Secretory Protein Translocation in a Yeast Cell-free System Can Occur Posttranslationally and Requires ATP Hydrolysis

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Abstract. We describe an in vitro system with all components derived from the yeast *Saccharomyces cerevisiae* that can translocate a yeast secretory protein across microsomal membranes. In vitro transcribed prepro-α-factor mRNA served to program a membrane-depleted yeast translation system. Translocation and core glycosylation of prepro-α-factor were observed when yeast microsomal membranes were added during or after translation. A membrane potential is not required for translocation. However, ATP is required for translocation and nonhydrolyzable analogues of ATP cannot serve as a substitute. These findings suggest that ATP hydrolysis may supply the energy required for translocation of proteins across the endoplasmic reticulum.

THE first step in the secretion of proteins from eukaryotic cells, translocation of the secretory polypeptide across the rough endoplasmic reticulum (RER)[1] membrane, has been extensively investigated using cell-free, reconstituted systems (for review see reference 30). Rough microsomes (vesiculated derivatives of the RER) isolated from canine pancreas have been widely used as a source of translocation competent membranes (7). From these membranes, several components have been isolated and characterized: an 11 S ribonucleoprotein, termed signal recognition particle (SRP) (27, 29), its receptor in the RER, termed SRP receptor (14, 28) or docking protein (20), and more recently, signal peptidase, which was purified as a complex of several polypeptide chains (11). SRP and SRP receptor serve in the targeting of proteins to the RER membrane (for summary see reference 30). How the polypeptide is then translocated across the membrane is not known. Models have been proposed for translocation to proceed either directly through the lipid bilayer (10, 26, 32) or through a proteinaceous pore (5, 6). Recent data suggest a role for proteins in this process (11, 13).

Despite the progress that has been made with the biochemical analysis of the translocation system of canine pancreas microsomal membranes, it is desirable to extend these studies to another eukaryotic system that could be genetically manipulated (23). As a first step in this direction we describe here an in vitro system, reconstituted entirely from yeast components, that translocates and glycosylates the yeast secretory protein prepro-α-factor. Translocation can occur posttranslationally in this system. Uncoupling of the translation and translocation steps allowed us to study the translocation process alone. In contrast to translocation in bacteria, we found that a membrane potential is not required for translocation. However, ATP is needed for translocation and nonhydrolyzable analogues of ATP, such as 5′-adenylimidodiphosphate (AMP-PNP), cannot serve as a substitute. These results suggest that ATP hydrolysis may supply the energy for protein translocation across the endoplasmic reticulum.

Materials and Methods

Materials

SP6 Transcription

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1. *Abbreviations used in this paper:* AMP-PNP, 5′-adenylimidodiphosphate; DTT, dithiothreitol; Endo H, endo-β-N-acetylglucosaminidase H; RER, rough endoplasmic reticulum; SRP, signal recognition particle; S100, 100,000 g$_{av}$ supernatant; S100-G25, S100 sieved through Sephadex G-25; YPD medium, 1% yeast extract, 2% peptone, 2% dextrose.

ethanol, and then LiCl precipitations. The concentration, as judged by A_{260}, was adjusted to 100 ng/µl.

Subfractionation of Yeast Spheroplasts

Cell growth, collection, and spheroplast preparation were done at room temperature (25°C–27°C). *S. cerevisiae*, strain SKQ2N, was grown in 1% yeast extract, 2% peptone, 2% dextrose (YPD medium) to an A_{660} of 1.5–2.0. For a typical preparation we used six 3-liter batches in 6-liter Erlenmeyer flasks. The cells were concentrated with a Millipore pellicon cassette system to ~2 liters, and then collected by centrifugation in a Sorvall GS3 rotor at 4,200 rpm (3,000 g) for 5 min. The supernatant was decanted and the cells washed with ¹⁄₂₀th of the original culture volume of YPD medium. The cells were collected as above and weighed. The average yield ranged from 4.2 to 5.6 g/liter depending on the A_{660} of the culture.

For the preparation of spheroplasts, the cells were resuspended in YPD medium that contained 1 M sorbitol (YPD/sorbitol) to a final concentration of 0.3 g/ml. The suspension was adjusted to pH 7 with 5 N NaOH, using pH test papers as indicators. Dithiothreitol (DTT) was added to a final concentration of 10 mM and the suspension incubated for 5 min. Zymolyase 100T was then added to 0.125 mg/ml and incubation was continued for 15 min. The spheroplasts were harvested as above, washed with ¹⁄₂₀th culture volume of YPD/sorbitol to remove zymolyase, and collected again. The spheroplasts were allowed to recover for 1 h by incubation in ¹⁄₂₀th culture volume of YPD/sorbitol.

All manipulations hereafter were done at 4°C. The spheroplasts were collected, washed with ¹⁄₂₀th culture volume of 1 M sorbitol, harvested, resuspended in buffer A (20 mM Hepes-KOH, pH 7.4/100 mM KOAc/2 mM Mg(OAc)₂/2 mM DTT) (added 0.5 ml/g of cells), and lysed in a Dounce homogenizer by 40 strokes with the "A" pestle. The lysate was centrifuged in a Sorvall SS34 rotor at 15,500 rpm (30,000 g) for 15 min. The supernatant was decanted and centrifuged in a Beckman Ti50.2 rotor at 33,000 rpm (100,000 g_{av}) for 30 min after reaching speed. After centrifugation the preparation had a distinctive appearance. At the bottom of the tube was a well packed transparent pellet. Above this pellet was a layer of dense flocculent material that occupied ~¹⁄₁₀th of the tube, followed by a clear supernatant with a thin layer of turbid material on top, presumably lipid. The lipid layer was withdrawn and discarded. The clear supernatant zone, referred to as S100, was collected without disturbing the flocculent material at the bottom of the tube. The A_{260} of the S100 used for the work described here was 189. We obtained ~0.35 ml of S100 per gram of cells. The S100 was then sieved (see below) and used for translation. The flocculent material and the pellet were used to prepare membranes for translocation.

