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In Vitro Protein Translocation across the Yeast Endoplasmic Reticulum: ATP-Dependent Post-translational Translocation of the Prepro-α-Factor

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Summary

The in vitro synthesized precursor of the α-factor pheromone, prepro-α-factor, of Saccharomyces cerevisiae was translocated across yeast microsomal membranes in either a homologous or a wheat germ cell free system. Translocated prepro-α-factor was glycosylated, sedimented with yeast microsomal vesicles, and was protected from digestion by added protease, but was soluble after alkaline sodium carbonate treatment. Thus prepro-α-factor was properly sequestered within yeast microsomal vesicles, but was not integrated into the lipid bilayer. In marked contrast to protein translocation across mammalian microsomal membranes, translocation of prepro-α-factor across yeast microsomal membranes could occur posttranslationally. This reaction required protein components in the yeast microsomal fraction that could be inactivated by alkylation or proteolysis, was ATP-dependent, and was insensitive to the presence of a variety of uncouplers and ionophores.

Introduction

An early event in the biosynthetic pathway of secretory, lysosomal, and a variety of integral membrane proteins in eukaryotic cells is the selective translocation of these proteins or certain of their domains (as is the case for integral membrane proteins) across the lipid bilayer of the endoplasmic reticulum (ER) membrane (for review see Walter et al., 1984). Much of the knowledge about the molecular mechanism of this phenomenon is derived from the reconstitution of this process in vitro from components derived from a variety of higher eukaryotic cells. From these studies a model evolved in which protein translation and its translocation across the membrane are strictly coupled (Blobel and Dobberstein, 1975). Two components, signal recognition particle (SRP) and SRP receptor, of the cellular machinery promoting this process have been purified (Walter and Blobel, 1980; Gilmore et al., 1982). These components function primarily to target the ribosomes that are synthesizing secretory proteins to the ER membrane. In vitro their molecular function can be described as a sequence of the following steps. First, signal recognition by SRP involves decoding the targeting information contained in the signal peptide as part of the nascent chain. As a result SRP binds with high affinity to the translating polysome (Walter et al., 1981) and transiently arrests elongation (Walter and Blobel, 1981; Siegel and Walter, 1985). Second, targeting to the ER is mediated through direct interaction of the SRP bound to the ribosome with an integral membrane protein, the SRP receptor or docking protein (Gilmore et al., 1982; Meyer et al., 1982), of the ER membrane (Hortsch and Meyer, 1985). Interaction of SRP with its receptor causes the elongation-arrest to be released (Walter and Blobel, 1981) and a loss of the high affinity of SRP for the ribosome–nascent chain complex. Third, translocation of the nascent polypeptide across the membrane occurs by an uncharacterized, but cotranslational mechanism (or mechanisms). SRP and SRP receptor are recycled after the ribosome binds to the membrane to form a functional ribosome–membrane junction (Gilmore and Blobel, 1983).

Because of the nature of the biochemical approach to the problem, the precise role of the various steps outlined above in protein secretion in vivo remain to be established. For this reason we are beginning to explore yeast as an experimental system in which it should be possible to link the results of biochemical exploration with the physiological requirements of living cells. Our approach is first to identify components of the yeast protein translocation machinery using in vitro assays analogous to those that were used to characterize the mammalian components. Because of the small size of the genome, it is comparatively easy to clone the genes for isolated yeast proteins. Modern genetic techniques make it then possible to delete or selectively alter the cloned genes and to study the consequences of such perturbations in vivo.

We chose as a model secretory protein the yeast mating factor produced by α cells (α-factor). A genetic approach to the study of the secretory process in yeast has produced conditional mutants that affect protein processing and secretion (for review see Schekman and Novick, 1982). Consequently, the biosynthetic pathway of α-factor is one of the best understood for a peptide hormone. α-Factor is synthesized in yeast cells of the α mating type initially as a larger precursor molecule, prepro-α-factor (pαF), of 18.5 kd that contains four copies of the 13 amino acid long α-factor at its carboxy-terminal end (see schematic drawing in Figure 1) (Kurjan and Herskowitz, 1982; Julius et al., 1983). The amino terminus of pαF shows a stretch of 20 nonpolar amino acids that is likely to function as a noncleavable signal sequence; no processing by signal peptidase upon translocation across the ER membrane is observed in vivo (Julius et al., 1984a). In the lumen of the ER, pαF becomes glycosylated at three asparagine residues (Julius et al., 1984a). We have used the change in mobility on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) that is associated with this covalent modification to monitor the translocation of pαF across yeast ER membranes and to establish an assay that allows us to characterize this process in vitro. Our data indicate that translocation of pαF can occur posttranslationally.

* The order of the first two authors is arbitrary.

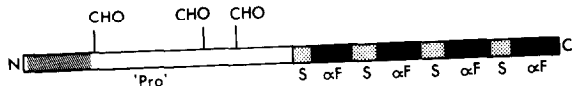

Figure 1. Structure of pαF

pαF contains a 20 hydrophobic amino acid signal peptide (shaded) at the amino terminus as part of an 80 amino acid "prepro"-region that contains three N-glycosylation sites (indicated by CHO). Carboxy-terminal to the "prepro"-region are four tandem repeats of the α-factor peptide (black) separated by spacer peptides, 6–8 amino acids long (marked S) (Kurjan and Herskowitz, 1982). pαF is translocated into the lumen of the ER without cleavage of its signal peptide and becomes modified by the addition of three core-oligosaccharides to asparagine residues (Julius et al., 1984a). It then traverses the yeast secretory pathway. Late in the Golgi apparatus or in secretory granules, pαF becomes processed by the KEX 2 gene product to α-factor, still containing the spacer peptide at the amino-terminal end (Julius et al., 1984b). Further processing by membrane-bound dipeptidyl-aminopeptidase A results in the production of mature α-factor that is secreted from the cell (Julius et al., 1983).

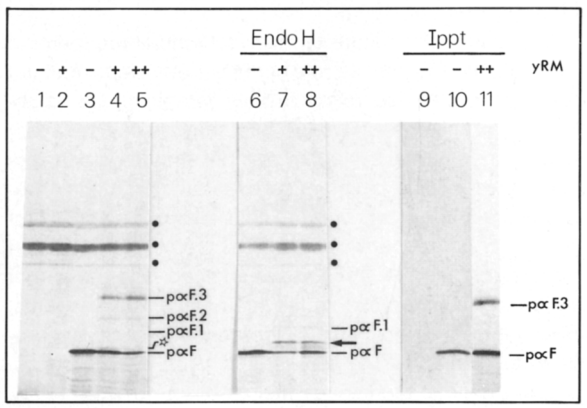

Figure 2. In Vitro Translation of pαF

Translations in a yeast cell free extract (see Experimental Procedures) were performed either in the absence (lanes 1, 2) or in the presence (lanes 3–11) of mRNA coding for pαF. yRM were present at 0.025 A_{280} units/20 μl (lanes 5, 8, 11; marked ++) or 0.0075 A_{280} units/20 μl (lanes 2, 4, 7; marked +), or omitted (lanes 1, 3, 6, 9, 10; marked −).

