

Secretion in Yeast: Preprotein binding to a Membrane Receptor and ATP-dependent Translocation Are Sequential and Separable Events In Vitro

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Abstract. We have used a cytosol-free assay in which efficient translocation and signal peptide cleavage is achieved when the affinity-purified precursor of OmpA (proOmpA) is diluted out of 8 M urea into a suspension of yeast rough microsomes. This aspect of protein targeting and transport occurs in two discernible steps: (a) in the absence of ATP and cytosolic factors, the precursor binds to the membranes but is not translocated; (b) addition of ATP results in the translocation of the bound precursor and its processing to the mature form. The binding to microsomes of radiolabeled proOmpA is saturable and inhibited by the addition of unlabeled proOmpA but not by mature OmpA or other proteins. The binding of radiolabeled prepro- α -factor is also effectively competed by other preproteins, but

not by mature ones. Scatchard analysis showed the K_d of proOmpA to be 7.5×10^{-9} M. Binding is most likely protein mediated as treatment of the microsomes with the protease papain was found to be inhibitory. These results represent the first functional characterization of secretory protein precursor binding to membranes. Alkylation of the microsomes with NEM, washing the membranes with urea or using membranes from the (translocation) mutant *ptll* at the nonpermissive temperature, did not affect binding, but did eliminate the subsequent ATP-dependent translocation. The ability to subdivide translocation into individual reactions provides a more precise means of determining the membrane components involved in this process.

PROTEIN translocation across the membrane of the ER has been studied both in heterologous and homologous in vitro systems. Both cytosolic and membrane components have been isolated that are required for recognition, targeting, and processing (11, 22). In yeast, numerous studies have taken advantage of the fact that translocation can be uncoupled from translation (for review, see reference 24). In such cases, the precursor of the yeast pheromone α -factor can be translocated across rough microsomes in the total absence of ongoing protein synthesis. Cytosolic factors are nonetheless required in these posttranslational assays (1, 7, 19, 20).

We have recently described an assay whereby translocation across eukaryotic membranes can be studied not only in the absence of translation, but also in the absence of any cytosolic factors (20). This system is a modified version of one previously described for use in studying translocation in prokaryotes (5). In it, only pure preprotein, membranes and ATP are required for the efficient translocation, processing (and glycosylation) of proOmpA or prepro- α -factor (20). The two translocation-relevant reactions that occur in the cytosol in yeast in vitro appear to be translation of the precursor and its maintenance in a competent conformation (for review, see reference 12). This conformation has been correlated with a more loose, open, or unfolded configuration of the precursor. In the cell-free assay that we use here, pure

preproteins are maintained in a translocation-competent state in 8 M urea. Upon addition to membranes, precursors that are diluted out of urea can be efficiently translocated. The basis of this reaction is the ability to chemically mimic a "nascent" state by urea denaturation.

The advantage of this system is that direct interactions of preproteins with the membrane can be probed in the absence of cytosolic factors. Translocation of precursors diluted out of urea is biologically relevant as it displays the same sensitivity to inhibitors and cofactor requirements as is seen using more complex assays. We report here that translocation across yeast microsomes can be dissected into two distinct steps that are amenable to biochemical as well as genetic analysis. The first step is an ATP-independent binding of the preprotein to a cytoplasmically disposed receptor on the membrane. The second step is the actual translocation of the precursor across the membrane. A number of inhibitory treatments were analyzed for their effect on the former or the latter step.