The S100 was passed through columns that contained 10 ml Sephadex G-25 medium equilibrated with buffer A. Tapered 12-ml Bio-Rad Econo-columns were placed in plastic tubes such that the outlet was ~3 cm from the bottom. The columns were loaded with 0.5 ml of S100 and centrifuged in a Sorvall RT6000 refrigerated tabletop centrifuge at ~2,000 rpm for 1 min. The material, referred to as S100-G25, was pooled, frozen in 200-µl aliquots in liquid nitrogen, and stored at −80°C. There was no significant loss of translation activity after two freeze/thaw cycles.

The procedures described above are similar to those of Gasior et al. (12, 21) but differ in several respects. The cells are grown in YPD medium instead of YM-1 medium to about twice the A_{660}. Before lysis the cells are constantly exposed to YPD media instead of water or sorbitol alone, thereby avoiding starvation. For the same reason we have spheroplasted the cells with zymolyase in YPD/sorbitol, while Gasior et al. have used glusulase in sorbitol alone. In addition, our recovery step was done in YPD/sorbitol instead of YM-5 with 0.4 M MgSO₄.

Preparation of Yeast Microsomal Membranes

The pellet and the flocculent zone from the 100,000 g_{av} centrifugation step were homogenized by five strokes in a small Dounce homogenizer. About 5 vol of buffer B (50 mM triethanolamine acetate, pH 7.5/1 mM DTT) were added, and the suspension was overlayed on a cushion of buffer B that contained 14% glycerol (load/cushion, 3:1). Centrifugation was done in a Beckman Ti50.2 rotor at 41,000 rpm (150,000 g_{av}) for 1 h. The supernatant and as much of the cushion as possible were removed without disturbing the loose pellet. The pellet was resuspended in the remaining cushion by five strokes in a small Dounce homogenizer. The optical density was measured in 1% SDS and the concentration adjusted to 250 A_{280} units with buffer B that contained 14% glycerol, yielding crude microsomal membranes at a concentration of 5 equivalents (eq)/µl as previously defined (27). The yield from the membrane preparation used in this work was ~0.74 ml/g of cells.

To degrade mRNA that would contribute to background protein synthesis upon addition of membranes to translations, we treated the membranes with Staphylococcal nuclease. 2 µl of 10,000 U/ml nuclease in 100 mM CaCl₂ were added to 200 µl of 5 eq/µl microsomal membranes. The material was incubated at 20°C for 10 min. To terminate the digestion, 4 µl of 100 mM EGTA were added, yielding nuclease-treated microsomal membranes at 5 eq/µl. Before use in translations, the membranes were diluted to the appropriate concentration with buffer B that contained 14% glycerol.

Yeast Translation

A 70-µl aliquot of S100-G25 was incubated for 10 min at 20°C with 7.7 µl of 2,000 U/ml Staphylococcal nuclease in 9.7 mM CaCl₂. After incubation, 6.3 µl of 35.7 mM EGTA were added to stop the digestion. For each S100-G25 preparation the concentration of nuclease was titrated such that the background protein synthesis was reduced, but the system still gave at least twofold stimulation of [³⁵S]methionine incorporation upon addition of prepro-α-factor mRNA (as judged by trichloroacetic acid insoluble cpm).

A "master mix" was prepared which contained per 25 µl translation reaction: 0.38 µl of water, 2.50 µl of compensation buffer (154.3 mM Hepes-KOH, pH 7.5/1.267 M KOAc/25.34 mM Mg(OAc)₂/2.34 mM DTT), 0.50 µl of 0.1% Nikkol, 0.30 µl of protease inhibitor mix (pepstatin A 25 µg/ml, chymostatin 25 µg/ml, antipain 25 µg/ml, leupeptin 25 µg/ml, Trasylol 2500 U/ml), 0.60 µl of 0.1 A_{280}/ml human placental RNase inhibitor, 0.60 µl of 10 mg/ml deacylated yeast tRNA, 2.00 µl of "energy mix" (0.5 mM of each amino acid except methionine/6.25 mM ATP/1.25 mM GTP/312 mM creatine phosphate/29 mM DTT), 0.63 µl of 8 mg/ml creatine kinase, and 1.50 µl of [³⁵S]-methionine of the highest specific activity available. The recipe for the "master mix" was multiplied by the appropriate factor depending on the number of translations to be done.

Each translation reaction contained 7.0 µl of nuclease-treated S100-G25, 9.0 µl of "master mix", and water and/or other components (see below) to 24 µl. The reactions were started by addition of 1 µl (100 ng) of mRNA. Incubations were done for 1 h at 20°C. The reactions were stopped by chilling on ice, and 15-µl aliquots were prepared for SDS PAGE.