The products of 20 μl translation reactions (see Experimental Procedures) were resolved on 10%–15% SDS polyacrylamide gels (lanes 1–5). The samples shown in lanes 6–8 have been treated with endoglycosidase H prior to SDS-PAGE (see Experimental Procedures). Lanes 10 and 11 show immunoprecipitations of translation products shown in lanes 3 and 5, respectively, using a rabbit serum raised against the secreted form of α-factor (M. Poritz and P. W., unpublished). Lane 9 is identical to lane 10, except that preimmune serum was used.

The bands marked with dots are unidentified. They are not related to pαF (see lanes 10–11), but rather are major Coomassie blue staining bands in the translation system that become labeled even in the absence of ongoing protein synthesis (see Figure 5A, lane 1). Their presence is variable between different batches of translation extract.

Results

Homologous In Vitro Translocation Assay

Our first goal in defining the molecular components involved in protein translocation across the ER membrane of Saccharomyces cerevisiae was to establish an assay that, in analogy to the systems described for higher eukaryotes, would faithfully reproduce this process in vitro. Yeast cells were fractionated to yield a high speed supernatant fraction that would promote protein synthesis directed from an exogenously added mRNA template and

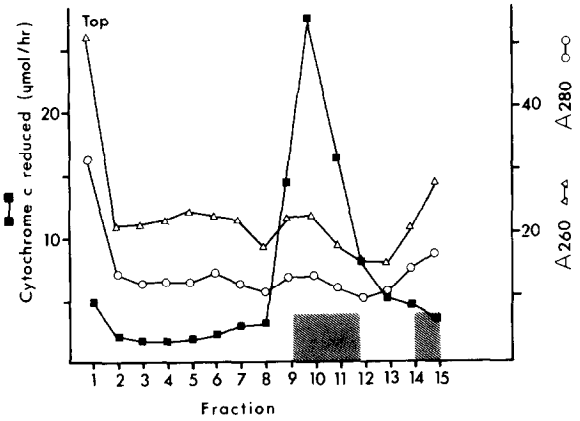

Figure 3. Equilibrium Density Gradient Fractionation of a Yeast Postmitochondrial Supernatant Fraction

Three milliliters of the S-10 fraction (derived from 1400 OD_{600} units of cells) were subfractionated on a Percoll density gradient as described in Experimental Procedures. After centrifugation a volume corresponding to the load was removed from the top of the sample. The remainder was fractionated using a Buchler gradient fractionator; 14 fractions of 1.3 ml were collected. The fractions were assayed for NADPH–cytochrome c reductase (solid squares) (Kubota et al., 1977), and the absorbances at 280 nm (open circles) and 260 nm (open triangles) were determined after dilution into 1% SDS. The approximate positions of two visibly turbid bands are indicated by shaded bars.

a membrane vesicle fraction that would promote translocation of secretory proteins synthesized in vitro.

For the reasons outlined in the Introduction, we chose α-factor as a model secretory protein. A plasmid containing the pαF gene cloned behind an SP6 RNA polymerase promoter (a generous gift from Dr. D. Julius) was linearized and then transcribed with SP6 RNA polymerase under conditions that produced transcripts terminating at the restriction site and containing a GpppG cap at their 5′ ends (see Experimental Procedures). The data in Figure 2 demonstrate that the synthetic mRNA directs the synthesis of pαF as a single protein band of the correct molecular weight (Figure 2, lane 3) when translated in a yeast cell free extract. The identity of the primary translation product as pαF is confirmed by immunoprecipitation with a rabbit antibody raised against authentic α-factor (Figure 2, lane 10).

A simple procedure was devised to prepare microsomal membranes from S. cerevisiae (see Experimental Procedures). A postmitochondrial supernatant was layered on top of a Percoll solution (a colloidal silica suspension) and centrifuged to generate a density gradient containing membranes banded at their equilibrium densities. After the brief centrifugation, two distinct turbid bands were visible in the gradient, which are indicated in Figure 3 as shaded bars. We assayed NADPH–cytochrome c reductase activity (an ER marker enzyme) (Kubota et al., 1977) across the gradient (Figure 3, solid squares). The bulk of this activity cosedimented with the band of lighter density, which was collected. In this membrane fraction we recovered about 30% of the NADPH–cytochrome c reductase activity that was present in the crude homogenate.

Addition of this fraction to the yeast translation system

in the absence of exogenous mRNA frequently produced a considerable background of translation products. The extent of this cofractionating mRNA activity was variable from one preparation to another (data not shown). We therefore proceeded to remove RNA by treatment with micrococcal nuclease, followed by EDTA extraction of the microsomes. We further concentrated the membranes by banding them onto a high density Percoll cushion. The resulting membrane suspension contributed no detectable mRNA activity (Figure 2, lane 2) and had lost over 70% of the absorbance at 260 nm (most likely because of loss of bound or adsorbed ribosomes or polysomes) that was originally present in the Percoll banded material. Henceforth, we will refer to this fraction of nuclease-treated and EDTA-stripped yeast rough microsomes as yRM.

When pαF was translated in the presence of increasing concentrations of yRM, we observed translocation of the protein across the microsomal membrane as indicated by the attachment of core-oligosaccharides (Figure 2, lanes 4 and 5). In particular, three additional bands of higher molecular weight were visible that correspond to the addition of one, two, or three core-oligosaccharide moieties, and are labeled pαF.1, pαF.2, and pαF.3. Increasing the membrane concentration caused a shift toward the fully glycosylated form (pαF.3). This may indicate that there is only a limited pool of assembled dolichol-linked core-oligosaccharides present in the microsome fraction that becomes depleted at the low membrane concentration. At higher concentrations of yRM we also observed some general inhibition of protein synthesis.

Yeast glycoproteins are susceptible to digestion with endoglycosidase H, which removes all of the N-linked core-oligosaccharides or high mannose oligosaccharides, except for the N-acetylglucosamine bound to the asparagine residues (Chu et al., 1978). Digestion of the translation products produced in the presence of membranes with endoglycosidase H resulted in removal of the core-oligosaccharides and production of correspondingly faster migrating bands (Figure 2, lanes 7 and 8, marked with arrow). Presumably because of the N-acetylglucosamine residues that remain attached to the polypeptide after endoglycosidase H cleavage, these forms still show reduced mobility compared to that of unglycosylated pαF (Julius et al., 1984a). In addition to the three core-glycosylated forms of pαF, we observed a minor species that was only slightly retarded in its mobility on SDS-PAGE (Figure 2, lanes 4 and 5, marked with asterisk). We do not know the precise molecular nature of this modification. This form may result from a transfer reaction of an incomplete core-oligosaccharide to pαF after depletion of the properly assembled oligosaccharide precursor pools. Transfer of incomplete core-sugars has been observed in vivo by Huffaker and Robbins (1983). This interpretation is consistent with the observation that this form is most abundant in the reactions containing low yRM concentrations. The protein is clearly translocated, as indicated by resistance to proteases and cosedimentation with vesicles (see below).

