP and ATPγS (Figure 2B). Accordingly, when microsomes were photolyzed in the presence of 8-N_3ATP alone, all αSSR was shifted compared with mock-treated membranes (Figure 2B, compare lanes 2 and 3). Moreover, when microsomes were photolabeled in the presence of both 8-N_3ATP and 10 mM ATPγS, the amount of αSSR cross-linked was greatly reduced (Figure 2B, compare lanes 3 and 4).

ER-p180 is proteolyzed from trypsinized microsomes

ER-p180 has a large cytoplasmic domain that is extremely sensitive to proteolysis (Savitz and Meyer, 1990; Nunnari *et al.,* 1991). To further characterize this protein with respect to 8-N_3ATP labeling, we used mild proteolysis conditions to cleave this domain from the membrane. Puromycin-treated, salt-washed microsomal membranes (pKRMs) were treated with a low concentration of trypsin, and the membranes were fractionated away from soluble proteolytic fragments by salt extraction and centrifugation. The protein composition of both fractions was analyzed with respect to ER-p180

by Western blotting with antibodies raised against this protein (Nunnari *et al.,* 1991; Zimmerman and Walter, unpublished data) (Figure 4). All of ER-p180 was recovered in the membrane pellet after mock treatment of the microsomes (Figure 4, compare lanes 2 and 3), whereas three proteolytic fragments were recovered in the supernatant fraction after trypsin treatment (Figure 4, lane 4). Moreover, neither intact ER-p180 nor any detectable degradation products pelleted with the microsomes after trypsinization (Figure 4, lane 5).

To map the site of [α^{32}P]8-N_3ATP cross-linking to ER-p180, microsomes were photolabeled with [α^{32}P]8-N_3ATP and then treated with trypsin as described. The [α^{32}P]8-N_3ATP label was found to be cross-linked to the trypsin-derived fragments (Figure 4, lane 8). Thus, the site of [α^{32}P]8-N_3ATP binding to ER-p180 is in the protease-sensitive cytoplasmic domain. In contrast to ER-p180, many other sites cross-linked by 8-N_3ATP are unaffected by mild trypsinization (Figure 4, compare lanes 6 and 7). For example, αSSR, which is less sensitive to proteolysis than ER-p180, is photolabeled in trypsinized pKRMs (TpKRMs) (Figure 4, compare lanes 9 and 10).

Trypsinized microsomes are sensitive to 8-N_3ATP

We showed above that TpKRMs no longer have the 8-N_3ATP-binding domain of ER-p180, and thus, this protein should no longer be a target for 8-N_3ATP in TpKRMs. Therefore, if photolabeling of ER-p180 leads to the inhibition of translocation activity that we observe, then TpKRMs should not be inhibited for translocation activity by 8-N_3ATP. To test this, the TpKRMs and mock-treated pKRMs that were depleted of proteolytic fragments of ER-p180 as described above (Figure 4, lanes 1–5) were photolabeled as described and were assayed for translocation activity (Figure 5). Because the α subunit of the SRP receptor is required for translocation (Walter *et al.,* 1979), but is itself very protease sensitive, we used an assay that allows activity to be restored to membranes depleted of SRα by mild trypsinization (Walter *et al.,* 1979; Andrews *et al.,* 1989). Thus, TpKRMs were inactive for translocation compared with pKRMs, as assessed by protection of prolactin by exogenously added protease (Figure 5, compare lanes 2 and 7), but when TpKRMs were supplemented with SRα translated from synthetic RNA, translocation activity was restored (Figure 5, lane 9). A similar result was observed when UV-irradiated TpKRMs were supplemented with SRα RNA (data not shown).