This translation protocol is modified from Gasior et al. (12, 21). The final conditions are as previously described (12, 21), except that we have added RNase inhibitor, protease inhibitors, and the non-ionic detergent Nikkol to a final concentration of 0.002%. Nikkol was included at low concentration to stabilize putative yeast SRP, by analogy to stabilization of canine SRP by Nikkol (27). RNase inhibitor and protease inhibitors were added as prophylactic measures against degradation. We have not tested the effect of omission of these components from the system.

Cotranslational Translocation

Instead of water, 2 µl of the appropriate concentration of nuclease-treated microsomal membranes or of buffer B that contained 14% glycerol were added to translations before starting the reaction with mRNA.

Posttranslational Translocation

After completion of translation, 1 µl of 50 mM cycloheximide was added to a 15-µl aliquot of a translation reaction and the reaction placed on ice. Water (or other components), and then up to 8 µl of the appropriate concentration of nuclease-treated microsomal membranes, were added so that the final volume was 25 µl. The reaction was then incubated at 20°C for 30 min. The reaction was stopped by chilling on ice and the entire reaction prepared for SDS PAGE.

Protease Protection

3 µl of 8.0 mM CaCl₂ were added to a 15-µl aliquot of a translation reaction. 3 µl of water or 8% (wt/vol) Triton X-100 were then added to either leave intact or destroy the membrane barrier, respectively. 3 µl of 800 µg/ml trypsin were then added and the reaction incubated on ice for 30 min. To terminate the digestion, 3 µl of 50 mM phenylmethylsulfonyl fluoride in dimethyl sulfoxide were added, followed by a 10-min incubation on ice. The entire reaction was then prepared for SDS PAGE. For analysis of posttranslationally translocated products a 25-µl aliquot was used (instead of 15 µl) and therefore all subsequent volumes were increased accordingly to maintain the same final conditions.

Endo H Digestion

1.7 µl of 10% SDS/0.5 M Tris-HCl, pH 7.4/0.5 M DTT was added to a 15-µl aliquot of a translation reaction. After boiling for 2 min, 33.3 µl of 0.3 M Na

citrate, pH 5.5 and then 2.5 μl of 20% Trasylol/20 mM phenylmethylsulfonyl fluoride/4 mM L-1-tosylamido-2-phenylethylchloromethyl ketone were added. 3 μl of either water or a 1 U/ml solution of Endo H were added and the reaction incubated for 18 h at 37°C. The entire reaction was then prepared for SDS PAGE.

Energy Requirements for Posttranslational Translocation

To study the effect of ionophores on translocation, standard posttranslational assays were done, except that before addition of membranes 2 μl of three concentrations of several ionophores were added. A stock solution of 12.5 mM carbonyl cyanide m-chlorophenyl hydrazone in absolute ethanol was diluted to obtain solutions of 1.25 mM, 125 μM, and 12.5 μM in 10% ethanol. Stock solutions of 6.25 mM valinomycin or monensin in absolute ethanol were diluted to obtain solutions of 625 μM, 62.5 μM, and 6.25 μM valinomycin or monensin in 10% ethanol. 10% ethanol was used as a control in the posttranslational assay.

Demonstration of the ATP dependence of translocation was done with potato apyrase (see Fig. 4, legend). This enzyme hydrolyzes ATP to ADP and P_i, and ADP to AMP and P_i (17). Apyrase was obtained as material that was partly protein and partly salts of potassium succinate, pH 6.5. Using data supplied from the manufacturer, a solution of 1.25 U/μl was calculated to contain ~30 mM potassium succinate, pH 6.5. Therefore, in control reactions without apyrase, the appropriate buffer was used to maintain identical conditions.

To determine if hydrolysis of ATP was required for translocation, we used gel filtration to remove small molecules from the translation before the posttranslational addition of membranes and potential energy supplying compounds. A 500-μl translation was passed through a 10-ml Sephadex G-15 column equilibrated in 20 mM Hepes-KOH, pH 7.5/150 mM KOAc/3 mM Mg(OAc)₂/0.1 mM EGTA/0.002% Nikkol. Fractions of 0.22 ml were collected and a 5-μl aliquot of each was used to determine trichloroacetic acid precipitable cpm. The three fractions with the highest cpm, which eluted with the void volume, were pooled. 15 μl of this material was used in standard posttranslational translocation assays, except that 2 μl of solutions of various compounds (ATP, GTP, CTP, UTP, dATP, creatine phosphate, AMP-PNP, α-β-methyleneadenosine 5′-triphosphate, and β-γ-methyleneadenosine 5′-triphosphate) were added to test for restoration of translocation. All compounds were prepared as 100 mM stock solutions in water, neutralized with KOH just before use, and then diluted to the appropriate concentration.

SDS PAGE

12% separating gels with 5% stacking gels were used throughout. The material in all lanes was ultimately derived from 15 μl of a translation reaction regardless of subsequent manipulations. Samples were precipitated by adding an equal volume of ice cold 20% trichloroacetic acid and incubating on ice for 15 min. The precipitates were collected by centrifugation in a microfuge for 5 min at 4°C. The samples were resuspended in 20 μl of 0.5 M Tris base/5% SDS by incubation at 50°C for 30 min. Finally, 15 μl of 40% glycerol/200 mM DTT/0.002% bromphenol blue were added and the samples boiled for 5 min.

After electrophoresis the gels were fixed in 35% methanol/10% acetic acid, treated with Enlightning, dried, and exposed to preflashed Fuji RX x-ray film for 16–48 h at −80°C.