We used two different criteria to confirm that the glycosylated forms of pαF are translocated into the lumen of sealed microsomal vesicles. First, we found that the glycosylated forms of pαF were protected from exogenously added protease (Figure 4A, lanes 5 and 6), whereas unglycosylated pαF was completely degraded (Figure 4A, lane 4). When the microsomal vesicles were dissolved by the inclusion of detergent during proteolysis, glycosylated pαF was also degraded (Figure 4A, lanes 8 and 9). This indicates that these forms were not intrinsically resistant to protease, but rather this resistance was due to their sequestration within closed vesicles.

The second criterion for proper translocation was to show that the glycosylated pαF can be selectively sedimented with the microsomal vesicles. Lanes 1–3 in Figure 4B show a series of translation reactions with increasing concentrations of yRM. After translation these reactions were subjected to a brief high speed centrifugation to pellet the membranes. Comparison of the supernatant fractions (Figure 4B, lanes 4–6) with the corresponding pellet fractions (lanes 7–9) revealed that the glycosylated forms of pαF were quantitatively recovered in the pellet fractions whereas most of the unmodified pαF was recovered in the supernatant fractions. The small amount of pαF that was detected in the pellet fractions is likely to be an artifact of the fractionation procedure because it is found even in the samples from which yRM were omitted (Figure 4B, lane 7) and is degraded upon proteolytic digestion (Figure 4A, lanes 5 and 6).

Because pαF retains its signal sequence upon translocation across the ER, it was suggested (Julius et al., 1984a) that its hydrophobic nature allows the signal sequence to serve as a membrane anchor and thus retain translocated and glycosylated pαF as an integral membrane protein in the lipid bilayer. To test this possibility we performed an alkaline sodium carbonate extraction of the reaction products. Under these conditions the vesicles are converted into sheets and only bona fide integral membrane proteins sediment with these membrane remnants (Fujiki et al., 1982; Davis and Model, 1985). The data shown in Figure 4C demonstrate that the glycosylated forms of pαF were only recovered in the supernatant fraction (Figure 4C, lanes 5 and 6), indicating that they were not integrated into the lipid bilayer of yRM, but rather were released from the lumen of the vesicles.

Posttranslational Translocation of pαF

The data presented above demonstrate that pαF is translocated across yeast microsomal membranes with fidelity when these membranes are present during translation. To our surprise we discovered that translocation still occurred when the membranes were added after protein synthesis had been terminated. This is in striking contrast to the translocation of secretory proteins across mammalian microsomal membranes, where translation and translocation appear strictly coupled. Posttranslational translocation of pαF was demonstrated as follows. mRNA coding for pαF was translated in the yeast translation system for 1 hr. We then added mRNA coding for globin, which was also translated upon further incubation (Figure 5A, lane 2, arrow). However, when cycloheximide was

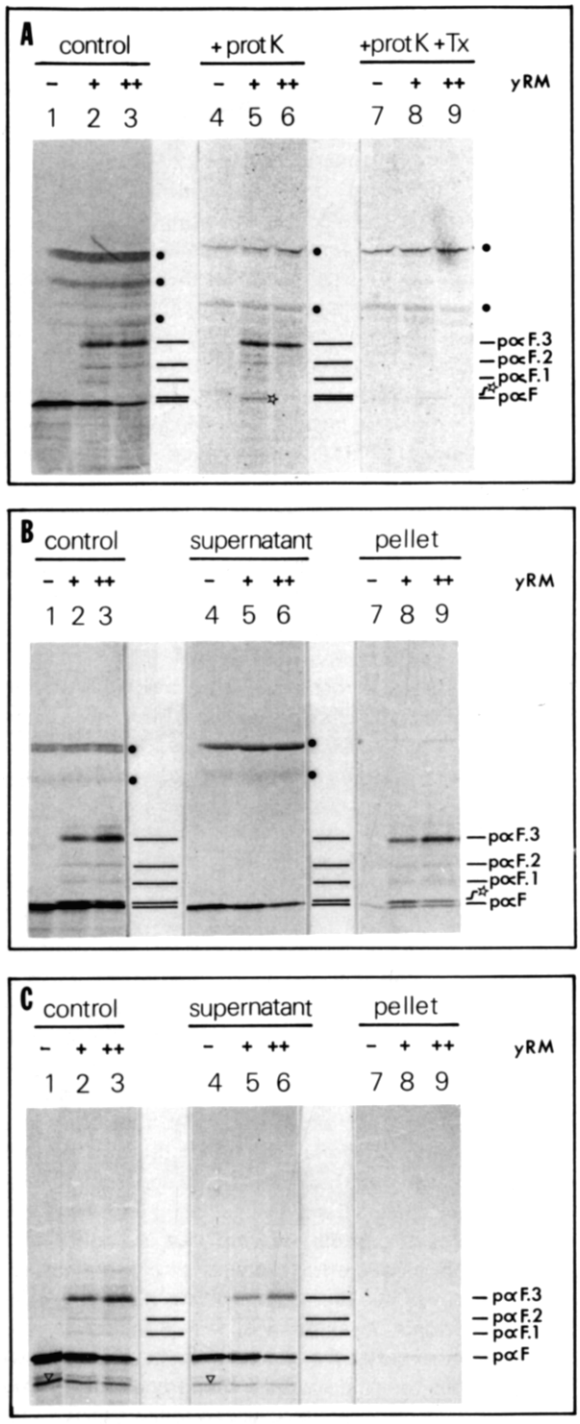

Figure 4. Verification of pαF Translocation

(A) Proteinase K protection. pαF was translated in 20 μl reactions in the absence of yRM (lanes 1, 4, 7; marked −), in the presence of 0.0075 A_{280} units yRM (lanes 2, 5, 8; marked +), or in the presence of 0.025 A_{280} units yRM (lanes 3, 6, 9; marked ++). After 1 hr of translation the samples were either left untreated (lanes 1–3) or incubated with proteinase K (Perara and Lingappa, 1985) in the absence (lanes 4–6) or presence (lanes 7–9) of 1% Triton X-100 (see Experimental Procedures). The bands marked with dots are not related to pαF (see Figure 2).

(B) Sedimentation assay. Translation reactions were performed as in A. They were then subjected to a brief centrifugation to generate a supernatant and a pellet fraction containing microsomal vesicles (see Experimental Procedures). Shown are the total translation products (lanes 1–3), the supernatant fractions (lanes 4–5), and the translation products that pellet with the microsomal membranes (lane 7–9).

