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Secretion in yeast: translocation and glycosylation of prepro-α-factor *in vitro* can occur via an ATP-dependent post-translational mechanism

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In an *in vitro* system comprising a yeast cell-free translation system, yeast microsomes and mRNA encoding prepro-α-factor, the translocation of this protein across the membrane of the microsomal vesicle and its glycosylation could be uncoupled from its translation. Such post-translational processing is dependent upon the presence of ATP in the system. It is not, however, affected by a variety of uncouplers, ionophores or inhibitors, including carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), valinomycin, nigericin, dinitrophenol (DNP), potassium cyanide (KCN) or N-ethyl maleimide (NEM). This mechanism of translocation is significant as it indicates that a protein of 18 000 daltons is capable of crossing an endoplasmic reticulum-derived membrane post-translationally. For the moment, this phenomenon seems to be restricted to prepro-α-factor in the yeast *in vitro* system. Neither invertase nor IgG κ light chain could be translocated post-translationally in yeast, nor was such processing observed for prepro-α-factor in a wheat germ system supplemented with canine pancreatic microsomes.

Key words: secretion/protein translocation/endoplasmic reticulum/membrane biogenesis/*Saccharomyces cerevisiae*

Introduction

The initial step in the process of secretion is the translocation of the nascent secretory protein from the cytoplasm into the lumen of the rough endoplasmic reticulum (ER) (for a review, see Hortsch and Meyer, 1986). A significant body of evidence has been accumulated that indicates that translocation, at least *in vitro*, is initiated prior to completion of the nascent chain (for a review, see Kreil, 1981). Only in exceptional cases has post-translational translocation across microsomal membranes been observed (Watts *et al.*, 1983).

We have recently described an *in vitro* assay for studying protein translocation in the yeast *Saccharomyces cerevisiae* (Rothblatt and Meyer, 1986). In this system, the yeast secretory proteins prepro-α-factor and pre-invertase were correctly translocated and glycosylated and, in the case of invertase, accurate removal of the signal sequence was observed. Time course analyses indicated that the rate of translation of prepro-α-factor was roughly paralleled by its translocation and glycosylation. However, when rough microsomes were added post-translationally, i.e. after a significant fraction of completed prepro-α-factor had been synthesized and further translation was blocked by cycloheximide, the system had a limited capacity for its translocation and glycosylation.

In this report we present data which not only demonstrate that, *in vitro*, prepro-α-factor translocation can occur post-translationally, but that there is a strict requirement for ATP. This process is not inhibited by any of the agents known to disrupt the membrane potential required for post-translational translocation in mitochondria (Schleyer *et al.*, 1982; Gasser *et al.*, 1982; Kolansky *et al.*, 1982) or *Escherichia coli* (Date *et al.*, 1980). Although this type of processing may be a unique property of prepro-α-factor in a yeast system, it demonstrates that a polypeptide of 18 000 daltons can be translocated across a membrane derived from ER and correctly glycosylated in the absence of translation.

Results

Prepro-α-factor is post-translationally translocated

In order to study the vectorial transfer of nascent proteins across the membrane of the rough ER, the yeast *in vitro* system described previously (Rothblatt and Meyer, 1986) was employed. To determine whether the translocation of prepro-α-factor observed in this system was strictly coupled to nascent chain elongation, a post-translational assay was set up. Translation of *in vitro*-transcribed prepro-α-factor mRNA was allowed to proceed for 30 min (see Materials and methods). At this point, further translation was blocked by the addition of cycloheximide to a final concentration of 100 μM. The effectiveness of cycloheximide in arresting translation was verified by the fact that the addition of another mRNA did not result in any translation whatsoever (data not shown). Five minutes after cycloheximide addition, rough microsomes, with or without various factors (see