The standards used to calculate the M_r of the translation products were: cytochrome C, 12.3 kD; soybean trypsin inhibitor, 21.5 kD; carbonic anhydrase, 30 kD; ovalbumin, 43 kD; pyruvate kinase, 57 kD; bovine serum albumin, 68 kD; and phosphorylase A, 94 kD.

Results

In vitro translation of 100 ng of SP6 derived prepro-α-factor mRNA in the yeast cell free system yielded about threefold to sixfold stimulation of [³⁵S]methionine incorporation into protein over the minus mRNA control (data not shown). Analysis of the reactions by SDS PAGE showed that background protein synthesis was low (Fig. 1A, lane 1) and that addition of mRNA produced one major product that migrated at 19 kD in our gel system (Fig. 1A, lane 2), consistent with the known M_r of prepro-α-factor of 18,580 (18). In addition to the major product there was also a minor mRNA-specific product that migrated slightly slower, at ~20 kD. This product was probably due to a small amount of microsomal membrane contamination in the translation system (see below).

Addition of yeast microsomal membranes to the translation system yielded three more polypeptides of slower mobilities than the primary translation product (Fig. 1A, lanes 3–6). The polypeptides have molecular masses of 24, 27, and 32 kD. In addition, the minor 20-kD polypeptide that was pres-
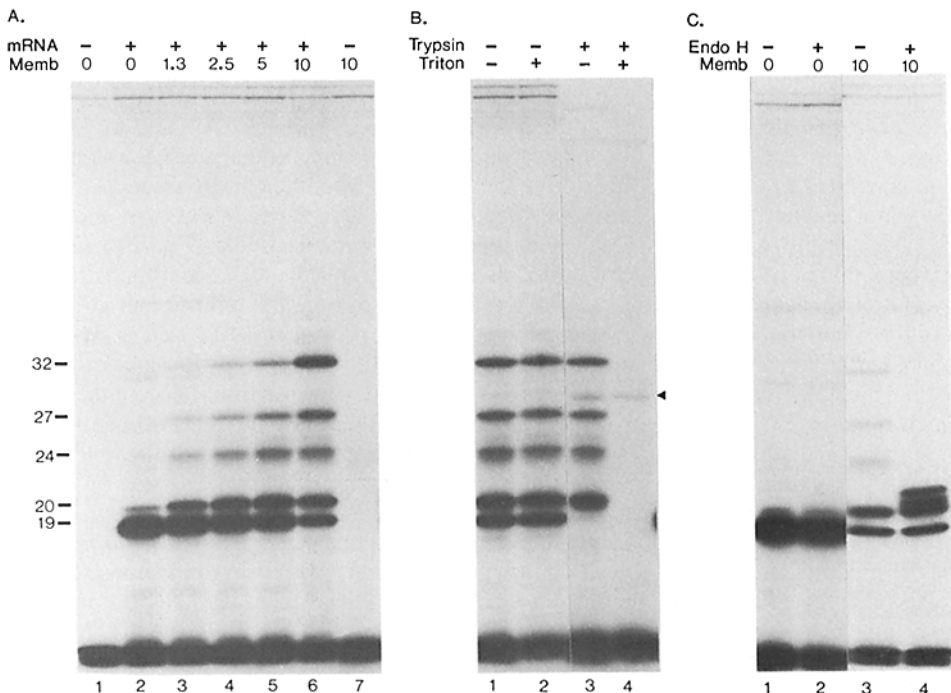

Figure 1. In vitro translation, translocation, and core glycosylation of prepro-α-factor. (A) Translations were done as described in Materials and Methods except that the final reaction volumes were 65 μl and the reactions were supplemented with buffer B (lanes 1 and 2) or rough microsomes at the indicated concentrations (lanes 3–7). The apparent M_r (kD) of the polypeptides are shown at the left. (B) Membranes were added to a translation at 10 eq/25 μl. After incubation aliquots were subjected to the protease protection protocol described in Materials and Methods. The final trypsin concentration was 100 μg/ml and that of Triton X-100 was 1% (wt/vol). The arrowhead indicates a degradation product from a high M_r product (see text). (C) Aliquots of the same reactions used in Fig. 1A, lanes 2 and 6, were either incubated in the presence or absence of Endo H as described in Materials and Methods. The arrowheads indicate new bands appearing after Endo H digestion of the 32- and 27-kD products. All membrane concentrations are expressed as eq/25 μl of reaction.

ent without added membranes (Fig. 1 A, lane 2) became more prominent when membranes were supplemented (Fig. 1 A, lanes 3-6). The relative abundance of the five polypeptides was dependent on the microsome concentration (Fig. 1 A, lanes 3-6). At low concentration (1.3 eq/25 μl) the low M_r polypeptides were abundant, whereas at high membrane concentration (10 eq/25 μl) the 32-kD polypeptide was one of the major products. Due to nuclease treatment of the membranes there is no significant increase in background protein synthesis upon addition of even high concentrations of membranes (Fig. 1 A, lane 7). Without nuclease treatment background protein synthesis can be quite high leading to a reduction in prepro-α-factor synthesis (data not shown).

