Prepro-carboxypeptidase Y and a truncated form of pre-invertase, but not full-length pre-invertase, can be posttranslationally translocated across microsomal vesicle membranes from Saccharomyces cerevisiae.

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**Abstract.** We have determined that prepro-carboxypeptidase Y and a truncated form of pre-invertase can be translocated across the yeast microsomal membrane post-translationally in a homologous in vitro system. The yeast secretory protein prepro-α-factor which was previously shown to be an efficient posttranslational translocation substrate is therefore not unique in this regard, but rather the yeast ER protein translocation machinery is generally capable of accepting substrates from a ribosome-free, soluble pool. However, within our detection limits, full-length pre-invertase could not be translocated posttranslationally, but was translocated co-translationally. This indicates that not every fully synthesized pre-protein can use this pathway, presumably because normal or aberrant folding characteristics can interfere with translocation competence.

**Materials and Methods**

**Construction of Plasmids for In Vitro Transcription-Translation**

Carboxypeptidase Y, pTS8, a pBR322 plasmid containing a fragment of yeast genomic DNA including the PRC1 gene (the generous gift of Dr. Tom...
Endoglycosidase H. Digestion. Removal of asparagine linked oligosaccharide residues was carried out as described previously (Hansen, et al., 1986).

Translocation Across Microsomal Vesicles. Co-translational incubation of in vitro synthesized proteins was carried out as described previously (Hansen, et al., 1986). Post-translational incubations including microsomal vesicles were performed as described previously, except that ATP, GTP, creatine phosphate and creatine phosphokinase were included at concentrations of 1 mM, 80 µM, 20 mM and 0.2 mg/ml, respectively.

Results

Fully Synthesized Prepro-carboxypeptidase Y can be Translocated across Yeast Microsomal Membranes In Vitro

We wondered whether the posttranslational translocation of ppCF across the yeast ER membrane reflected a general mechanism in yeast which can be used by a large number of substrates. We therefore investigated the behavior of prepro-carboxypeptidase Y (ppCPY), the 59-kD precursor of the vacuolar protease carboxypeptidase Y. Translation of a synthetic mRNA in a yeast translation extract (see Materials and Methods) resulted in the synthesis of a protein of the expected molecular weight which could be immunoprecipitated with antibodies recognizing CPY (Fig. 1 A, lane 1). A second translation product of smaller molecular weight which is also immunoreactive is an incompletely synthesized form of CPY. If yeast microsomal vesicles were present after ppCPY was fully synthesized, and any further translation was prevented by the addition of cycloheximide, new protein species of higher molecular weight were observed (Fig. 1 A, cf. lanes 1 and 3). Since ppCPY receives four asparagine-linked core oligosaccharide moieties upon translocation into the lumen of the ER in vivo (Stevens et al., 1982; Valls et al., 1987), this increase in molecular weight resulting from transfer of the core oligosaccharides would be expected upon translocation of ppCPY into yeast microsomal vesicles. As expected, the glycosylated bands were also generated if the yeast microsomal vesicles were added to the translation extract during the synthesis of ppCPY (Fig. 1 A, lane 2). Note that the efficiency of translocation is comparable (in either case estimated to be ~10%) whether the membranes were added during or after synthesis of ppCPY.

To ascertain that the protein species of higher molecular weight were truly translocated across the membrane of the vesicles, we tested whether they were sensitive to degradation upon addition of protease. As expected, the glycosylated bands formed upon incubation of ppCPY with yeast microsomal membranes (Fig. 1 B, lane 4) are resistant to trypsin digestion (Fig. 1 B, lane 5), unless the membrane was rendered permeable by the addition of nonionic detergent (Fig. 1 B, lane 6). In contrast, the not translocated, unglycosylated species were completely digested. To confirm that the protein species protected by the membrane were indeed core glycosylated forms of pCPY, the core sugars were removed by digestion with endoglycosidase H, resulting in the expected increase in electrophoretic mobility (Fig. 1 B, lane 7).

Translocation of ppCPY was found to be less efficient than that of ppCF. One possible explanation for this observed difference was the potential for disulfide bond formation in the case of ppCPY which contains 13 cysteines, while ppCF contains no cysteines (Valls et al., 1987; Karjun and Herkowitz, 1982). Increasing the dithiothreitol concentration to

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Translocation of ppCPY into yeast microsomal vesicles can occur in the absence of polypeptide elongation. (A) A yeast in vitro translation reaction was programmed with mRNA encoding ppCPY and incubated for 60 min in the absence (lane 1) or in the presence of yeast microsomal vesicles (lane 2). In lane 3, microsomal membranes were added after 60 min of translation and incubated in the translation reaction for an additional 60 min after elongation had been completely blocked by addition of cycloheximide to a final concentration of 2 mM. (B) Cycloheximide inhibited translation reactions containing ppCPY were incubated for 60 min in the absence, lanes 1-3, or in the presence, lanes 4-7, of yeast microsomal vesicles. Reactions shown in lanes 1 and 4 received no other additions. Reactions shown in lanes 2, 3, 5, 6, and 7 were incubated with trypsin as described in Materials and Methods. In addition to the added protease reactions shown in lanes 3, and 6 also contained the detergent Nikkol. After the added protease was inactivated (see Materials and Methods), the reaction shown in lane 7 was digested with endoglycosidase H. Proteins immunoprecipitated by antibody recognizing ppCPY were then subjected to SDS-PAGE and visualized by autoradiography. The mobility of preproCPY and glycosylated proCPY are marked by lines at the side of the autoradiogram with an arrow pointing up between them to indicate the mobility shift which occurs upon glycosylation of the protein.