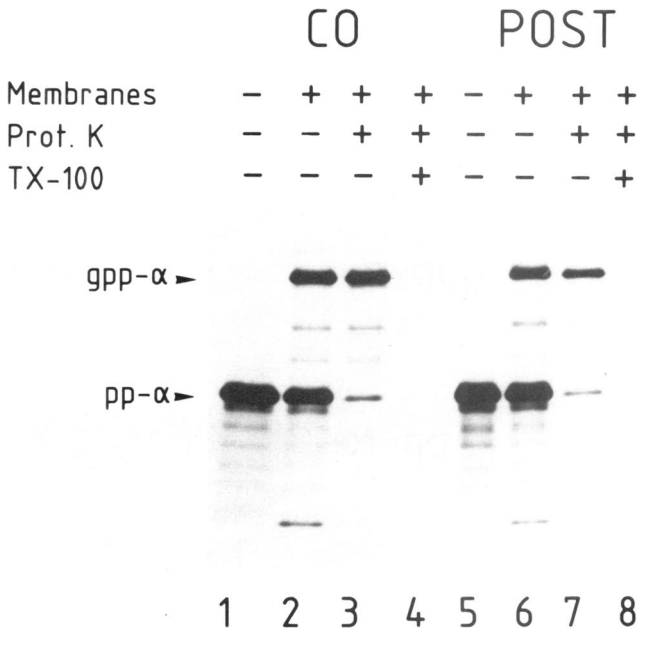

Fig. 1. Prepro-α-factor can be post-translationally translocated in a yeast cell-free system. mRNA encoding prepro-α-factor was translated *in vitro* as described in Materials and methods. Yeast microsomes were present from the onset of translation (**lanes 2-4**) or added after translation had been stopped by the addition of cycloheximide (**lanes 6-8**). **Lanes 1** and **5** represent samples to which no membranes were added. pp-α and gpp-α represent prepro-α-factor and its glycosylated form, respectively.

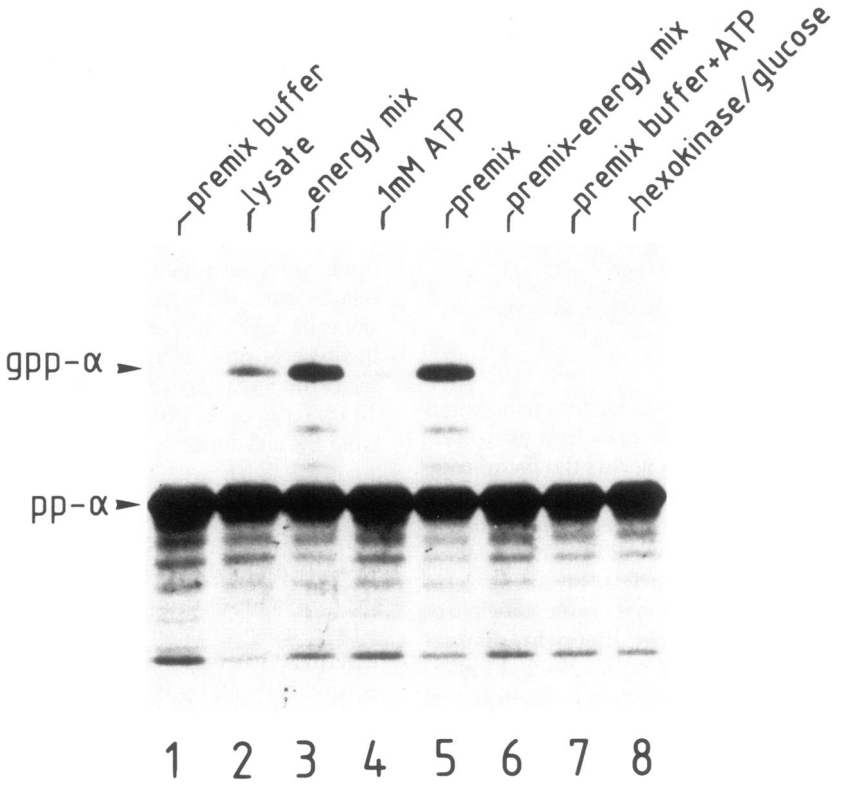

Fig. 2. Post-translational processing of prepro-α-factor requires ATP. Post-translational assays were carried out as described in Materials and methods. The components indicated on the top of the figure were added simultaneously with the yeast microsomes. A more detailed description of the components appear in the text. pp-α and gpp-α refer to prepro-α-factor and glycosylated prepro-α-factor.