To investigate whether any of the translation products were sequestered in microsomes we did protease protection experiments (Fig. 1 B). We found that all of the membrane-specific polypeptides, that is, the four highest M_r species, were protected from degradation by trypsin (Fig. 1 B, lane 3). The primary translation product was trypsin sensitive. Addition of the non-ionic detergent Triton X-100 to destroy the integrity of the membrane barrier resulted in proteolysis of the formerly protected proteins (Fig. 1 B, lane 4). The new band that appeared after addition of protease probably represents a degradation product of a higher M_r polypeptide from background protein synthesis (Fig. 1 B, lanes 3 and 4, arrowhead). These data suggest that all the membrane-specific polypeptides are sequestered in intact microsomal vesicles.

It has previously been shown that prepro-α-factor has three potential asparagine-linked glycosylation sites (18) and an uncleaved signal sequence (15). To determine if any of the high M_r membrane-specific polypeptides were glycosylated we did translation reactions in the presence or absence of microsomes and then digested aliquots with Endo H (Fig. 1 C). This endoglycosidase has been shown to remove the core oligosaccharide units from asparagine-linked glycoproteins, leaving one N-acetyl glucosamine residue on the protein backbone (24). The results indicated that the 32-, 27-, and 24-kD polypeptides contain asparagine-linked oligosaccharides because the corresponding bands disappeared after Endo H treatment (Fig. 1 C, lane 3). Concurrently two new bands appeared at 21 and 22 kD (Fig. 1 C, lane 4, arrowheads). We believe that the 32-kD polypeptide was converted to the 22-kD polypeptide by removal of three core oligosaccharide units, leaving three asparagine-linked N-acetyl glucosamine residues. Likewise, the 27-kD product, containing two asparagine-linked oligosaccharide chains, was probably converted to the 21-kD product. Finally, we believe that the 24-kD polypeptide contains only one core oligosaccharide unit, and that its removal by Endo H results in a polypeptide with only one N-acetyl glucosamine residue which migrates at 20 kD. Since none of the products of Endo H digestion migrate faster than the primary translation product, our results confirm that the signal sequence of prepro-α-factor is not removed (15).

The 20-kD band, whose synthesis is stimulated by addition of membranes, most likely represents a modified form of translocated prepro-α-factor (Fig. 1 C, lane 3; see also Fig. 1 A, lanes 3-6). The polypeptide appears to be unaffected by Endo H treatment (Fig. 1 C, lane 2).

A phenomenon that deserves comment is an imbalance in the substrate/product ratios upon Endo H treatment. For example, the 22-kD product of Endo H digestion (Fig. 1 C,

lane 4) appears to be more abundant than the corresponding 32-kD substrate (Fig. 1 C, lane 3). It is possible that the observed imbalance is due to trimming of terminal sugar residues (22) or to transfer of incompletely assembled oligosaccharides (25). As a result there could be a number of less abundant, more or less trimmed products that migrate slower or faster than the 32-kD band. In either case, digestion with Endo H converts the variously trimmed polypeptides to only one product (24), allowing co-migration upon electrophoresis and subsequently a more intense band after fluorography. The same explanation would apply to the imbalance between the 27-kD (Fig. 1 C, lane 3) and the 21-kD (Fig. 1 C, lane 4) bands. It should also be noted that the intensity of the glycosylated bands after incubation in the absence of Endo H (Fig. 1 C, lane 3) is considerably reduced compared to the corresponding bands in the substrate material (Fig. 1 A, lane 6). The reason for this is not clear.

The corresponding three glycosylated forms of prepro-α-factor, produced by translocation into canine pancreas microsomal vesicles (from a wheat germ translation system), migrated slower, by ~1-3 kD (data not shown), than their counterparts translocated into yeast microsomes (from a yeast translation system). This provides support for the notion that either trimming or transfer of incompletely assembled core sugars occurs in yeast microsomes.

To investigate whether translocation occurs coupled to translation, as it does, by and large, in systems derived from higher eukaryotic cells, we did the following experiment (Fig. 2). Two translation reactions were prepared, the first lacked mRNA (Fig. 2 A, lane 1) and the second contained mRNA (Fig. 2 A, lane 2). These were incubated for 60 min, chilled, and a portion of each divided into four aliquots. The first aliquot from each reaction received no addition (Fig. 2 B, lanes 1 and 5), the second received cycloheximide (Fig. 2 B, lanes 2 and 6), the third aliquot received microsomal membranes (Fig. 2 B, lanes 3 and 7), and the fourth cycloheximide and microsomal membranes (Fig. 2 B, lanes 4 and 8). Finally, the aliquots from the minus mRNA reaction received mRNA (Fig. 2 B, lanes 1-4) while the samples from the plus mRNA reaction received water (Fig. 2 B, lanes 5-8), and the incubation was continued for 30 min. When mRNA was present only during the second incubation a small amount of translation still occurred (Fig. 2 B, lane 1) but was effectively inhibited by addition of cycloheximide (Fig. 2 B, lane 2). Addition of membranes under the same conditions also inhibited the small amount of translation that occurred during the second incubation (Fig. 2 B, lane 3), and served as a control that showed that introduction of ribosomes with the membranes did not stimulate translation. Because the translation was already negligible in the presence of membranes, addition of cycloheximide did not have a noticeable effect (Fig. 2 B, lane 4). For reactions that had mRNA present during the first incubation there was no significant translation during the second incubation (compare Fig. 2 B, lane 5 to Fig. 2 A, lane 2), confirming the previous result. Since translation in the second incubation was negligible, the addition of cycloheximide had no apparent effect (Fig. 2 B, lane 6). The presence of membranes during the second incubation resulted in the translocation of prepro-α-factor synthesized in the first incubation (Fig. 2 B, lanes 7 and 8), indicating that in vitro translocation of prepro-α-factor into yeast microsomes can
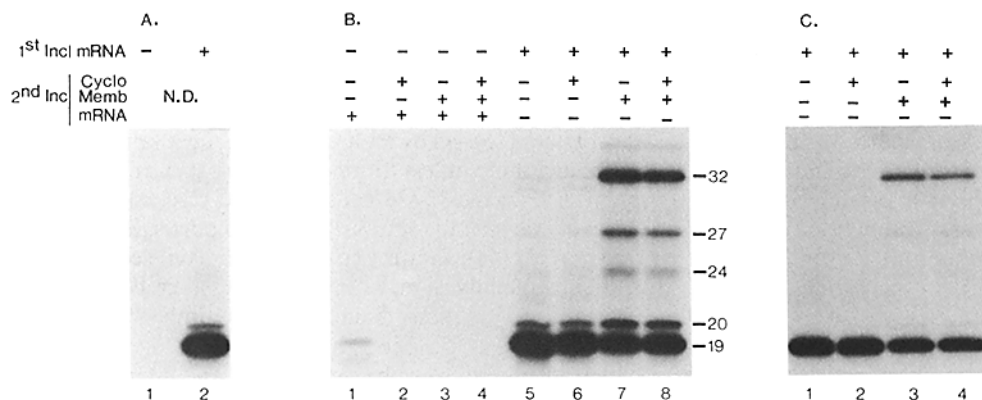
occur posttranslationally.