(C) Alkaline carbonate extraction. Translations were performed as in A. After translation 7 μl of translation products was analyzed directly by SDS-PAGE (lanes 1–3). Aliquots of 10 μl of the same translation reactions were carbonate extracted as described in Experimental Procedures. The supernatants are shown in lanes 4–6, and the pellet fractions in lanes 7–9. The arrowheads indicate the position of globin included as a soluble protein control. The recovery is not complete, because of losses in sample preparation from the relatively dilute supernatant fractions. Because the pellets have been directly dissolved in sample buffer for PAGE, no losses could have occurred in the pellet fractions.

Depending on the translation extract used, the translocation efficiency of in vitro synthesized pαF ranged from 10% to 40% in a 1 hr incubation period. The reasons for this variability are unknown.

added with the globin mRNA, no globin was synthesized (Figure 5A, lanes 3 and 4), indicating that cycloheximide effectively inhibited protein synthesis. Addition of yRM to the cycloheximide-inhibited translation system resulted in the formation of fully glycosylated pαF (Figure 5A, lanes 5 and 6). To ascertain that the ribosome is not participating in the observed translocation, we prepared a ribosome-depleted supernatant containing in vitro synthesized pαF (prepared by a 1 hr spin in a Beckman airfuge, see Experimental Procedures). This supernatant no longer promoted protein synthesis (Figure 5A, lane 7, note the absence of globin); however, pαF was still translocated when yRM were added in the absence of cycloheximide (Figure 5A, lanes 8 and 9) or in its presence (Figure 5A, lanes 11 and 12). Posttranslationally translocated pαF was properly sequestered inside the yRM vesicles, as shown by protease protection (Figure 5B, lanes 3 and 4). Kinetic analysis (not shown) of the reaction showed that after a short lag phase of about 3 min the posttranslational translocation of pαF was linear with time for roughly 60 min. From a quantitative comparison of the data from Figures 4A and 5A we conclude that the posttranslational translocation reaction of pαF occurred with efficiency comparable to that of the cotranslational process (3.2 fmol pαF translocated per 0.025 A_{280} units of yRM cotranslationally, compared to 2.8 fmol posttranslationally).

The experiments shown in Figure 6 demonstrate that the posttranslational translocation of pαF is not a spontaneous process, as suggested for membrane proteins (for review, see Wickner, 1979) or for secretory proteins (von Heijne and Blomberg, 1979), but rather requires the participation of membrane proteins, as well as the presence of an energy source. As shown in Figure 6 (lanes 1–3) alkylation of yRM with N-ethylmaleimide inhibited the reaction. Furthermore, yRM could be inactivated by trypsin digestion (not shown, digestions were for 30 min at 0°C at 500 μg/ml trypsin; thereby providing additional support for the conjecture that cytoplasmically exposed membrane proteins are essential. We presently cannot rule out the possibility that core-oligosaccharide transferase contains a cytoplasmically exposed domain that renders the enzyme susceptible to alkylation or proteolytic inactivation. However, no translocated unglycosylated pαF was detected (not shown).

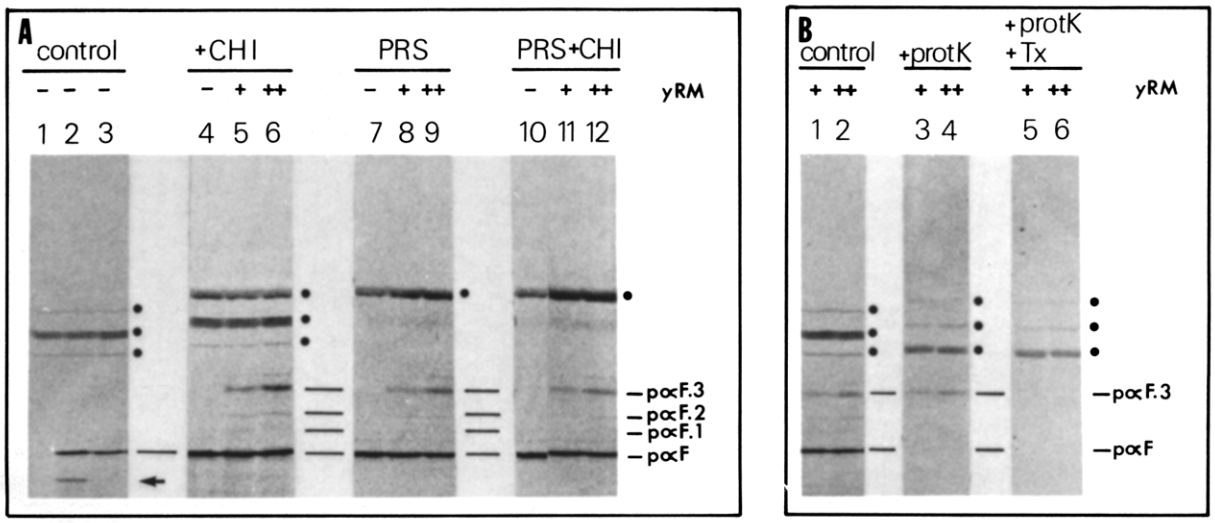

Figure 5. Posttranslational Translocation of pαF

(A) pαF mRNA was translated for 1 hr as described in Experimental Procedures. After this time further translation was inhibited by adding cycloheximide to 2 mM (lanes 3–6, marked CHI), or removing the ribosomes by centrifugation (lanes 7–9, marked postribosomal supernatant PRS), or both (lanes 10–12, marked PRS+CHI). After these treatments yRM were added to 0.025 A_{280} units/20 μl (lanes 6, 9, 12; marked ++), or to 0.0075 units/20 μl (lanes 5, 8, 11, marked +), or no yRM were added (lanes 1, 2, 3, 4, 7, 10; marked −). At the same time, globin mRNA was also added in order to ascertain that further protein synthesis was inhibited. The reactions were then incubated for an additional hour at 20°C.

Lanes 1–3 show control reactions in the absence of yRM. In lane 1, cycloheximide was added at the beginning of the translation, showing that it abolishes pαF synthesis. In lane 2, translation was carried out for 1 hr, then globin mRNA was added and the incubation was continued for an additional hour. Translated globin is marked with an arrow. Lane 3 is identical to lane 2, except that cycloheximide was added together with globin mRNA.

(B) Protease protection of posttranslationally translocated pαF. The reactions shown in lanes 1 and 2 correspond to reactions shown in A, lanes 5 and 6. After the posttranslational incubation the samples were treated with proteinase K under the same conditions as described in Figure 4A either in the absence (lanes 3 and 4) or in the presence (lanes 5 and 6) of 1% Triton X-100.

The bands marked with dots are not related to pαF (see Figure 2).