Materials and Methods

Membrane Treatments

ABYS66 yeast membranes were prepared as described (17). Yeast mem-

branes (20 μ l, 40 A₂₈₀ units/ml) were treated with 20 mM *N*-ethylmaleimide (NEM)¹ (final concentration) for 15 min at 25°C, and the reaction was stopped by adding DTT to 40 mM (final concentration). Microsomes treated with papain were digested with 500 μ g/ml (final concentration) for 1 h at 0°C, and the reaction was stopped by adding leupeptin to 1 μ g/ml and TLCK to 1 mM (final concentration). Untreated and treated membranes were brought to 0.5 M KOAc and spun at 100,000 *g* for 1 h at 4°C through a 0.5 M sucrose cushion in buffer 1 (40 mM Hepes-KOH pH 7.4, 0.5 M KOAc, 5 mM Mg(OAc)₂, 1 mM DTT). Pellets were resuspended in membrane buffer (20 mM Hepes-KOH pH 7.4, 0.25 M sucrose, 50 mM KOAc, 1 mM DTT), and the membrane concentration was adjusted to 40 A₂₈₀ U/ml in all cases. Urea-treated microsomes were prepared by treatment with 6 M urea (final concentration) for 30 min at 0°C. The mixture was centrifuged through a 0.5 M sucrose, 6 M urea cushion. Pellets were resuspended in membrane buffer.

ProOmpA Binding and Translocation Assays

Synthesis and immunopurification of proOmpA and prepro- α -factor was carried out in the same way as described by Sanz and Meyer (20). Affinity-purified ³⁵S-proOmpA (3 μ l in 8 M urea, 30,000 cpm/ μ l) was mixed with 37 μ l of buffer 2 (40 mM Hepes-KOH pH 7.4, 162 mM KOAc, 5 mM Mg(OAc)₂, 1 mM DTT) and 4 μ l of salt-washed yeast microsomes (40 U A₂₈₀/ml) and incubated at 25°C for 10 min. Samples were then spun at 100,000 *g* for 1 h at 4°C through a 0.5 M sucrose cushion in buffer 2. Pellets were resuspended in 20 μ l of membrane buffer and, where indicated, 1 μ l of energy mix (30 mM ATP, 1.8 M creatine phosphate, 2.4 mg/ml creatine phosphokinase) was added. Samples were incubated at 25°C for 10 min, and the reaction was stopped by boiling the samples in electrophoresis buffer. Binding and translocation were then quantified by SDS-PAGE, fluorography, and densitometry using an ultrascan laser densitometer (model 2202; LKB Instruments Inc., Bromma, Sweden).

Protease Protection Assay

Samples were treated with proteinase K (0.4 mg/ml, final concentration) for 30 min at 0°C. PMSF (1 mM final concentration) was added, and the samples were left at 0°C for 5 min. They were then boiled in electrophoresis sample buffer and analyzed by SDS-PAGE and fluorography.

Saturation of the Binding Capacity of the Membranes

Different amounts of a mixture of labeled and unlabeled proOmpA (2,740 cpm/ μ l, 20 ng/ μ l, in 8 M urea) were mixed with 4 μ l of membranes in a final volume of 50 μ l. Samples were incubated at 25°C for 10 min and then spun at 100,000 *g* for 1 h at 4°C through a 0.5 M sucrose cushion in buffer 2. Radioactivity was measured in the supernatant (unbound) and in the pellet (bound). Parallel samples were run without membranes to calculate the background.

Competition of proOmpA Binding by Mature Proteins

Buffer 2 (37 μ l) containing 3 μ l of labeled proOmpA (30,000 cpm/ μ l) and either unlabeled proOmpA (10 ng, 100 ng, or 1 μ g), unlabeled OmpA (1 μ g), or lysozyme (1 μ g) was added to salt-washed membranes (4 μ l) and incubated in a final volume of 50 μ l at 25°C for 10 min. The membranes were collected as described above and analyzed by SDS-PAGE and fluorography. The corresponding bands were quantitated using an ultrascan laser densitometer (model 2202; LKB Instruments Inc.).

Prepro- α -factor Binding Assay

Affinity-purified prepro- α -factor (20 μ l in 8 M urea, 1,000 cpm/ μ l) was mixed with 180 μ l of buffer 2 and 8 μ l of yeast membranes. The mixture was incubated at 25°C for 10 min and then brought to 2.1 M sucrose (final concentration). This solution was transferred to an ultracentrifuge tube and 1.5 ml of a 1.7 M sucrose solution in buffer 3 (20 mM Hepes-KOH pH 7.4, 50 mM KOAc, 1 mM DTT) was layered on top, followed by 1.5 ml of a 1.0-M sucrose solution in buffer 3. Samples were centrifuged at 50,000 rpm at 4°C for 2 h 30 min in a rotor (model SW-60; Beckman Instruments, Inc., Palo Alto, CA). Floated membranes were collected in the interface between the 1.0 M and the 1.7-M sucrose solutions. Aggregates and unbound prepro- α -factor remained in the 2.1-M sucrose cushion. Radioactivity was then mea-

sured along the sucrose gradient. Radioactivity found in the 1.0-M sucrose step and in the interface was considered as the bound fraction. Radioactivity found in the rest of the gradient was considered as the unbound fraction.