We have also shown that posttranslational translocation occurs in this system by using differential centrifugation to remove the ribosomes that synthesized the prepro-α-factor in the first incubation. Addition of membranes to a ribosome-free supernatant from a translation that had synthesized prepro-α-factor during the first incubation (Fig. 2A, lane 2) resulted in translocation of the polypeptide (Fig. 2C, lanes 3 and 4), again demonstrating posttranslational translocation.

As expected, the amount of posttranslationally translocated product obtained is proportional to the membrane concentration (Fig. 3A, lanes 1–7). As is the case for cotranslational translocation, the glycosylated products and the membrane specific 20-kD polypeptide are resistant to externally added trypsin (Fig. 3B, lane 2), whereas the primary translation product is almost totally degraded. The presence of 1% Triton X-100 during the trypsin digestion resulted in complete degradation of the formerly protected products (Fig. 3B, lane 3).

Since it was possible to uncouple translation from translocation we were able to investigate the energy requirements for translocation. The possible role of a membrane potential was tested by using various ionophores. The protonophore carbonyl cyanide m-chlorophenyl hydrazone (from 1 μM to 100 μM), the potassium ionophore valinomycin (from 0.5 μM to 50 μM), and the calcium ionophore monensin (from 0.5 μM to 50 μM) all had no effect on posttranslational translocation (data not shown). These results suggested that a potential across the membrane was not required for translocation. However, ATP was required because enzymatic degradation of ATP with apyrase before the addition of membranes completely abolished translocation (Fig. 4A, lanes 1 and 2). This result, however, did not rule out the possibility that ATP was required for glycosylation but not for translocation. If this were the case, apyrase treatment would be expected to result in a significant amount of translocated, nonglycosylated product which would be resistant to trypsin degradation. We found however, that trypsin digestion of an apyrase-treated reaction resulted in almost complete degradation of the primary translation product (Fig. 4A, lane 3), indicating that the translocation step required ATP. (If ATP was needed for both translocation and glycosylation, the same result would be
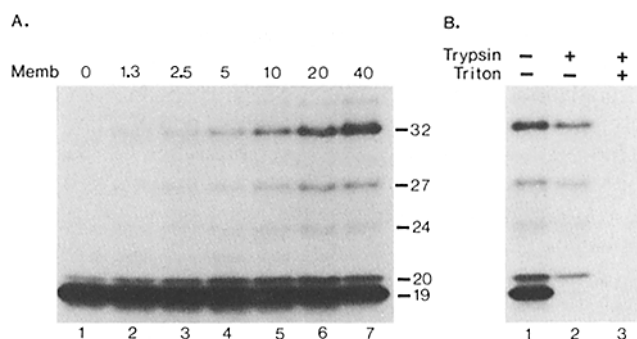

Figure 3. The amount of posttranslationally translocated product is dependent on membrane concentration. (A) Posttranslational translocation, with the indicated concentration of membranes, was done as described in Materials and Methods. The apparent M_r (kD) of the polypeptides are shown at the right. (B) The membrane concentration used was 20 eq/25 μl. The protease protection protocol is described in Materials and Methods.

expected. Therefore our data does not rule out the possibility that ATP was also required for glycosylation.) The small amount of trypsin-resistant primary translation product (Fig. 4A, lane 3) was probably due to aggregated polypeptide (rather than sequestered polypeptide) because this material remained resistant even in the presence of Triton X-100 (Fig. 4A, lane 4). The 20-kD product that was protected from degradation (Fig. 4A, lane 3) in the absence (but not in the presence) of detergent, was probably translocated before apyrase treatment into the small amount of membranes that contaminated the system (see Fig. 1A, lane 2).