As expected, unproteolyzed membranes were inhibited for translocation by 8-N_3ATP treatment (Figure 5, lanes 3–5), whereas TpKRMs were inactive for translocation activity both before and after treatment with 8-N_3ATP (Figure

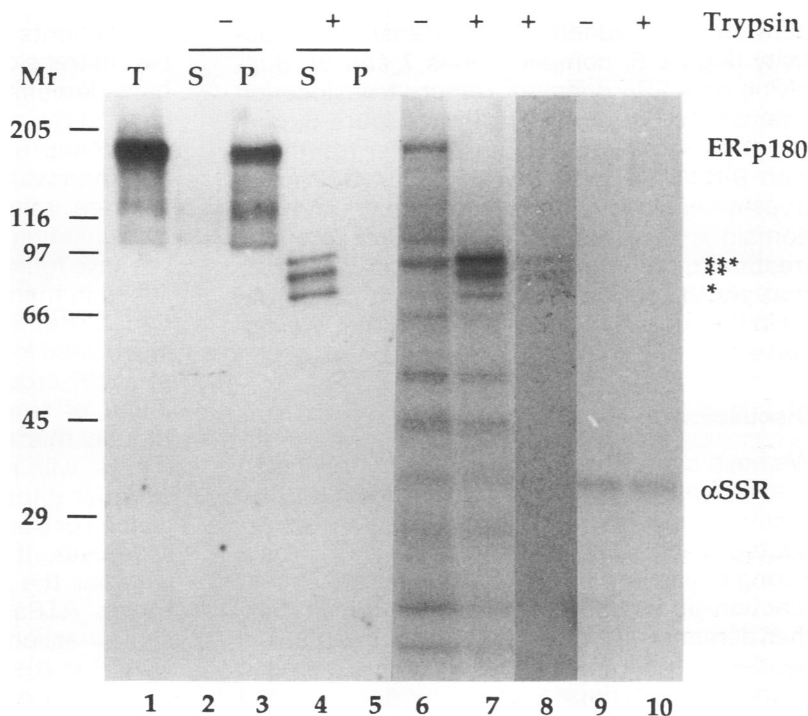

***Figure 4.* ER-p180 is sensitive to mild proteolysis.** Material derived from 10 eq of pKRMs (lane 1) or of supernatant (S) and pellet (P) fractions of mock-treated (lanes 2 and 3) or trypsin-treated pKRMs (lanes 4 and 5) was analyzed by Western blot with antibodies against ER-p180. pKRMs were photolabeled with [α^{32}P]8-N_3ATP and were prepared for SDS-PAGE (lane 6) or immunoprecipitation with antibodies against αSSR (lane 9). [α^{32}P]8-N_3ATP-labeled pKRMs were treated with trypsin and were prepared for SDS-PAGE (lane 7) or immunoprecipitation with antibodies raised against ER-p180 (lane 8) or αSSR (lane 10). αSSR and ER-p180 are indicated. Trypsin-derived fragments of ER-p180 are indicated by asterisks. Protein standards are indicated (Mr) $\times 10^{-3}$.

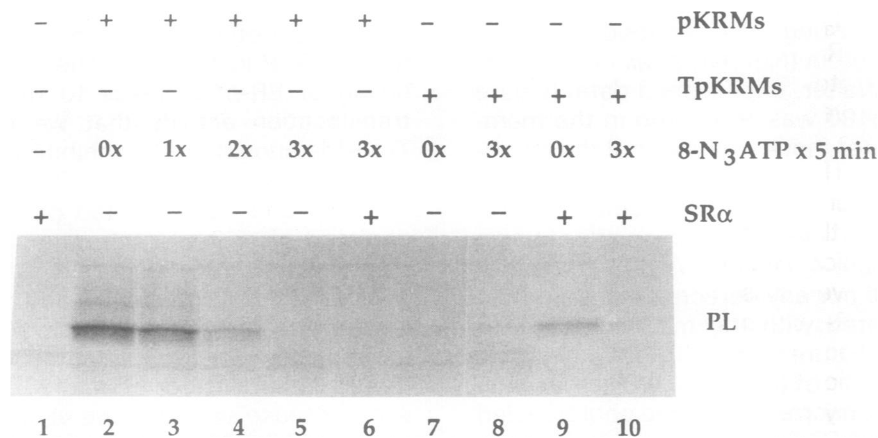

Figure 5. **Trypsinized microsomal membranes are inhibited for protein translocation activity by 8-N_3ATP.** Translocation reactions were carried out in the absence (lane 1) or in the presence of pKRMs (lane 2); pKRMs treated with 5 mM 8-N_3ATP for 5 min one (lane 3), two (lane 4), and three times (lanes 5 and 6); trypsinized pKRMs (lanes 7 and 9); or trypsinized pKRMs treated for a total of 15 min with 8-N_3ATP (lanes 8 and 10). Translation reactions were supplemented with SRα where indicated. All reactions were treated with proteinase K before being prepared for SDS-PAGE. The processed form of prolactin (PL) is indicated.