When the competition assay was carried out, 20 μ l of prepro- α -factor (1,000 cpm/ μ l) were mixed with 180 μ l of buffer 2 containing unlabeled proOmpA (0.5 μ g or 5 μ g), unlabeled mature OmpA (5 μ g) or lysozyme (5 μ g). Yeast membranes (8 μ l) were added to the mixtures and incubated in a final volume of 200 μ l at 25°C for 10 min. Membranes were then floated as described above, and the radioactivity measured in the corresponding bound and unbound fractions.

Materials

ProOmpA and OmpA were kindly provided by the W. Wickner group (University of California at Los Angeles). Proteinase K and papain were purchased from E. Merck (Darmstadt, FRG). ATP, NEM, DTT, urea, lysozyme, IgG, leupeptin and TLCK were from Sigma Chemical Co. (St. Louis, MO), creatine phosphate and creatine phosphokinase from Boehringer-Mannheim (Mannheim, FRG) and PMSF from Eastman Kodak Co. (Rochester, NY).

Results

Receptor-mediated Binding of proOmpA to Yeast Microsomes

The components of the translocation assay (see Fig. 1) consist of affinity-purified precursor of OmpA (proOmpA) and yeast microsomes (20). Radiolabeled proOmpA was prepared by *in vitro* transcription and translation in an *Escherichia coli* system followed by its purification to homogeneity on an antibody column eluted with 8 M urea (4). In the absence of any cofactors, proOmpA was diluted into buffer containing yeast microsomes. Incubation was carried out at 25°C for 10 min. Preliminary experiments indicated that the amount of binding of proOmpA was unchanged during incubations as short as 5 min or as long as 40 min (data not shown). The membranes were then pelleted by centrifugation through a sucrose cushion, resuspended and divided into

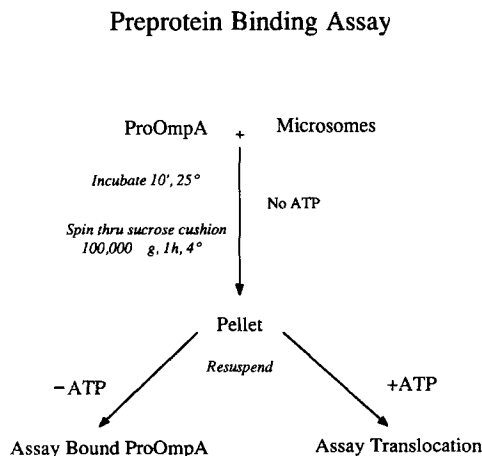


Figure 1. Preprotein binding assay. Affinity-purified ³⁵S-proOmpA was mixed with salt-washed yeast membranes and incubated at 25°C for 10 min. Samples were then spun at 100,000 *g* for 1 h at 4°C through a 0.5-M sucrose cushion. Pellets were resuspended, and, where indicated, ATP was added. Samples were incubated at 25°C for 10 min and binding and translocation were then quantified by SDS-PAGE, fluorography, and densitometry using an ultrascan laser densitometer (model 2202; LKB Instruments Inc.). See Materials and Methods for details.