To confirm the result that ATP is required for translocation, and to rule out the possibility that a contaminant in the apyrase preparation (for example, a protease) was abolishing translocation, we attempted to restore translocation by readdition of ATP. Since no specific inhibitor of apyrase was available we did the following experiment. Synthesis of prepro-α-factor was terminated with cycloheximide and the reaction was either treated with apyrase (Fig. 4B, lanes 6–10) or a mock treatment was done (Fig. 4B, lanes 1–5). A low concentration of apyrase (10-fold lower than in Fig. 4A) was chosen so as to eliminate most of the subsequent translocation
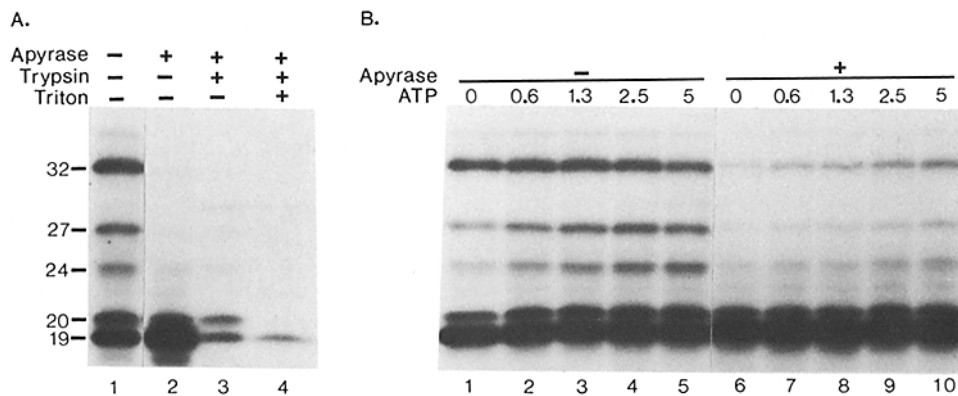
A.

Apyrase	−	+	+	+
Trypsin	−	−	+	+
Triton	−	−	−	+

32—
27—
24—
20—
19—

1 2 3 4

B.

| Apyrase | | − | | | | | + | | | |
| ATP | 0 | 0.6 | 1.3 | 2.5 | 5 | 0 | 0.6 | 1.3 | 2.5 | 5 |

1 2 3 4 5 6 7 8 9 10

Figure 4. Translocation across the microsomal membrane requires ATP. (*A*) A translation was done and four 15-μl aliquots prepared. The reaction in each aliquot was stopped by addition of 1 μl of 50 mM cycloheximide. One reaction then received 1 μl of 30 mM potassium succinate, pH 6.5 and the other three received 1 μl of 1.25 U/μl of apyrase (see Materials and Methods). The reactions were then incubated at 20°C for 5 min. 4 μl of water or nuclease-treated yeast membranes were then added and the standard posttranslational assay was done, after which the reactions were subjected to the protease protection protocol described in Materials and Methods. The membrane concentration was 20 eq/25 μl. The apparent M_r (kD) of the polypeptides are shown at the left. (*B*) To each of ten 15-μl aliquots of translations 1 μl of 50 mM cycloheximide was added, followed by 1 μl of either 0.125 U/μl of apyrase in 3 mM potassium succinate or the buffer alone. The reaction was incubated at 20°C for 5 min. 4 μl of nuclease-treated microsomes was then added followed by 4 μl of either water or 3.9, 7.8, 15.6, or 31.3 mM ATP in water. The standard posttranslational assay was then done. The final membrane concentration was 20 eq/25 μl. The final ATP concentration (mM) is shown.
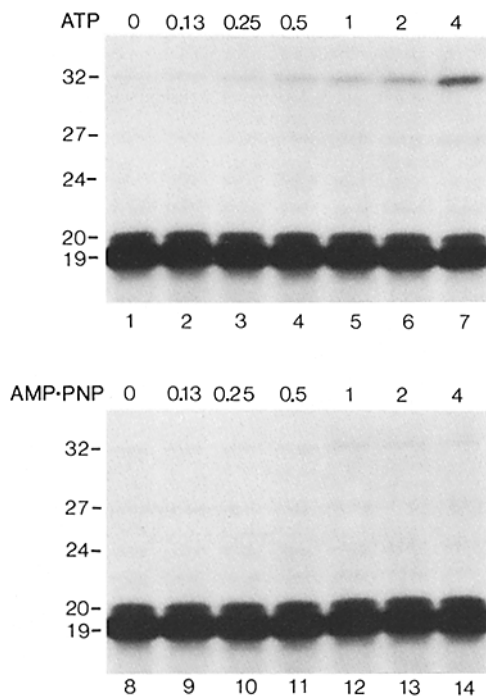

| ATP | 0 | 0.13 | 0.25 | 0.5 | 1 | 2 | 4 |

32—
27—
24—
20—
19—

1 2 3 4 5 6 7

| AMP·PNP | 0 | 0.13 | 0.25 | 0.5 | 1 | 2 | 4 |

32—
27—
24—
20—
19—

8 9 10 11 12 13 14

Figure 5. ATP hydrolysis is required for posttranslational translocation of prepro-α-factor. ATP was removed after completion of translation by sieving the translation through Sephadex G-25. The ^{35}S-protein peak was pooled and used in a posttranslational translocation assay. ATP or AMP-PNP was supplied at the indicated final concentrations (mM). The membrane concentration was 40 eq/25 μl. The apparent M_r (kD) of the polypeptides are shown at the left.