5, lanes 7 and 8). In contrast to uncross-linked TpKRMs, translocation competence was not restored to 8-N_3ATP-treated TpKRMs when SRα was added back to them (Figure 5, compare lanes 8 and 10). Thus, TpKRMs that no longer have the 8-N_3ATP binding site of ER-p180 are still sensitive to 8-N_3ATP treatment, and it is unlikely that photolabeling of this protein is responsible for the inhibition of translocation activity that we observe.

We showed above that TpKRMs are dependent on newly added SRα for translocation activity (Figure 5, compare lanes 7 and 9). Supplying new SRα does not restore translocation competence to either TpKRMs (Figure 5, lane 10) or pKRMs (Figure 5, lane 6) after treatment with 8-N_3ATP. Because SRα binds GTP in its trypsin-sensitive cytoplasmic domain and this domain is restored to TpKRMs after 8-N_3ATP treatment, then these data further demonstrate that photolabeling of SRα does not cause the inhibition of translocation activity that we observe.

Discussion

We have shown that microsomes photolabeled with 8-N_3ATP are inactive for translocation. The requirements for photolabeling are those expected if inhibition is due to 8-N_3ATP cross-linking to one or more ATP-binding proteins that function during translocation. Our results further demonstrate that the target protein(s) is a resident membrane protein of the ER, because microsomes stripped of all ribosomes and loosely bound cytosolic factors are sensitive to 8-N_3ATP treatment. We find that there are upward of 20 substrates for 8-N_3ATP, any of which could be responsible for the inactivation observed. However, it is intriguing that two of the major targets for 8-N_3ATP are proteins previously proposed to have roles in translocation: αSSR and ER-p180.

αSSR is an integral membrane glycoprotein that forms a complex with another 25-kDa glycoprotein (Görlich *et al.,* 1990). Two types of experiments have implicated this protein complex in translocation. Photocross-linking studies have demonstrated that αSSR is in close proximity to the nascent chain during translocation (Wiedmann *et al.,* 1987, 1989; Krieg *et al.,* 1989) and monovalent F_{ab}-fragments produced from antisera raised against αSSR block protein translocation in vitro (Hartmann *et al.,* 1989). We have found that αSSR is quantitatively cross-linked in membranes inactivated for translocation. Moreover, when microsomes are partially inactivated for translocation activity, the amount of αSSR cross-linked correlates with the inhibition of translocation activity observed. Although this result is intriguing, more experiments will need to be done to determine whether photolabeling of αSSR causes the inhibition of translocation activity that we observe. Moreover, it will be important to determine whether the purified SSR complex has an intrinsic ATPase activity or whether another closely associated protein actually binds to 8-N_3ATP in the membrane, putting the photoactivatable azido group in close proximity to αSSR.

The second 8-N_3ATP-binding protein that we have identified, ER-p180, has a putative role as a ribosome receptor (Savitz and Meyer, 1990). However, we have demonstrated that microsomes that have been mildly trypsinized no longer have the ATP-binding domain of ER-p180 yet they remain sensitive to 8-N_3ATP treatment. Thus, it is unlikely that photolabeling of ER-p180 causes the inhibition that we observe after photolabeling. Moreover, these results raise doubts about whether ER-p180 plays an essential role in protein translocation in general. As shown above, translocation competence is restored to TpKRMs when they are supplemented with SRα. Thus, under the conditions used, the only trypsin-sensitive protein required for translocation is SRα. Because the proteolytic products derived from ER-p180 that we can detect range in size from 70–100 kDa (Figure 4, lane 4), it can be concluded that proteolysis of at least half of this protein does not impair microsomes for translocation activity.