1. Abbreviation used in this paper: NEM, *N*-ethylmaleimide.

PRE-PROTEIN BINDING ASSAY

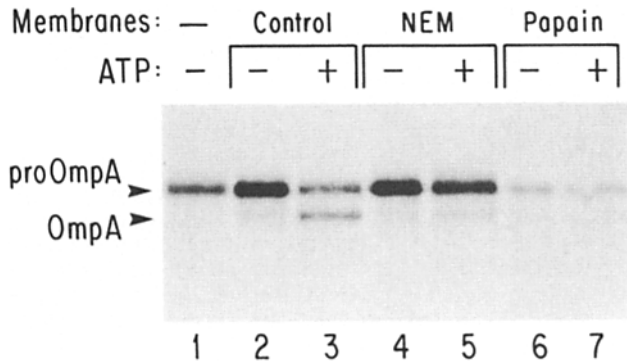


Figure 2. Translocation of proOmpA through yeast microsomes can be dissected into two steps. Affinity-purified ^{35}S -proOmpA was mixed with control or treated yeast microsomes (see Materials and Methods). The binding and the translocation reactions were carried out as described in Fig. 1. Membranes were treated with 20 mM NEM (final concentration) or treated with 500 $\mu\text{g}/\text{ml}$ of papain (final concentration). Lane 1, assay without membranes; lanes 2 and 3, assay with salt-washed untreated membranes; lanes 4 and 5, assay with NEM-treated membranes; lanes 6 and 7, assay with papain-treated membranes.

two portions. From the first sample the amount of proOmpA that specifically bound to the membranes was determined (Fig. 2, lanes 1 and 2). The second sample received ATP, was further incubated and assayed for the proportion of proOmpA that was translocated and processed to OmpA (lane 3). Protease protection experiments confirmed that the processed form was indicative of translocation in this assay (data not shown). In this way, both the initial binding step and the subsequent translocation step could be characterized independently.

If the binding observed represents specific interaction of the precursor with a putative proteinaceous receptor, several predictions can be made: binding of radiolabeled proOmpA

will be saturable, and will be competitively inhibited by unlabeled proOmpA, but not by mature OmpA or other proteins. If the receptor mediates the translocation of many precursors, one can expect that the binding of prepro- α -factor to the microsomes would also be competed by proOmpA, but not by mature proteins. Moreover, proteolysis of the yeast microsomes should result in the inhibition of binding.

The data presented in Fig. 3 A indicate that the binding of radiolabeled proOmpA to microsomes is a saturable process. A Scatchard analysis of these data is shown in Fig. 3 B and reveals a dissociation constant of 7.5×10^{-9} M. This represents the first calculation of the dissociation constant of a preprotein for the membrane in a functional assay. As the yeast microsomal fractions are rather heterogeneous, and antigenic markers for rough ER in yeast have not yet been identified, it is at this stage impossible to determine the number or density of such receptors.

Labeled proOmpA binding could be competed by the addition of increasing amounts of unlabeled proOmpA, but not by mature OmpA, lysozyme (Fig. 4 A) or IgG. (Under the conditions used in our system, half-maximal inhibition of binding occurred at a concentration of unlabeled proOmpA of 49 nM.) ProOmpA was unable to bind to the microsomes after it had been diluted out of urea and incubated in buffer at 0° for 15 h (data not shown), a treatment known to render the preprotein translocation incompetent (20). This supports the notion of a specific receptor-mediated interaction that depends, at least in part, on the leader peptide, and the proper conformation of the proOmpA.

ProOmpA binds to the same receptor as prepro- α -factor. One can perform similar experiments using affinity-purified prepro- α -factor, where dilution out of urea results in its translocation, processing, and glycosylation (20). In the absence of ATP, saturable binding, but not translocation, took place. The binding could be competed with unlabeled proOmpA, but not by OmpA, lysozyme (Fig. 5), or IgG. Similar to what was observed above, the concentration of unlabeled proOmpA needed for half-maximal competition of prepro- α -factor binding was about 50 nM.

We have previously shown that papain treatment of yeast microsomes inhibits translocation (20). It would be consistent with the data already presented if such proteolysis were