(Fig. 4*B*, lane 6). After the apyrase or mock treatment, membranes and various concentrations of ATP were added and incubated for posttranslational translocation. Addition of up to 2.5 mM ATP to the mock-treated reactions caused a small increase in translocation (Fig. 4*B*, lanes *1–3*, 24- and 27-kD products), indicating that the amount of residual ATP from the translation was limiting for translocation. 5 mM ATP caused a slight inhibition of translocation, probably due to acidification of the reaction by ATP (Fig. 4*B*, lane *5*). When ATP was restored to the apyrase-treated reactions the amount of translocation was proportional to the final ATP

concentration (Fig. 4*B*, lanes *6–10*). Complete restoration of translocation to the original level cannot be expected because the apyrase was still active during the translocation reaction. These data suggest that translocation requires ATP in this cell-free system.

To determine whether hydrolysis of ATP, or simply the presence of ATP, was required for translocation, we removed ATP from a translation by gel filtration, and then added back either ATP or the nonhydrolyzable analogue AMP-PNP. Incubation of the sieved, ATP-depleted, translation with membranes yielded no translocation. Re-addition of ATP to the posttranslational translocation reaction resulted in translocation of prepro-α-factor. The amount of translocated product was proportional to the ATP concentration (Fig. 5, lanes *1–7*). This finding confirms the result with apyrase. When the nonhydrolyzable ATP analogue AMP-PNP was added to the reaction, no translocation above background was detected (Fig. 5, lanes *8–14*). Likewise, the nonhydrolyzable analogues α,β-methyleneadenosine 5′-triphosphate and β,γ-methyleneadenosine 5′-triphosphate did not restore translocation (data not shown). In addition, GTP, CTP, UTP, dATP, or creatine phosphate did not stimulate translocation (data not shown). These results suggest that hydrolysis of ATP is required for the posttranslational translocation of prepro-α-factor into microsomes in vitro.

Discussion

We have developed an efficient cell-free system for the translocation of secretory proteins, with all components derived from yeast. A Staphylococcal nuclease-treated supernatant fraction from yeast served as a source of ribosomes and translation factors. Prepro-α-factor mRNA was generated by in vitro transcription of the gene and was used to program translation of a yeast secretory protein. A microsomal membrane fraction from yeast added to the translation system yielded translocation of prepro-α-factor accompanied by core glycosylation at all the glycosylation sites. The extent of translocation and glycosylation depended on the concentration of added microsomal membranes. At high membrane concentration a high proportion of prepro-α-factor molecules synthesized were translocated and fully core glycosylated.

In contrast to similar cell-free translocation systems derived from higher eukaryotic cells where translation and translocation appear to be obligatorily coupled (30, for exception see 31), translocation in the yeast cell-free system can occur posttranslationally, at least for prepro-α-factor. As yeast, like higher eukaryotic cells, contain an RER, the close physical association of the ribosome with the ER membrane suggests that at least some translocation is occurring co-translationally in vivo. Therefore, posttranslational translocation in yeast may be facultative, not obligatory. It is possible that some yeast secretory proteins can posttranslationally translocate whereas others cannot, and that the proteins that can post-translationally translocate can also traverse the membrane co-translationally.

Since prepro-α-factor can be posttranslationally translocated in our in vitro system, a direct interaction between the yeast ribosome and the ER (via a putative ribosome receptor) is not required. The results, however, do not rule out that a ribosome receptor is involved in translocation in vivo. The existence of a ribosome receptor in the ER has been surmised based on disassembly (1) and cross-linking (16) studies with rough microsomes from higher eukaryotic cells. However, direct evidence for the requirement of a ribosome receptor in translocation of proteins across the ER is still lacking (3).

The fact that translocation can occur uncoupled from translation allowed us to investigate the energy requirements for translocation. Unlike translocation across the prokaryotic plasma membrane (2, 8), a membrane potential is not required for translocation across the yeast microsomal membrane. Ionophores, such as carbonyl cyanide m-chlorophenyl hydrazone, valinomycin, or monensin, had no effect. However, enzymatic depletion of ATP completely abolished translocation. Re-addition of ATP restored translocation. These data indicate that ATP is required for translocation of prepro-α-factor across the yeast microsomal membrane.

To determine if ATP was acting as an allosteric effector or if hydrolysis was occurring, we removed ATP after completion of prepro-α-factor synthesis by gel filtration and then added back ATP or the nonhydrolyzable analogue AMP-PNP. Translocation only occurred when ATP was restored, suggesting that ATP hydrolysis is essential for the posttranslational translocation of prepro-α-factor into yeast microsomes in vitro. It is possible that ATP hydrolysis is performed by a protein (a "translocase") that acts as a mechano-chemical transducer by coupling the energy released upon ATP hydrolysis to movement of prepro-α-factor across the membrane. Alternatively, ATP could be required for phosphorylation of a protein required for translocation.

With the cell-free translocation system at hand, it should be possible to isolate analogues of canine SRP, SRP receptor, and signal peptidase complex from yeast. The opportunity to complement biochemical analyses with genetic approaches should eventually lead to a more complete description of the events in protein translocation across the endoplasmic reticulum.

Note Added in Proof. Similar systems for in vitro translocation of yeast secretory proteins have recently been developed in two other labs (Hansen, W., P. D. Garcia, and P. Walter, 1986, *Cell*, in press; and Rothblatt, J. A., and D. I. Meyer, 1986, *Cell*, 44:619–628). In addition, an ATP requirement for posttranslational translocation into *E. coli* membrane vesicles has been demonstrated (Chen, L., and P. C. Tai, 1985, *Proc. Natl. Acad. Sci. USA*, 82:4384–4388).

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