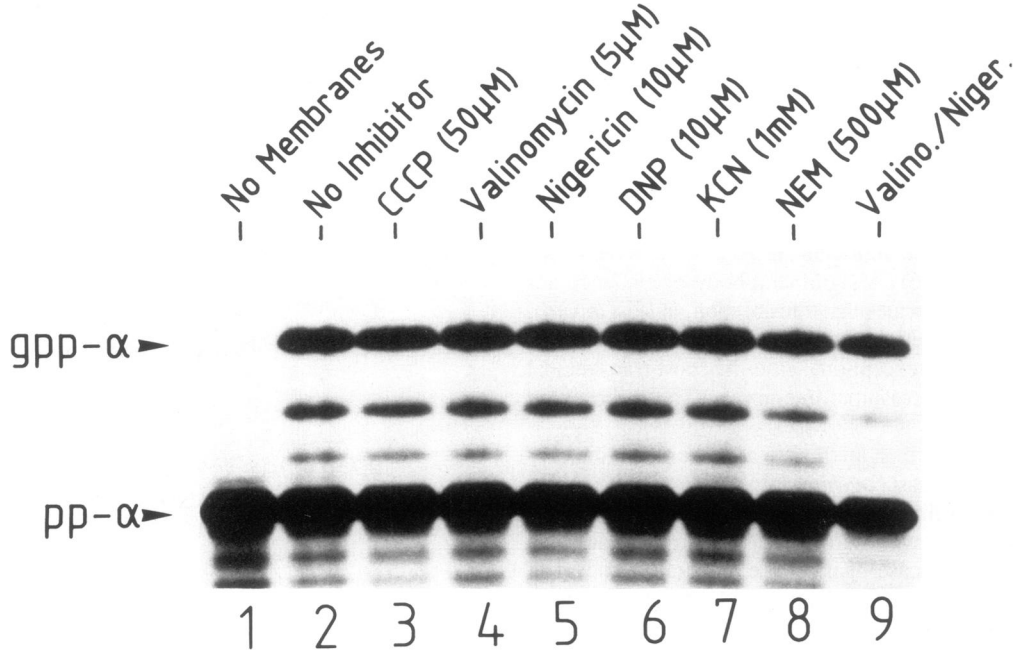

Fig. 3. Uncouplers, ionophores and inhibitors do not affect post-translational prepro-α-factor translocation. Assays are described in Materials and methods. Inhibitors, and their final concentrations in the assay system are indicated on the top of the figure. pp-α and gpp-α refer to prepro-α-factor and glycosylated prepro-α-factor.

below) were added, and incubation was continued for a further 30 min. Subsequently, samples were analyzed for the amounts of prepro-α-factor that had been translocated and glycosylated.

Shown in Figure 1 is the fact that prepro-α-factor can be translocated and glycosylated post-translationally. Lanes 2–4 correspond to experiments where membranes were present from the onset of translation, lanes 6–8 are post-translational assays carried out as described above. Lanes 1 and 5 are controls where