Previous studies have shown that ATP is required for protein translocation across the membrane of mammalian ER when pre-elongated nascent chains are used as a substrate (Mueckler and Lodish, 1986; Perara *et al.,* 1986; Garcia and Walter, 1988). However, these studies could not distinguish whether the ATP requirement involved a cytosolic component or a membrane protein. Thus, our results are the first demonstration that a putative ATP-binding protein in the ER membrane is required for translocation.

Connolly and Gilmore (1986) found that, in contrast to the longer chains used in the other studies, an 86 amino acid truncated form of preprolactin requires GTP, but not GTP hydrolysis, for translocation. Because GTP is needed for nascent chain targeting and signal sequence insertion (Connolly *et al.,* 1991), then proper delivery of these chains to the membrane may be sufficient to ensure their subsequent translocation into the lumen. Thus, it might be expected that 8-N_3ATP treatment of membranes would not affect translocation of short nascent chains. In contrast, we found that 8-N_3ATP-treated membranes are blocked for translocation at the level of signal sequence insertion (data not shown). Thus, it is possible that the ATP-binding protein(s) that is cross-linked is required for translocation of both long and short nascent chains. However, more steps might be required at the level of the membrane to translocate the longer chains, and ATP binding and hydrolysis may not be required until a later step.

Our results demonstrate that cross-linking of an ER protein by 8-N_3ATP renders microsomal membranes inactive for translocation activity. Thus, it might be possible to restore translocation competence to 8-N_3ATP-treated membranes by adding back uncross-linked protein, thereby providing an assay to purify the protein involved. We are currently using affinity chromatography to purify the ATP-binding proteins from the ER membrane and will use the reconstitution assays currently available (Yu *et al.,* 1989; Nicchitta and Blobel, 1990; Zimmerman and Walter, 1990) to try to complement 8-N_3ATP-inactivated microsomes with the purified proteins and identify the required component.

Materials and methods

Reagents

8-N_3ATP, [α^{32}P], was purchased from ICN Biomedicals (Irvine, CA); 8-N_3ATP and puromycin were from Sigma (St. Louis, MO); the ECL Western blotting detection system was from Amersham (Arlington Heights, IL).

Preparation of microsomal membranes

Salt-washed and EDTA-stripped microsomes were prepared as previously described (Walter and Blobel, 1983) except that stocks of microsomes were stored in a buffer containing 10 mM triethanolamine (TEA)-HOAc, pH 7.5, 250 mM sucrose, 100 μM $Mg(OAc)_2$ (buffer A) at a concentration of 3 eq/μl. One equivalent is defined as the material derived from 1 μl of rough microsomal membranes that are at a concentration of 50 A 280 units/ml (Walter and Blobel, 1983).

Preparation of pKRMs was adapted from a procedure by Adelman *et al.* (1973). Rough microsomes were brought to a final volume of 0.5 eq/μl in a buffer containing 50 mM TEA, 250 mM sucrose, 10 mM $Mg(OAc)_2$, 500 mM KOAc, pH 7.5, 1 mM dithiothreitol (DTT), 1 mM puromycin and incubated on ice for 1 h, followed by successive incubation for 10 min at 37°C and room temperature. The membranes were loaded on top of a 2-ml cushion [1.8 M sucrose, 50 mM TEA, 1 mM DTT, 100 mM KOAc, pH 7.5, 5 mM $Mg(OAc)_2$] and centrifuged at 4°C for 20 h at 40 000 rpm in a SW-40 rotor (Beckman, Palo Alto, CA). The membranes sedimenting at the interface were collected and resuspended in twice their original volume in a buffer containing 50 mM TEA, 250 mM sucrose, 1 mM DTT (buffer B). The membranes were pelleted to remove excess sucrose and were resuspended to their original volume in buffer B. Rough microsomes were extracted twice with this procedure.