Our ability to uncouple translocation from protein synthesis allows us to characterize directly the energy requirements of the translocation reaction. We depleted a translation extract containing in vitro synthesized pαF of small molecules by gel filtration. Upon subsequent addition of yRM no translocation was observed (Figure 6, compare lanes 4 and 5). Readdition of ATP and a regenerating system restored translocation (Figure 6, lane 7), whereas nonhydrolyzable ATP analogues did not support the reaction (Figure 6, lane 6). This reaction was completely inhibited by the addition of E. coli glycerol kinase (which is absolutely ATP-specific; Hayashi and Lin, 1967; Thorner and Paulus, 1973) (Figure 6, lane 8), demonstrating conclusively that ATP is essential; however, these experiments cannot prove that hydrolysis of the ATP is concomitant with pαF translocation. The addition of 1 mM ATP alone did promote pαF translocation (not shown), albeit at reduced efficiency, indicating that an ATP-regenerating system is required in order to maintain an adequate level of ATP in the complex reaction mixture. Interestingly, addition of the regenerating system alone, or addition of GTP (1 mM), also promoted translocation at much reduced efficiencies (not shown). This is likely to be due to the regeneration of ATP from residual protein-bound ATP or ADP, since inclusion of E. coli glycerol kinase also abolished these reactions.

In contrast to posttranslational protein translocation across the prokaryotic plasma membrane or the mitochondrial envelope, we found that a variety of uncouplers and ionophores has no effect on the translocation reaction. Specifically, translocation was not inhibited by the proton ionophores SF6847 and FCCP, the potassium ionophore valinomycin, or the proton/potassium ionophore nigericin, alone or in combinations (Figure 6, lane 9 and 10 and legend).

Translocation of pαF in Heterologous Systems

Translocation of secretory proteins across mammalian microsomal membranes occurs cotranslationally. Our finding of a posttranslational mechanism for pαF translocation therefore raises the question whether this apparent difference results from a special property of pαF or whether it is due to inherent differences in the translocation machineries of yeast and mammalian ER membranes. We therefore tested pαF as a substrate in a translocation assay containing canine SRP and canine potassium-extracted (i.e. SRP-depleted) rough microsomes (cKRM). pαF mRNA was translated in a wheat germ extract (Figure 7A, lane 2). As for other secretory proteins, translation was arrested if canine SRP was present cotranslationally (Figure 7A, lane 3). Translation of mRNA coding for the cytoplasmic protein globin was not affected (not shown). Addition of cKRM in the presence of SRP resulted in release of the elongation-arrest and translocation of the

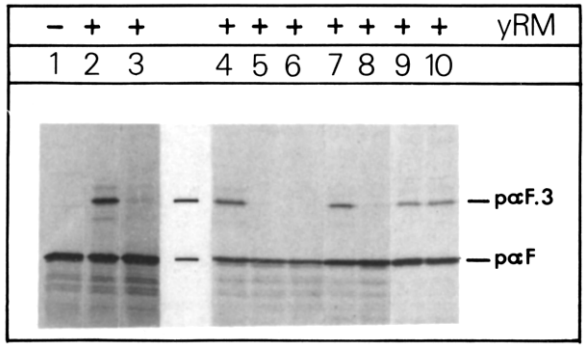

Figure 6. Requirements for Posttranslational Translocation

pαF was synthesized in a yeast translation extract at 20°C for 60 min, followed by inhibition of protein synthesis with 2 mM cycloheximide. Ten microliter aliquots were removed and incubated an additional 60 min in a final volume of 15 μl in the absence (lane 1) or presence (lanes 2–10) of 0.05 A_{280} units of yRM with the following variations: lanes 1 and 2, control incubations; lane 3, yRM were alkylated with N-ethylmaleimide (see Experimental Procedures); lane 4, control incubation. For the experiments shown in lanes 5–10 the translation extract was desalted on Sephadex G-25 (see Experimental Procedures) prior to posttranslational incubation in the presence of 0.05 A_{280} units of yRM and the following additions: lane 5, no additions; lane 6, 2 mM of the nonhydrolyzable ATP analogue adenosine-5'-[β,γ-imido]-triphosphate was included (identical results were obtained when the [β,γ-methylene] or the [γ-thio] derivatives of ATP were included; all ATP analogues were tested at 0.5 mM, 1 mM, and 2 mM [not shown]); lane 7, 1 mM ATP and 17.5 mM creatine phosphate were included; lane 8, same as lane 7 except that 2.5 μg of E. coli glycerol kinase was included; lane 9, control incubation containing 0.4% dimethylsulfoxide; lane 10, same as lane 9 except that 20 μM SF6847 (a benzylidenemalononitrile proton ionophore; Heytler, 1979; Grossman et al., 1980), 10 μM nigericin, 10 μM valinomycin, and 25 mM potassium acetate were included. (All of the listed ionophores were also ineffective in inhibiting pαF translocation when assayed by themselves or in pairwise combinations. Likewise, the addition of 50 μM FCCP had no effect [not shown].)

Quantitations for lanes 4–10 (amount pαF translocated in femtomoles): lane 4, 2.5; lane 7, 2.3; lane 9, 1.8; lane 10, 1.8; lanes 5, 6, 8, <0.2.

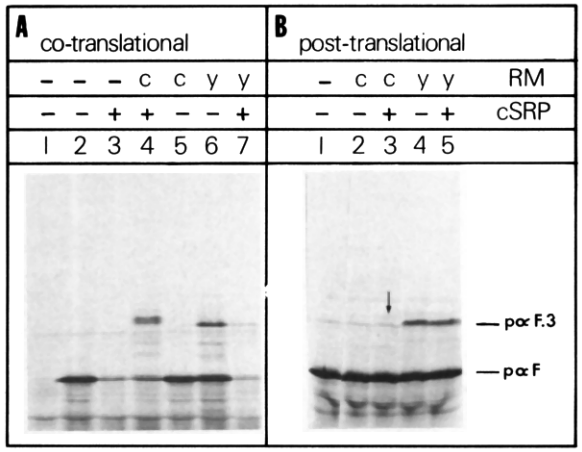

Figure 7. Translation and Translocation in Heterologous Systems

Translations in a wheat germ cell free extract (Walter and Blobel, 1980) were performed for 60 min at 20°C in the absence (A, lane 1) or in the presence (A, lanes 2–7; B, lanes 1–5) of mRNA coding for pαF.

(A) At the beginning of translation, 10 μl reactions were supplemented with 45 nM canine SRP (cSRP, lanes 3, 4, 7), 2 equivalents (Walter and Blobel, 1980) of canine potassium- and EDTA-extracted, i.e. SRP-depleted microsomes (cKRM, denoted c) (lanes 4 and 5) and 0.025 A_{280} units of yRM (denoted y) (lanes 6 and 7), respectively.

(B) Translation was terminated by the addition of 2 mM cycloheximide. Aliquots of 10 μl were further incubated for 60 min at 20°C with the following posttranslational additions: 45 nM cSRP (lanes 3 and 5), 2 equivalents of cKRM (lanes 2 and 3), and 0.025 A_{280} units of yRM (lanes 4 and 5), respectively.