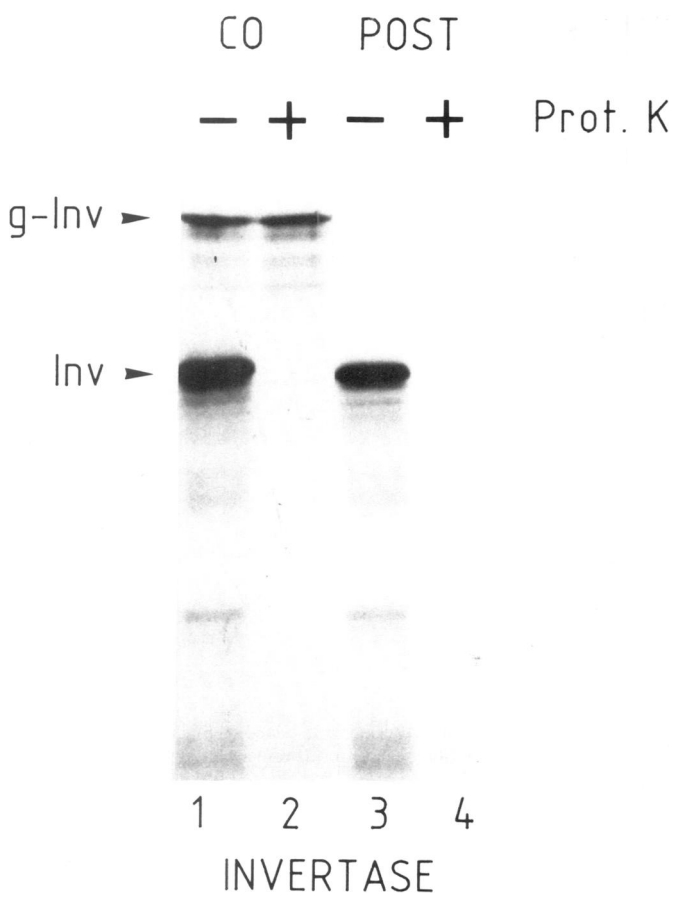

Fig. 4. Invertase is not post-translationally translocated in the yeast system. mRNA encoding the N-terminal half of invertase was translated in the yeast system as described in Materials and methods. In **lanes 1** and **2** microsomes were present from the onset of translation, whereas **lanes 3** and **4** show the results of a post-translational assay. Inv and g-Inv refer to pre-invertase and glycosylated invertase, respectively.

no membranes were added. In these cases only prepro-α-factor was observed. When membranes were present either from the start of translation (lane 2) or added post-translationally (lane 6), conversion to the glycosylated form was seen. In both cases, translocation had indeed occurred as confirmed by protease protection experiments (lanes 3, 4, 7 and 8). This finding raises several interesting questions including: What are the requirements for post-translational uptake? Are all secretory proteins capable of post-translational translocation in the yeast system? Is prepro-α-factor capable of being post-translationally translocated in other *in vitro* systems as well? The remainder of this study is devoted to answering these questions.

Post-translational translocation requires ATP

In the experiments whose results are shown in Figure 1, achieving post-translational translocation involved the simultaneous addition of one original volume of complete fresh translation system, inactivated with cycloheximide for obvious reasons. This indicated the need to replenish some factor which had become inactive or exhausted during the first 30 min translation period. To establish the nature of this component, the translation system was dissected into individual parts, and each was tested for its ability to support post-translational translocation of prepro-α-factor. The results are shown in Figure 2.

The addition of cycloheximide-treated lysate alone resulted in the translocation of a small amount of prepro-α-factor (Figure 2, lane 2). On the other hand, the addition of fresh pre-mix stimulated prepro-α-factor translocation and glycosylation considerably (lane 5). Pre-mix is composed of an energy-regenerating system comprising 1 mM ATP, creatine phosphate (CP) and creatine phosphokinase (CPK); 0.1 mM GTP; dithiothreitol (DTT); tRNA; 19 amino acids (−methionine) buffered with Hepes. It was, therefore, of interest to ascertain which of the aforementioned components of pre-mix contributes to the translocation process. Deletion of the energy-regenerating system (ATP, CP, CPK) completely abolished the translocation-promoting activity of the pre-mix (lane 6). Restoration of ATP alone (lane 4) or in combination with the pre-mix buffer (lane 7) was insufficient. The re-addition of the energy-regenerating system, CP and CPK, to 1 mM ATP (final concentration) enabled restoration of translocation activity to the level observed with complete pre-mix (lane 3). The necessity for replenished levels of ATP was further confirmed by the result using an energy depletion system consisting of hexokinase in the presence of glucose (lane 8). In this case no post-translational translocation of prepro-α-factor could be observed, even at 10 times higher initial levels of ATP (10 mM). From these data, one can conclude that some aspect of the translocation of prepro-α-factor, at least in this post-translational *in vitro* assay, requires a continuous presence of ATP. Control experiments using protease protection indicated that in the absence of ATP no prepro-α-factor had been translocated post-translationally. This ruled out the possibility that it is merely glycosylation that requires ATP, not translocation (data not shown).