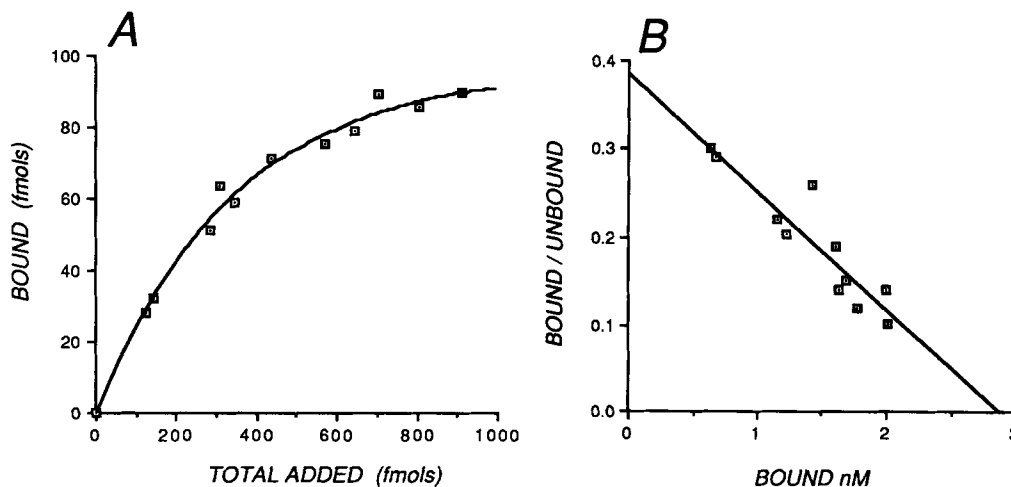


Figure 3. Binding of proOmpA to yeast microsomes is saturable. A, mixtures of labeled and unlabeled proOmpA were incubated with membranes and assayed for binding as described in Fig. 1. Radioactivity was measured in the supernatant (unbound) and in the pellet (bound). Parallel samples were run without membranes to calculate the background. B, Scatchard analysis of the data shown in A.

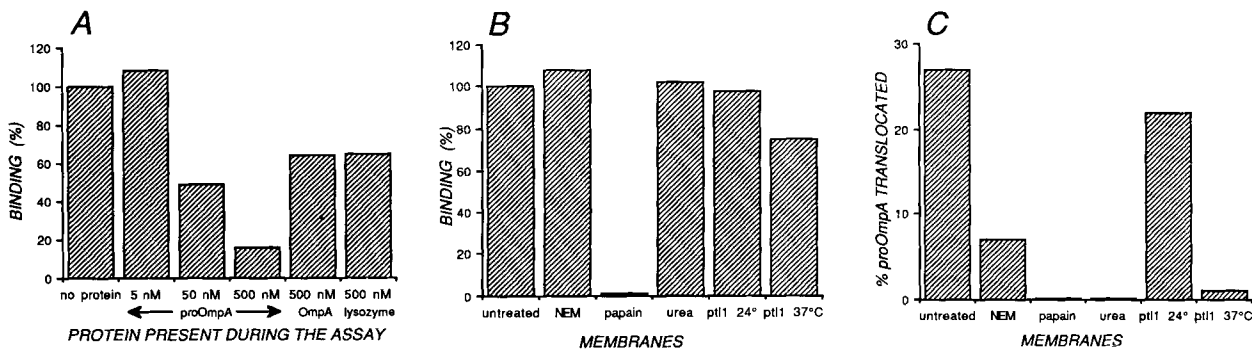


Figure 4. Binding and translocation of proOmpA through yeast microsomes are affected by different treatments. *A*, Binding of labeled proOmpA is inhibited by unlabeled proOmpA. Competition experiments between labeled proOmpA and unlabeled proOmpA as well as OmpA and lysozyme were carried out as described in Materials and Methods. The histogram shows the quantification of fluorograms using an ultrascan laser densitometer (model 2202; LKB Instruments Inc.). Binding is expressed as a percentage of proOmpA bound by control membranes. *B*, binding of labeled proOmpA is abolished by treating the membranes with papain. Wild-type membranes treated with 20 mM NEM, or 6 M urea (30 min 0°C), or 500 μg/ml papain or membranes obtained from the ptt1 mutant (20) were analyzed for their ability to bind proOmpA as described in Fig. 1. This histogram shows the amount of labeled proOmpA recovered in the membrane pellet with respect to the control (100%). In the case of ptt1 membranes, these were preincubated at 25°C or 37°C for 5 min, then the labeled proOmpA was added, and the membranes were incubated again at 25°C or 37°C, respectively, for an additional 10 min. *C*, translocation of labeled proOmpA is affected by various treatments. Aliquots of the membrane pellets obtained as in *B* were resuspended and assayed for translocation in the presence of ATP. Histogram shows the translocation efficiency (amount of proOmpA translocated/total added) under the different conditions.