Note that the autoradiogram shown in B was exposed four times longer. The absolute amounts of pαF translocated across yRM (0.025 A_{280} units) cotranslationally versus posttranslationally were as follows: A, lane 6, 15 fmol; B, lane 4, 2.7 fmol. In B, lane 3, the glycosylated form of pαF is marked with an arrow.

The difference between the electrophoretic mobility of the glycosylated pαF factor produced by canine membranes and that produced by yeast membranes could result from the absence of glucose residues from the core-oligosaccharides, either due to trimming of the glucose residues after transfer, or due to transfer of nonglucosylated or partially glucosylated oligosaccharide. Yeast core-oligosaccharide transferase is less strict than the mammalian enzyme in its requirement for the presence of glucose residues on the dolichol-linked oligosaccharide (Trimble et al., 1980). A minor species migrating slightly above glycosylated pαF with mobility similar to that produced by cKRM is also visible in most other figures.

synthesized pαF across the membrane. The resulting glycosylated form of pαF (Figure 7A, lane 4) migrated slightly slower on the gel than glycosylated pαF produced in the presence of yRM (see legend to Figure 7). The translocation was dependent on the presence of SRP, since cKRM in the absence of SRP were not sufficient to effect translocation (Figure 7A, lane 5). If yRM were present cotranslationally, pαF synthesized in the wheat germ system was efficiently translocated (Figure 7A, lane 6). However, when translocation across yeast microsomal membranes was assayed in the presence of SRP, the yRM fraction was apparently not able to release the SRP-induced elongation-arrest, and as a consequence correspondingly less translocated pαF was obtained (Figure 7A, lane 7). Thus the mechanism of pαF translocation across the mammalian microsomal membrane seems to be indistinguishable from that of other secretory proteins. yRM can function in the heterologous wheat germ system, yet are unable to interact productively with mammalian SRP.

If cKRM alone was added posttranslationally to the wheat germ extract containing pαF, no translocation was detected (Figure 7B, lanes 1 and 2). However, if canine SRP was present during this incubation, a trace amount of glycosylated pαF was observed (Figure 7B, lane 3, arrow). The identity of this band was confirmed by immunoprecipitation and cosedimentation with the membrane vesicles (not shown). In contrast, yRM were able to translocate pαF more efficiently when added posttranslationally to the wheat germ extract, both in the presence and in the absence of canine SRP (with SRP added posttranslationally) (Figure 7B, lanes 4 and 5), although the absolute amount of translocated pαF was less than that in the cotranslational incubation (Figure 7A, lane 6). The posttranslational translocation of pαF appears to occur efficiently only across yeast microsomal membranes; we conclude that this reflects a special (or at least significantly enhanced) property of the yeast translocation system.

Discussion

We have established an in vitro assay for the translocation of pαF across the lipid bilayer of microsomal membranes of the yeast S. cerevisiae. Similar assays were recently developed by Waters and Blobel (1986) and Rothblatt and Meyer (1986). To verify that translocation occurred with fidelity in vitro, we applied three independent criteria: acquisition of endoglycosidase H-sensitive core-oligosaccharides on pαF; resistance of pαF to proteolysis after having been sequestered inside the microsomal vesicles; and cosedimentation of the translocated products with the microsomal vesicles. pαF has a very well characterized biosynthetic pathway (Julius et al., 1984a, 1984b; Schekman, 1985) that closely resembles that of classical mammalian secretory proteins. We were therefore surprised to discover that the translocation of pαF across the yeast ER membrane can occur posttranslationally and thus does not require the strict coupling between protein synthesis and membrane translocation that is obligatory for mammalian secretory proteins.

To determine whether these differences are due to a special property of pαF per se or whether the translocation machineries in yeast and mammalian microsomal membranes function differently, we performed a series of heterologous cross mixing experiments using a wheat germ translation system cotranslationally supplemented with mammalian SRP and/or mammalian microsomal vesicles (Figure 7A). Like other presecretory proteins, cotranslational translocation of pαF across this membrane system strictly required targeting of pαF by the SRP–SRP receptor system. In contrast to other presecretory proteins, however, pαF was also able to cross mammalian microsomal membranes in an SRP-dependent reaction posttranslationally. In spite of the low efficiency of this reaction, we have to conclude that pαF differs from other presecretory proteins in this respect.

A variety of proteins can be secreted posttranslationally across the plasma membrane of prokaryotic cells. For example, this has been documented for β-lactamase both in vivo (Koshland and Botstein, 1982) and in vitro (Muller and Blobel, 1984). However, as for pαF, efficient translocation of β-lactamase across mammalian microsomal membranes required SRP and microsomal vesicles to be present during translation; no translocated β-lactamase was detected when translocation was assayed posttranslationally (Muller et al., 1982). To work efficiently, the mammalian translocation machinery requires that translation of both proteins be coupled to translocation. In contrast, pαF could be translocated across yeast microsomal membranes posttranslationally with an efficiency comparable to that observed cotranslationally. Posttranslational translocation also was independent of whether pαF was synthesized in the homologous yeast system or in the wheat germ extract; therefore, it is unlikely that the yeast translation system contributes specialized, soluble factors required for this process. yRM, however, have the ability to catalyze this process very efficiently.

It has been demonstrated that the "prepro"-region of pαF is sufficient to cause other, unrelated proteins to become translocated. DNA encoding the "prepro"-peptide of pαF has been fused to genes encoding many secretory proteins (for review see Smith et al., 1985), as well as cytoplasmic proteins (e.g. superoxide dismutase; P. Valenzuela, personal communication), and shown to cause secretion of the resulting fusion proteins by yeast in vivo. This strongly suggests that the machinery used for pαF translocation is not specific for this particular protein, but rather is sufficiently pliable to accept completely heterologous, even cytoplasmic, proteins. However, the demonstration that translocation of pαF can occur posttranslationally in vitro does not necessarily imply that this translocation mode is operating in vivo (or even in vitro if yRM are present during translation). Rather, the degree of coupling between protein synthesis and translocation may depend on the relative in vivo rates of the two respective processes and, if translocation is rapid, may result in a strictly cotranslational translocation. Our in vitro assay conditions, in which the system is artificially deprived of microsomal membranes, may have allowed us to uncouple the two processes. Therefore, our finding that pαF can be translocated posttranslationally does not necessarily suggest that the above mentioned fusion proteins (or other yeast secretory proteins that have not yet been tested) will show the same property.