Uncouplers and ionophores do not inhibit translocation

Post-translational uptake of certain proteins into mitochondria and translocation of secretory and membrane proteins in bacteria has been shown to be dependent upon a membrane potential generated by the translocation of protons (for reviews, see Randall and Hardy, 1984; Harmey and Neupert, 1985; Wickner and Lodish, 1985). This process is energy requiring and thus dependent upon ATP. It was therefore of interest to see if the ATP requirement for post-translational prepro-α-factor translocation reflected the necessity of a membrane potential in yeast microsomes. Accordingly, we treated the microsomes with various uncouplers and ionophores well above the concentrations known to inhibit protein translocation in mitochondria or *E. coli*. These included carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) (Figure 3, lane 3), valinomycin (lane 4), nigericin (lane 5), dinitrophenol (DNP, lane 6) and KCN (lane 7). None of these ionophores or known inhibitors of translocation into mitochondria or secretion in *E. coli* had any effect in our system.

Recently, the presence of a proton-translocating ATPase was discovered and characterized in microsomes derived from rat liver (Rees-Jones and Al-Awqati, 1984). Proton transport in these membranes was found to be completely inhibited by sulfhydryl reagents such as N-ethyl maleimide (NEM). The post-translational translocation of prepro-α-factor by yeast membranes was unaffected, however, by NEM, even at 10-fold higher concentrations than necessary to eliminate proton translocation in rat liver microsomes (Figure 3, lane 8). These data indicate that if a membrane potential is required for translocation in yeast microsomes, it is not created by a mechanism having inhibitor sensitivities similar to characterized systems.

There are, of course, several other ATP-dependent functions which may be of importance in translocation, including ATPases, phosphorylation reactions or other unknown enzymatic or regulatory phenomena. Clearly further experiments are necessary to establish the role played by ATP in this process. We have

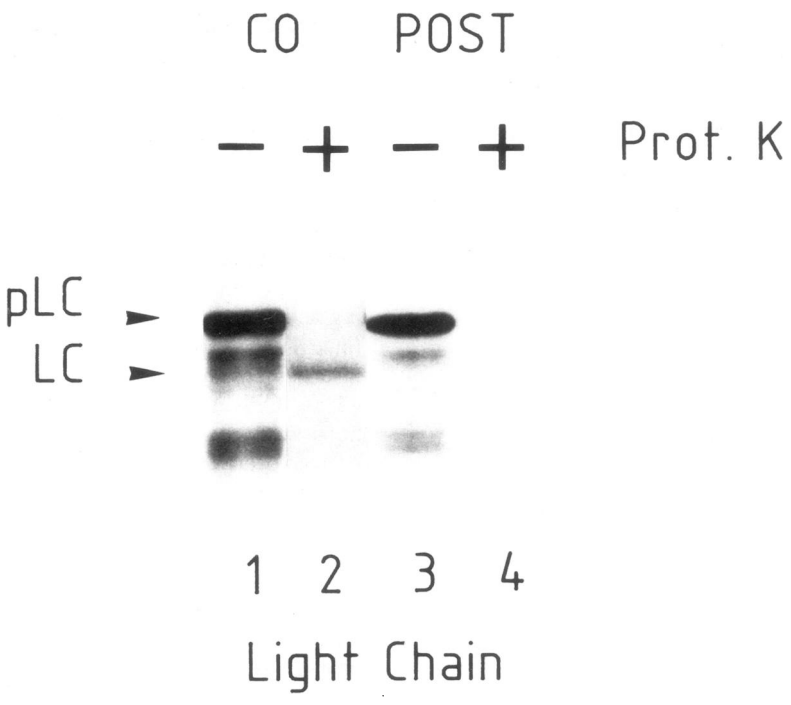

Fig. 5. IgG κ light chain is not post-translationally translocated in the yeast system. Experiments were identical to those described in Figure 4, only MOPC-41 poly(A)$^+$ RNA was used instead of invertase mRNA. pLC and LC refer to pre-light chain and the mature form, respectively.