20 mM did not yield increased translocation (not shown), indicating that artifactual oxidation of cysteine residues was not impeding translocation in our assay as suggested by others (Maher and Singer, 1986).

**Fully Synthesized Yeast Invertase Cannot be Translocated In Vitro**

The observation that full length ppCPY could be translocated indicated that ppaF is not unique in this respect. To further evaluate the generality of this type of translocation reaction, we examined the behavior of pre-invertase (pINV), the precursor of another yeast secretory protein. pINV is nearly identical in molecular weight to ppCPY and, like CPY, invertase is glycosylated upon its translocation into the ER.

Translation of pINV mRNA in the absence of microsomal vesicles produced a 62-kD protein (Fig. 2, lane 1, pINV) which was precipitated with anti-invertase antibodies and could be degraded by added protease (Fig. 2, lanes 2 and 3). If microsomal vesicles were present during translation, new immunoreactive bands of higher molecular weight were produced (Fig. 2, lane 4) as the result of core glycosylation of translocated invertase. Addition of protease preferentially degraded the nonglycosylated form, while the higher molecular weight forms were more resistant, confirming their luminal disposition in the vesicles (Fig. 2, lane 5). Digestion of the proteins protected by the microsomal vesicles with endoglycosidase H resulted in the expected molecular weight shift confirming that the high molecular weight bands were core glycosylated (Fig. 2, lane 6). Hence, according to these criteria pINV could be translocated co-translationally in the homologous yeast in vitro system with fidelity.

However in marked contrast to the translocation of ppCPY, addition of microsomal vesicles to the translation system after pINV was fully synthesized did not yield any detectable glycosylated products (Fig. 3 compare lanes 1-5 with the corresponding lanes in Fig. 2). Since the co-translation translocation of pINV resulted in the appearance of a heterogeneous population of glycosylated species, it was possible that a small amount of translocation occurred, yet went undetected because the signal was divided amongst several bands. For this reason the products of the protease protection assay were incubated with endoglycosidase H to convert any heterogeneously glycosylated invertase to one species (see for example the case for co-translation translocation of pre-invertase (Fig. 2, lanes 5 and 6)). However, we did not detect any increase of intensity of the band corresponding to the unglycosylated invertase under these conditions (Fig. 3, cf.
lanes 5 and 7). Thus, although pINV could be translocated in vitro co-translationally, once synthesized to its full-length, the pINV molecule did not retain translocation competency.

A Truncated Invertase Protein Remains Translocation Competent after its Synthesis is Complete

There are several possible explanations for the lack of translocation competence of full-length pINV. First, it could be a property of the signal sequences themselves that enables ppctF and ppCPY, but not pINV to engage with the translocation machinery after they have been fully elongated. Alternatively, it could be structural properties of the fully synthesized proteins that renders pINV, but not ppctF and ppCPY translocation incompetent.

To distinguish between these two possibilities, we decided to alter the structure of pINV by inserting a termination codon into the middle of the coding sequence. When mRNA encoding this truncated pINV was translated in vitro, a protein of the predicted molecular weight was synthesized (Fig. 3, lane 8). In contrast to the complete pINV, some of the truncated product (pINV8) retained its translocation competence after it was fully elongated. (This result and results discussed below regarding ppCPY are in conflict with previous reports where it was claimed that a similarly truncated invertase fragment (Rothblatt and Meyer, 1986b) and ppCPY (Rothblatt et al., 1987), could not be translocated in the presence of inhibitors of polypeptide elongation, while they could be translocated co-translationally. However, given the requirements for posttranslational translocation of ppctF (see Introduction) it is likely that the concentrations of cytoplasmic protein factors, ATP and microsomal membranes present in experiments carried out by different investigators will all play a role in whether posttranslational translocation of a particular protein will be detected). If microsomal vesicles were added to a reaction containing pINV8 after further elongation was inhibited by cycloheximide, a ladder of four new glycosylated protein species was produced which could be precipitated with anti-invertase antibody (Fig. 3, lane II).