This ability to uncouple protein synthesis from translocation allowed us to characterize the translocation reaction of pαF across yeast microsomal membranes in more detail. For example, the ribosome itself is not directly involved in this process. It follows directly that it cannot be energy from the elongation process itself that drives the nascent chain across the membrane. Rather, the posttranslational translocation reaction is an ATP-dependent process (Figure 6). Because the addition of nonhydrolyzable ATP analogues did not support pαF translocation it is likely, but not proven (see Results), that ATP hydrolysis is required. It is also noteworthy that no electrochemical potential across microsomal membranes has been detected, and that various ionophores have no effect on in vitro protein translocation in yeast (Figure 6) or higher eukaryotic systems (P. Walter, unpublished). This is in contrast to the prokaryotic plasma membrane (Bakker and Randall, 1984) and the mitochondrial envelope (Gasser et al., 1982; Pfanner and Neupert, 1985), in which posttranslational protein translocation is driven by a protonmotive force and a membrane potential, respectively, but seems to resemble protein import into chloroplasts, which also is an ATP-dependent process (Grossman et al., 1980).

Recently other evidence has been provided that mammalian microsomal membranes in principle are able to accept and translocate proteins posttranslationally. The cytoplasmic protein globin fused to signal peptide was translocated across canine rough microsomal membranes posttranslationally (Perara and Lingappa, 1985; Perara et al., 1986). This reaction was shown to be efficient, but required that the nascent chain still be associated with the ribosome for proper targeting. Mueckler and Lodish (1986) demonstrated that the amino-terminal domain of the glucose transporter (an integral plasma membrane protein) can also translocate across mam-

malian microsomal membranes posttranslationally, albeit very inefficiently. For these cases, as well as for pαF, the way in which a completely synthesized protein crosses the membrane remains obscure. pαF contains no significantly hydrophobic regions (other than a typical signal sequence) or amphipathic stretches that might suggest a direct interaction with the lipid bilayer. The protein may become actively unfolded (or somehow be prevented from folding during synthesis) and then be translocated as a linear polypeptide chain. Alternatively, the translocation machinery may be able to accept whole domains of prefolded proteins. The latter case would imply that the signal sequence causes a large but selective pore to form in the membrane that facilitates protein translocation.

In summary, it is unlikely that protein translocation in yeast is fundamentally different from that in mammalian cells. The apparent differences may result from the degree of coupling between translation, targeting, and translocation. In addition, certain proteins may retain the ability to be translocated after they have been completely synthesized, while others may strictly require translocation before their synthesis has advanced beyond some critical point. Cotranslational translocation could be necessary if, for example, a particular folding or oligomerization renders a protein incompatible with subsequent translocation. Alternatively, complete synthesis of certain proteins (such as potentially harmful nucleases or proteases) in the cytoplasmic compartment could be detrimental to the cell. Thus different proteins, for a variety of reasons, may have more or less stringent requirements for coupling of translation to translocation. We speculate that the translocation machinery in the ER membrane has evolved means (such as the postulated SRP-mediated elongation-arrest) to cope with such particular requirements.

Experimental Procedures

In Vitro Transcription

The plasmid pDJ100 (obtained from Dr. D. Julius) was constructed by ligation of BamHI linkers at the upstream HinfI site closest to the initiating ATG and to the downstream SalI restriction sites of the pαF coding sequence, and insertion of this fragment into the BamHI restriction site of the pSP65 vector (D. Julius, personal communication). The plasmid was linearized by digestion with XbaI and transcribed in vitro by SP6 phage RNA polymerase (Promega Biotech) (Krieg and Melton, 1984). The transcription was carried out in a 20 μl reaction containing 40 mM Tris-HCl (pH 7.5), 6 mM magnesium chloride, 2 mM spermidine, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM UTP, 0.1 mM GTP, 0.5 mM GpppG (PL-Pharmacia), 10 mM DTT, 1000 U/ml of human placental ribonuclease inhibitor, 0.1 mg/ml of linearized plasmid, and 500 U/ml of SP6 RNA polymerase. The reactions were incubated at 40°C for 60 min, and were stopped by phenol:chloroform extraction. The nucleic acids were ethanol precipitated and dissolved in 40 μl of water. One microliter of this solution was sufficient to obtain translation products that were easily visible after overnight exposures of the SDS polyacrylamide gels without fluorography.

Yeast In Vitro Translation Assay

Yeast translation extracts were prepared by a modification of the method described by Gasior et al. (1979). The ade6 pep4-3 MATa strain of S. cerevisiae (which contains reduced levels of vacuolar proteases; Hemmings et al., 1981) was grown in 4 liters of YEP medium (Mortimer and Hawthorne, 1969) containing 2% glucose to 1 OD_{600}/ml (which corresponds to 107 cells/ml). Cells were collected by centrifugation at 3000 × g for 5 min, washed with distilled water, and resuspended in 200 ml of 50 mM potassium phosphate (pH 7.5), 40 mM 2-mercaptoethanol, and 1.4 M sorbitol. Zymolyase 5000 (Kirin Brewery, Japan) was added to 50 μg/ml, and the suspension was incubated at room temperature for 1 hr. These conditions were found to cause optimal spheroplasting for this particular strain. Spheroplasts were harvested by centrifugation at 3000 × g for 5 min and resuspended in 400 ml of YM-5 (Hartwell, 1967) medium containing 0.4 M magnesium sulfate and incubated at room temperature for 90 min. The culture was cooled to 0°C. All the following procedures were performed at 4°C. Regenerated spheroplasts were harvested by centrifugation at 3000 × g for 5 min, washed by centrifugation in 1.4 M sorbitol, and resuspended in 16 ml of lysis buffer (20 mM Hepes/KOH [pH 7.5], 0.1 M potassium or ammonium acetate, 2 mM magnesium acetate, 2 mM DTT, and 0.5 mM PMSF). The suspension was homogenized with 10 strokes in a motor driven Potter homogenizer and centrifuged at 27,000 × g for 15 min in a Beckman Ti50 rotor. The supernatant was collected and centrifuged at 100,000 × g for 30 min after reaching speed in the same rotor. The resulting supernatant was passed over an 80 ml Sephadex G-25 gel filtration column equilibrated with the lysis buffer containing 20% glycerol. Fractions with absorbances of more than 20 A_{260} units/ml were pooled. The pooled peak (12 ml) was adjusted to 0.1 mM calcium chloride, and 300 U/ml of micrococcal nuclease was added. After 15 min of incubation at 20°C, EGTA was added to a final concentration of 1.8 mM. The extract was aliquoted and quick-frozen in liquid nitrogen and could be stored at −80°C for several months without loss of activity.

The translation reactions (20 μl), containing 40%–50% by volume of the yeast extract above, were incubated for 1 hr (except where noted) at 20°C with the following additions: 1 mM ATP, 80 μM GTP, 17.5 mM creatine phosphate, 30 μM of each of the 19 amino acids excluding methionine, 200 U/ml of human placental RNAase inhibitor, 2 mM putrescine, 0.2 mg/ml creatine phosphate kinase, 2.4 mM DTT, 3.1 mM magnesium acetate, 150 mM potassium or ammonium acetate, 20 mM Hepes/KOH (pH 7.5), 8 μM S-adenosyl methionine, and 75 mM sucrose, 500 μCi/ml ^{35}S-methionine (Amersham, 1000 Ci/mmol), in vitro transcribed mRNA, and 100 μg/ml yeast tRNA (Sigma).