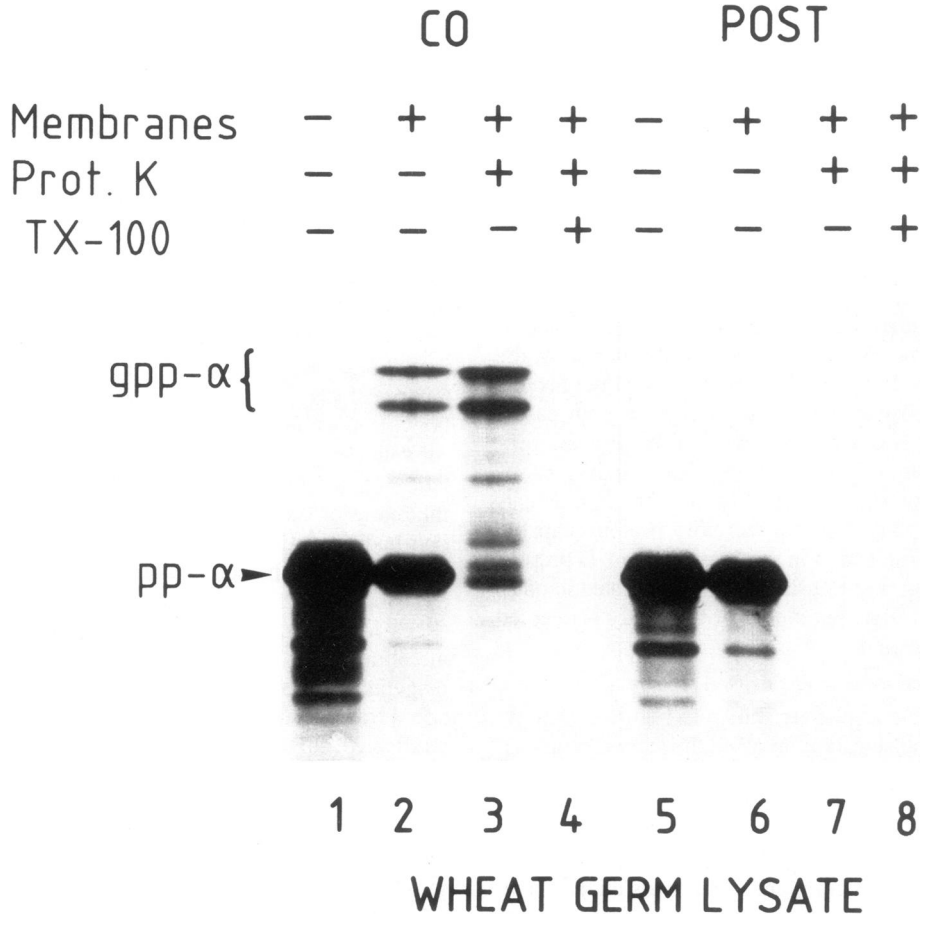

Fig. 6. Prepro-α-factor is not post-translationally translocated into pancreatic microsomes in wheat germ lysate. α-Factor mRNA was translated in a wheat germ lysate (**lanes 1** and **5**). Salt-washed canine pancreatic microsomes and SRP were either present from the onset of translation (**lanes 2–4**) or added after translation (30 min) had been arrested by cycloheximide (**lanes 6–8**). Translocation was determined by proteolysis with proteinase K (**lanes 3** and **7**). pp-α and gpp-α refer to prepro-α-factor and its glycosylated form, respectively.

recently shown that an as yet unknown component, required for translocation in pancreatic microsomes, can be alkylated by NEM resulting in a loss in translocation activity (Hortsch *et al.*, 1986). The minimum concentration of NEM which brought about this inhibition was 25 mM. We have subsequently found that the translocation of prepro-α-factor in the yeast system, both co-translational and post-translational, could be inhibited by pre-treating the microsomes with 5 mM NEM (data not shown).

Other secretory proteins are not post-translationally translocated

Are all secretory proteins translocated post-translationally in the yeast system? To answer this question, we examined the translocation of another yeast secretory protein, invertase, and IgG κ light chain. As can be seen in Figure 4, pre-invertase synthesized in yeast lysate is successfully translocated and glycosylated by yeast microsomes (lanes 1 and 2). We know from previous studies that correct cleavage of the signal sequence also takes place (Rothblatt and Meyer, 1986). Neither translocation nor glycosylation was observed post-translationally (lanes 3 and 4). In the case of IgG κ light chain, a protein which is poorly translocated and processed in yeast (Rothblatt and Meyer, 1986), a level of ~10–15% co-translational processing and protection was seen (Figure 5, lanes 1 and 2). As with invertase, no translocation was observed in the post-translational assay (lanes 3 and 4). This implies that, for the moment, prepro-α-factor is unique in its ability to be post-translationally translocated.