Photolabeling with 8-N_3ATP

Reaction volumes ranged from 50–200 μl in a buffer containing 10 mM TEA, 250 mM sucrose. $Mg(OAc)_2$ was equimolar with the final nucleotide concentration, and 0.5 mM GTP was included in all reactions. Microsomes were included in the reaction at a final concentration of 1.5 eq/μl. For each reaction all components except nucleotides and/or 8-N_3ATP were mixed together and kept on ice. GTP and/or ATPγS were added to the reaction mix just before addition of 8-N_3ATP. The samples were transferred to siliconized wells of a 1/16" S/P serological ring slide placed on ice and irradiated with UV light of 366 nm by a hand-held lamp (Min-

eralight model UVGL-25 from UVP, San Gabriel, CA) at a distance of 3 cm for 5 min. After UV irradiation the reactions were quenched by addition of an equal volume of buffer containing 10 mM TEA, 250 mM sucrose, 60 mM DTT and were transferred to centrifuge tubes fitting a TLA 100.2 rotor (Beckman). The ring slide plate was rinsed with an equal volume of buffer, and this was added to the corresponding sample. The membranes were pelleted by centrifugation at 70 000 rpm for 10 min (trypsinized/mock-trypsinized microsomes were centrifuged for 15 min). Pelleted microsomes were resuspended in 3 times their original volume in buffer A and pelleted again under the same conditions. The microsomes were resuspended to 3 eq/μl in buffer A and subjected to two more rounds of 8-N_3ATP treatment as described. Aliquots of microsomes were saved at each step for analysis.

Photolabeling with [α^{32}P]8-N_3ATP

Photocross-linking with [α^{32}P]8-N_3ATP was carried out as described above with the following differences. The final reaction volumes ranged from 5–20 μl, and microsomes were included in the reactions at a final concentration of 1.5–2 eq/μl. Before addition, an aliquot of anhydrous [α^{32}P]8-N_3ATP was dried under a gentle stream of nitrogen, resuspended at 4°C in buffer A to a final concentration of 100–200 μM, and immediately diluted into the reaction mixture to the appropriate final concentration. Where included, nucleotides were added to the reaction mix just before addition of [α^{32}P]8-N_3ATP. Samples were UV irradiated, and the reactions were quenched as described above. Samples were prepared for SDS-PAGE (Garcia and Walter, 1988) or immunoprecipitation as described (Krieg *et al.,* 1986).

Translation/translocation assays

Rabbit reticulocyte translation extracts were prepared as previously described (Jackson and Hunt, 1983). Translations were programmed with synthetic preprolactin RNA or SRα RNA as described (Andrews *et al.,* 1989). Translocation assays were as described (Andrews *et al.,* 1989). Reconstitution of trypsin-treated microsomes with SRα was as previously described (Andrews *et al.,* 1989).

Protease treatment of microsomes

pKRMs at a concentration of 2 eq/μl in buffer B were adjusted to 2 μg/ml of trypsin or 100 μg/ml proteinase K and incubated on ice for 1 h. The protease was inactivated by addition of phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 2 mM, and incubation was continued for an additional 15 min. Trypsinized membranes were pelleted by centrifugation at 4°C in a TLA 100.2 rotor at 75 000 rpm for 10 min and resuspended to 1 eq/μl in a buffer containing 50 mM TEA, pH 7.5, 100 mM sucrose, 1 mM PMSF. The membrane suspension was diluted with an equal volume of buffer containing 1 M KOAc, pH 7.5, 50 mM TEA, pH 7.5, 1 mM PMSF, underlayered with a cushion of 50 mM TEA, pH 7.5, 500 mM sucrose, and centrifuged for 1 h at 70 000 rpm. Membrane pellets were resuspended in buffer B to 0.5 eq/μl and pelleted again for 60 000 rpm for 1 h. The TpKRMs were finally resuspended in buffer B at a concentration of 3 eq/μl.

Immunoprecipitations and Western blotting

Immunoprecipitations (Krieg *et al.,* 1986) and Western blotting were performed as described (Fisher *et al.,* 1982) with the following exceptions. The primary antibodies were detected using the enhanced chemiluminescent Western blotting detection system (Amersham). Blots were incubated with horseradish peroxidase-labeled secondary antibodies at a dilution of 1:10 000 and were detected as described in the Amersham manual.

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