Preparation of Yeast Microsomes

The same S. cerevisiae strain used for preparation of the translation extract was grown in YEP medium containing 2% glucose to 2–3 OD_{600}/ml. The cells from a 1 liter culture were spheroplasted, regenerated, and homogenized as described above, except that the lysis buffer was 4 ml of 20 mM Hepes/KOH (pH 7.5), 500 mM sucrose, 1 mM DTT, 3 mM magnesium acetate, 1 mM EGTA, 1 mM EDTA, 100 U/ml Trasylol, 0.5 mM PMSF, and 2 μg/ml each of pepstatin A, chymostatin, antipain, and leupeptin. The homogenate was centrifuged in half-filled tubes in a Beckman JS-13 swinging bucket rotor at 8000 rpm (10,000 × g) for 10 min to obtain the S-10 supernatant. The pellet was resuspended in 4 ml of the lysis buffer, homogenized with 10 strokes in a Potter homogenizer, and centrifuged under the same conditions. Both S-10 supernatants were pooled and recentrifuged as above. The sample (3 ml) was loaded on top of 18 ml of a solution containing 35% Percoll in homogenization buffer, but without the protease inhibitors. The samples were centrifuged at 29,000 rpm (76,000 × g) for 1 hr in the Ti50.2 rotor. Two turbid bands were visible within the generated Percoll gradient, the upper one (containing the ER, see Figure 3) was collected with a Pasteur pipette. Calcium chloride (1.2 mM) and micrococcal nuclease (1 U/A_{280} units) were added and the mixture was incubated at 20°C for 20 min. The digestion was terminated by addition of 0.5 mM EGTA. After the addition of an equal volume of 20 mM Hepes/KOH (pH 7.5), 250 mM sucrose, 50 mM EDTA, and 1 mM DTT, the sample was incubated on ice for 15 min. Aliquots of 1.4 ml were loaded on top of a two-step gradient containing 0.3 ml of 50% Percoll, 20 mM Hepes/KOH (pH 7.5), 250 mM sucrose, and 1 mM DTT at the bottom, overlaid with 0.4 ml of 20 mM, Hepes/KOH (pH 7.5), 500 mM sucrose, and 1 mM DTT. The gradients were centrifuged in a swinging bucket rotor (TLS 55) in a Beckman TL 100 ultracentrifuge at 46,000 rpm (140,000 × g) for 50 min. The turbid band on top of the Percoll cushion was collected. The sample was diluted with 20 mM Hepes/KOH (pH 7.5), 250 mM sucrose, 1 mM DTT to a final concentration of 25 A_{280} units/ml (measured in a 1% SDS solution). One liter of culture yielded approximately 150 μl of this suspension. Small aliquots were frozen in liquid nitrogen and stored at −80°C. The membranes could be thawed and refrozen at least once without detectable loss of activity.

Alkylation of yRM

yRM (1.5 units/μl) were incubated at 20°C for 30 min in the presence of 5 mM N-ethylmaleimide followed by the addition of dithiothreitol to a concentration of 10 mM. Control membranes were prepared by addition of the dithiothreitol prior to N-ethylmaleimide addition. The translocation activity of these membranes was unaffected by this treatment.

Posttranslational Analyses of Translation Products

Quantitation of Translation Products

The bands corresponding to pαF and glycosylated pαF were quantitated by densitometry of the autoradiogram and comparison to standards of known radioactivity. Calculations of absolute amounts of pαF were based on an endogenous methionine concentration of 1 μM in the translation mixture, as determined by isotope dilution (Walter and Blobel, 1981).

Endoglycosidase H Digestions

SDS and DTT were added to final concentrations of 2% and 75 mM, respectively, to 20 μl translation reactions followed by boiling for 5 min. The samples were diluted to 400 μl and digestions were carried out at 37°C for 12 hr in the presence of 100 mM sodium citrate (pH 5.5), 5 mM sodium azide, 0.15% SDS, 100 U/ml Trasylol, and 0.25 μg/ml of endoglycosidase H (New England Nuclear). The reactions were terminated by addition of TCA to 15% on ice, and the precipitated proteins were solubilized in SDS-PAGE sample buffer.

Protease Protection

Translation reactions (20 μl) were chilled in an ice water bath to 0°C, and calcium chloride was added to 10 mM. A solution of proteinase K (1 mg/ml) was preincubated for 15 min at 37°C in Tris-HCl (pH 7.5) and 10 mM calcium chloride to degrade contaminating lipases. Two microliters of this protease solution was added to the translation samples. The digestions were incubated at 0°C for 30 min. The reaction was stopped by the addition of 5 μl of 0.2 M PMSF in ethanol and immediately transferred to boiling SDS-PAGE loading buffer. Under these conditions protection efficiencies of translocated pαF varied from 70% to 90%, whereas unglycosylated pαF was not detectable (i.e. >98% digested).

Sedimentation Analysis

Potassium acetate was added to 500 mM to 20 μl translation reactions. The samples were layered on top of a 100 μl cushion containing 350 mM sucrose, 500 mM potassium acetate, and 2 mM magnesium acetate. Centrifugation was in a Beckman Airfuge at 30 psi for 5 min (A-110 rotor). The supernatant including the upper half of the cushion was carefully removed from the top and TCA precipitated. The pellet fraction was directly solubilized in loading buffer.

Alkaline Sodium Carbonate Extraction

Translation reactions were diluted 100-fold with ice-cold 100 mM sodium carbonate (pH 11.5). After a 30 min incubation at 4°C, samples were centrifuged for 30 min at 100,000 rpm (360,000 × g, TLA-100 rotor) in a Beckman TL-100 ultracentrifuge. Seventy percent of the supernatant was carefully removed from the top, neutralized with acetic acid, and TCA precipitated. The remainder of the supernatant in the tube was discarded. The visible pellet containing ribosomes and membrane remnants was directly solubilized in SDS-PAGE sample buffer.

Energy Depletion

In vitro protein synthesis was carried out for 60 min as described above, followed by addition of cycloheximide to a final concentration of 2 mM. The reaction was then centrifuge desalted at 4°C by two successive passages over 10 sample volumes of Sephadex G-25 fine (3 min at 1600 × g) equilibrated in 150 mM ammonium acetate, 20 mM Hepes/KOH (pH 7.4), 3.1 mM magnesium acetate, 2.4 mM DTT, 0.5% bovine serum albumin, 8% glycerol, 2.2 mM putrescine, 2 mM cycloheximide, 17 μg/ml aprotinin, and 2 μg/ml each of chymostatin, antipain, leupeptin, and pepstatin. Equivalent fractions of the centrifugal eluate were incubated for 60 min at 20°C in the presence of yRM (0.05 A_{280} units/15 μl) in the absence of energy substrates or after additions as described in the legend to Figure 6.

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