Does this ability extend to other translocation systems? Prepro-α-factor has been successfully translocated across pancreatic microsomes and glycosylated co-translationally *in vitro* (Julius *et al.*, 1984). Attempts to achieve the post-translational translocation of prepro-α-factor across pancreatic microsomes in wheat germ lysate were unsuccessful (Figure 6). Prepro-α-factor was translocated into pancreatic microsomes and glycosylated co-translationally in wheat germ lysate (lanes 1–4). In a post-translational wheat germ assay system, identical to the yeast one, no prepro-α-factor was translocated (lanes 5–8), despite the addition of amounts of signal recognition particle (SRP) which we had previously shown to be sufficient to bring about a translation arrest and ensure the co-translational translocation of prepro-α-factor. The translation system used to synthesize prepro-α-factor was not crucial to its post-translational translocation across yeast microsomes. When prepro-α-factor mRNA was synthesized in rabbit reticulocyte lysate, post-translational uptake into yeast microsomes was observed (data not shown). From these data it appears as if the post-translational processing of prepro-α-factor which we have observed cannot be taken as a general feature of the *in vitro* translocation process in the yeast system.

Discussion

The results of this study indicate that in a yeast *in vitro* system, prepro-α-factor can cross the microsomal membrane after its translation has been completed. Similar findings have also been made in another laboratory (W.Hansen, P.Garcia and P.Walter, personal communication). This is the first instance in which a secretory protein has been translocated post-translationally in a eukaryotic system. Moreover, there is a strict requirement for ATP, whose function has not as yet been ascertained. In this system, neither another yeast secretory protein, invertase, nor IgG κ light chain could be translocated after their synthesis had been completed. Prepro-α-factor, which can be translocated and glycosylated by canine pancreatic microsomes in a wheat germ lysate, could not be transferred post-translationally. Thus, for the moment, the post-translational translocation of prepro-α-factor seems to be unique to the yeast system.

Even if post-translational translocation is not a widespread phenomenon, its mere existence necessitates the re-evaluation of existing models. The fact is that fully 18 000 daltons of protein is crossing the membrane of ER-derived vesicles in the absence of translation. One can no longer dismiss post-translational translocation of F1 phage procoat into rough microsomes (Watts *et al.*, 1983) as an exceptional case whose translocation was attributable to its small size. The obvious question is: how does the completed protein cross the membrane? The conformation of the polypeptide chain is certainly relevant. Conceptually, it is more difficult to imagine a folded, or even globular molecule crossing the bilayer, especially if proteinaceous pores or channels are involved (Blobel, 1980). On the other hand, if translocation occurs when the protein is in an unfolded or more open configuration, a mechanism must exist whereby such a configuration is maintained until translocation takes place. Retention of a 'translocation-conducive' conformation could be mediated by factors known to interact with nascent secretory and membrane proteins such as the signal recognition particle (for a review, see Hortsch and Meyer, 1986), or other, as yet unidentified, cytoplasmic components.

The fact that neither invertase nor IgGκ light chain could be translocated post-translationally in this system argues against the presence of components which keep secretory proteins in a favorable configuration. On the basis of this, and in the absence of data on other proteins, one must conclude that prepro-α-factor intrinsically possesses a conformation which, in the yeast system, allows its translocation. Such a notion would be consistent with models postulating 'domain-wise' translocation of secretory proteins. Recent evidence suggests that this is true for certain prokaryotic secretory proteins (Randall, 1983). In this case, prepro-α-factor could be considered as comprising one translocatable domain, whereas other secretory proteins, whose translocation is strictly co-translational, would be made up of more than one domain; the translocation of each domain requiring the previous translocation of its predecessor. By functional analogy, phage procoat would also consist of one such domain. It must be borne in mind, however, that prepro-α-factor could not be post-translationally translocated across canine-pancreatic microsomes. This implies differences between the specific requirements for translocation between yeast and higher eukaryotes.