to exert its effect on the preprotein receptor. Accordingly, microsomes were treated with papain and analyzed for their ability to bind proOmpA in our assay system. Preliminary controls indicated that papain treatment was without effect on membrane integrity (not shown). As can be seen in Figs. 2 and 4, only background levels of proOmpA were bound by papain-treated microsomes (Fig. 2, lanes 6 and 7, and Fig. 4 *B*). The line in a Scatchard analysis of the binding of proOmpA to papain-treated membranes was shifted substantially to the left, but had an identical slope to the control shown in Fig. 3 *B*. This indicates that papain treatment reduces the number of sites, not their affinity.

The background amount of proOmpA that was sedimented

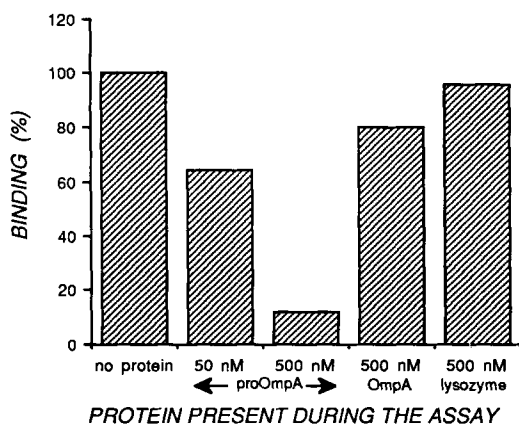


Figure 5. Binding of prepro- α -factor can be competitively inhibited by proOmpA, but not by mature proteins. Radiolabeled prepro- α -factor was mixed with proOmpA, OmpA, or lysozyme and binding assays were performed as described in Materials and Methods. Histogram shows the binding efficiency of the membranes expressed as percent of the control (binding of prepro- α -factor alone).

with papain-treated membranes shown in Fig. 2 (lanes 6 and 7) appears to be less than the control without membranes, shown in lane 1. It was possible that proOmpA was being digested by residual papain activity, and that failure to bind the precursor was because of its disappearance from the assay, not by the loss of receptors. Control experiments were performed to rule out this possibility. Incubation of proOmpA with papain-treated membranes, without the subsequent centrifugation step of the binding assay, showed that during the time it takes to perform such assays, no proOmpA was degraded. Supernatants generated by the binding assay, that normally contain the unbound proOmpA, were found to contain increased amounts of proOmpA in the case of the papain-treated membranes consistent with a decreased binding capacity and a lack of precursor degradation (data not shown).

These data demonstrate that binding is destroyed by proteolysis of the external surface of the microsomal membrane. We conclude, therefore, that before their translocation across membranes, preproteins bind specifically, and with high affinity to receptor proteins on the cytoplasmic face of the endoplasmic reticulum.

Translocation of Bound proOmpA across Yeast Microsomes

ProOmpA that had been bound and purified with the membranes by centrifugation was to a large extent competent for translocation. As can be seen in Fig. 2 (lane 3), the addition of ATP to such a reaction enabled the rapid translocation of proOmpA and its processing to OmpA. Not all of the bound material was translocated when ATP was added. This is most likely because of the time-dependent folding of proOmpA into a translocation-incompetent conformation that occurred during the incubation, centrifugation, and resuspension process. This phenomenon has been observed by several groups examining the translocation competence of

purified precursors (2, 4, 5, 16, 20). The data presented here show that the translocation reaction requires ATP. Its need to be hydrolyzed can be concluded from experiments showing that the nonhydrolyzable analogue, ATP γ S, could not substitute for ATP in this reaction (data not shown).

Translocation has been shown to be inhibited by treating the membranes with various reagents (10, 18), or by using membranes derived from the translocation mutant *ptll* when the assay is carried out at the nonpermissive temperature (21). In addition to protease, alkylation with NEM as well as washing with 6 M urea will block translocation. Having now separated translocation into two steps, one can ask: which of these steps is affected by such treatments?