The ability to post-translationally translocate prepro-α-factor in the yeast lysate, even if it is not representative of a generalized phenomenon, provides an excellent opportunity for studying translocation. In this system, translocation can be uncoupled from translation, and cytosolic and membrane-bound components can be dissected and/or inactivated without having to worry about the integrity of the translation process. Of tremendous advantage is the fact that this is occurring in a yeast system. The existence of secretory mutants (Ferro-Novick *et al.*, 1984) and the ease of genetic manipulation of this organism, in conjunction with a post-translational *in vitro* assay, will make a biochemical analysis significantly easier.

Materials and methods

Cell-free translation systems, construction and *in vitro* transcription of plasmids encoding secretory proteins, and all analytical methods have been described previously (Rothblatt and Meyer, 1986). Yeast microsomes were prepared essentially as described (Rothblatt and Meyer, 1986) with the exception that the post-nuclear supernatant was centrifuged for 15 min at 24 000 *g* instead of at 125 000 *g* for 90 min. The 24 000 *g* pellet was resuspended and used in these experiments as the microsomal fraction. The 24 000 *g* supernatant did not contain membranes competent for translocation. Ionophores, uncouplers, inhibitors, and cyclohex-

imide were all purchased from Sigma (St. Louis, MO). [^{35}S]Methionine was from Amersham-Buchler (Braunschweig, FRG).

Post-translational assays were carried out as follows. mRNA was translated at 25°C *in vitro* for a period of 30 min. Further translation was abolished by the addition of cycloheximide to a final concentration of 100 μM. Five minutes later, membranes (or buffer in the case of controls) were added and incubation was continued for an additional 25 min. To determine the extent of translocation, proteolysis was carried out using proteinase K as described (Rothblatt and Meyer, 1986).

Studies using uncouplers, ionophores and inhibitors were carried out by pre-incubating yeast microsomes with stock solutions whose ultimate addition (post-translational) to the system would achieve the final concentrations indicated in Figure 3.

Acknowledgements

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References

Blobel,G. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 1496–1500.
Date,T., Zwizinski,C., Ludmerer,S. and Wickner,W. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 827–831.
Ferro-Novick,S., Novick,P., Field,C. and Schekman,R. (1984) *J. Cell Biol.*, **98**, 35–43.
Gasser,S.M., Daum,G. and Schatz,G. (1982) *J. Biol. Chem.*, **257**, 13034–13041.
Harmey,M.A. and Neupert,W. (1985) In Martonosi,A. (ed.), *The Enzymes of Biological Membranes Vol. IV.* Plenum Publ. Corp. NY, pp. 431–464.
Hortsch,M. and Meyer,D.I. (1986) *Int. Rev. Cytol.*, **102**, 215–242.
Hortsch,M., Avossa,D. and Meyer,D.I. (1986) *J. Cell. Biol.*, in press.
Julius,D., Schekman,R. and Thorner,J. (1984) *Cell*, **36**, 309–318.
Kolansky,D.M., Conboy,J.G., Fenton,W.A. and Rosenberg,L. (1982) *J. Biol. Chem.*, **257**, 8467–8471.
Kreil,G. (1981) *Annu. Rev. Biochem.*, **50**, 317–348.
Randall,L.L. (1983) *Cell*, **33**, 231–240.
Randall,L.L. and Hardy,S.J.S. (1984) In Satir,B. (ed.), *Modern Cell Biology*. Alan R. Liss, NY, Vol. 3, pp. 1–20.
Rees-Jones,R. and Al-Awqati,Q. (1984) *Biochemistry*, **23**, 2236–2240.
Rothblatt,J.A. and Meyer,D.I. (1986) *Cell*, **44**, 619–628.
Schleyer,M., Schmidt,B. and Neupert,W. (1982) *Eur. J. Biochem.*, **125**, 109–116.
Watts,C., Wickner,W. and Zimmermann,R. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 2809–2813.
Wickner,W.T. and Lodish,H.F. (1985) *Science*, **230**, 400–407.

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