The binding of proOmpA to membranes was unaffected by treatment with NEM (Fig. 2, lanes 4 and 5; Fig. 4 B), or by a 6 M urea wash (Fig. 4 B). Binding of proOmpA was also normal to membranes derived from the translocation mutant *ptll*, even when the assay was carried out at the nonpermissive temperature for translocation (Fig. 4 B). In contrast, the subsequent translocation of proOmpA did not occur upon addition of ATP to these samples (Fig. 4 C). This suggests that a process that takes place after binding, and is involved in the translocation per se, is affected by these treatments or mutation.

From these data, we conclude that translocation across yeast microsomes is preceded by a specific binding of the protein precursor to the membrane, and that this process can be uncoupled from translocation by withholding ATP. Translocation may be mediated by another protein or set of proteins distinct from that mediating binding. One of these must include an ATPase, as the translocation step in the cytosol-free assay requires the hydrolysis of ATP.

Discussion

The data presented here represent the first functional characterization of the binding of a secretory protein precursor to the membrane across which it must be translocated. This has been facilitated through the use of a cytosol-free assay, in which translocation competence of preproteins is preserved by chemical denaturants. Moreover, we show that binding and translocation represent two distinct and separable steps that can be analyzed independently in vitro. Through such progressive biochemical dissections, it should be possible to understand the process of translocation in greater molecular detail.

Several common features of protein translocation across various membrane systems in different organisms are becoming evident. Mitochondrial proteins, encoded by nuclear genes, are imported into mitochondria in a two-step process similar to that reported here (8, 15). The binding step requires an energized inner membrane, and the translocation needs ATP. In the case of yeast microsomes, however, we and others have reported earlier that agents capable of uncoupling or dissipating membrane potentials had no effect on translocation (10, 18). A situation similar to mitochondria exists in bacteria, where translocation across inverted membrane vesicles is both potential- and ATP-requiring (9), but it remains to be seen if separate binding and translocation steps occur.

A putative outer membrane receptor for transit peptides has recently been implicated in protein import into chlo-

roplasts (14). In the case of mammalian microsomes, a similar situation may exist, although the inability to translocate most proteins posttranslationally (or in the absence of cytosolic factors) in this system makes its elucidation difficult. We have recently demonstrated, however, that affinity-purified proOmpA can be translocated across pancreatic microsomes (albeit less efficiently than in yeast) when diluted out of 8 M urea (20). This means that similar binding studies may be possible in the classical mammalian system.

Rapoport and co-workers have shown that signal sequences can be cross-linked to a protein in the microsomal membrane (23). This protein remains to be functionally characterized. Connolly and Gilmore have examined the initial interactions between nascent chain-bearing ribosomes and the membrane in the mammalian system, and demonstrated a requirement for GTP (3). In our ribosome-free assay system, no requirement for GTP was observed (data not shown). Zimmermann and colleagues have shown that engineered proteins, containing a COOH-terminal loop, can be bound by pancreatic microsomal membranes, and translocation will occur only upon reduction of the disulfide bonds that maintain the loop (13). Such modulated precursors may also be useful in identifying preprotein receptors in the ER membrane.

A tentative model, consistent with these and other data, can be put together for translocation across yeast ER. Translocation need not be coupled to translation, as we have recently demonstrated in *in vivo* studies using *ptll* (21). Cytosolic factors such as heat shock-like proteins, known to be required for translocation (1, 7), would thus be required for preservation of a translocation-competent structure, particularly if precursors are translocated some time after their synthesis. As no yeast cytosolic signal sequence recognition component (like signal recognition particle) has been characterized as yet, it is possible that recognition first occurs when precursors contact the membrane-associated receptor. This would, in the case of yeast microsomes, be the component susceptible to papain digestion. Translocation then involves the participation of one or several components identified biochemically by urea washing or a NEM treatment, and/or other components, identified genetically *ptll*, *sec6l*, and *sec62* mutations (6, 21). An ATPase is clearly involved at the membrane level, as suggested by the data based on the cytosol-free assay. Further genetic and biochemical studies are needed to enable a more precise determination of the number of components and their roles in translocation.

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Note added in proof: The results of a recent *in vitro* study suggest that nascent chain binding and translocation across mammalian microsomes are also distinct events (Nicchitta, C. V., and G. Blobel. 1989. *J. Cell Biol.* 108:789-795).

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