The protein species produced were protected from digestion by exogenous protease in the absence of added detergent (Fig. 3, lane J2), but were digested if detergent was added with the protease (Fig. 3, lane J3). A small amount of pINV8 remained undigested by protease in lanes 9 and J2, however we can be certain that some pINV8 is translocated because we have used both protection from digestion by added protease and addition of core oligosaccharides (see Fig. 5, A and B) as criteria to verify that translocation has occurred. Furthermore, the glycosylated bands were sensitive to endoglycosidase H digestion (not shown, see Fig. 5). Thus it appears that it is a structural feature of the complete pINV protein that renders it translocation incompetent and to thereby possess the observed requirement for translocation to occur co-translationally, i.e., before folding can occur.

The Translocation of Full-length ppCPY and pINVδ Can Occur Posttranslationally

The observation that ppCPY and pINVδ can be translocated in a reaction that is independent of polypeptide elongation does not necessarily prove that these proteins traverse the microsomal membrane by a posttranslational mechanism. The latter term implies that the polypeptide's synthesis be terminated and that the completed protein be released from the ribosome before translocation is initiated (see Discussion). We therefore investigated the nature of the polypeptide chains which were translocation competent under these assay conditions to determine to which degree these polypeptide chains might still be ribosome associated, i.e., fully elongated but not properly terminated.

To this effect, release from the ribosome of chains which were not terminated was evoked by treatment with puromycin. Alternatively, ribosome-associated pre-proteins were removed from the translation reaction by centrifugation prior to membrane addition. These experiments were performed in parallel with ppCPY (Fig. 4, A and B) and pINVδ (Fig. 5 A and 5 B) as translocation substrates. Polypeptide elongation directed by the respective mRNAs was inhibited by the elon-
Emetine was added to a 60-μL translation reaction synthesizing ppCPY to a final concentration of 100 μM to completely block polypeptide elongation and stabilize polysomes after it had been incubated 60 min at 20°C. Six equal aliquots were removed and puromycin was added to the reactions shown in lanes 4 and 6 followed by incubation of all samples for 15 min at 20°C. The reaction shown in lane 1 received no further additions. A peptide inhibitor of N-linked core glycosylation NYT (see Materials and Methods) was added to the reactions shown in lanes 5 and 6. The reactions in lanes 2–6 were then incubated for 60 min in the presence of microsomal vesicles followed by trypsin and proteinase K treatment (750 and 100 μg/ml, respectively) of reactions shown in lanes 3–6. Proteins immunoprecipitated by antibody recognizing carboxypeptidase Y were then subjected to SDS–PAGE and visualized by autoradiography. Puromycin was found to inhibit 90% of the translocation detected. Puromycin also effectively inhibited the translocation and glycosylation of the incompletely synthesized form of ppCPY, thus the majority of the translocation of this species required the context of the ribosome and was not truly posttranslational. (B) The conditions used in the reactions in B were identical to those in A in the corresponding lanes, except that instead of using an unfractionated translation reaction as the source of ppCPY a postribosomal supernatant was used.

As above, subsequent incubation with yeast microsomal vesicles resulted in the production of glycosylated and protease protected (i.e., properly translocated) pCPY and INVδ (Figs. 4 A and 5 A, lanes 2 and 3). If the emetine-treated reactions were incubated with puromycin to cause chain termination prior to membrane addition (Figs. 4 A and 5 A, cf. lanes 3 and 4), translocation of ppCPY or pINVδ did still occur, albeit at reduced efficiency. This indicated that these substrates can, in principle, cross the membrane posttranslationally. However, the translocation of ppCPY and pINVδ was markedly inhibited by the puromycin treatment, indicating that a significant portion of the observed translocation was contributed by ribosome-associated pre-proteins.

To further ascertain the conclusion and to rule out that the puromycin reaction might have been incomplete, we removed the ribosomes from the translocation assay before membrane addition by centrifugation. ppCPY and pINVδ present in the resulting post-ribosomal supernatant (Figs. 4 B, lane 1, and 5 B, lane 1) were converted to glycosylated (Figs. 4 B, lane 2, and 5 B, lane 2) and protease insensitive (Figs. 4 B, lane 3, and 5 B, lane 3) translocated proteins, when incubated with microsomal membranes. As expected and contrary to the results described above for the unfractionated translation system (Figs. 4 A and 5 A), the efficiency of translocation was now unaffected by the prior incubation of the post-ribosomal supernatant fractions with puromycin (Figs. 4 B and 5 B, cf. lanes 3 and 4). Finally, in a separate analysis by sedimentation velocity gradient centrifugation it was determined that precursor proteins displaying sedimentation coefficients less than 11S (and therefore not ribosome associated proteins) could be translocated (data not shown), again indicating that ribosome independent translocation could occur.

The relative contributions of the two discernible translocation modes, i.e., ribosome-dependent and posttranslational, were unaffected by conditions which inhibited glycosylation. For this purpose a tripeptide was added as a competitive core oligosaccharide acceptor (Fig. 4, A and B, lanes 5 and 6, and Fig. 5, A and B, lanes 5 and 6). Inhibition of glycosylation
allowed a quantitative comparison of the relative contributions of the ribosome-dependent and posttranslational translocation pathways by simplifying the pattern of translocated products to a single band in each case. Thus it was determined that ~80% of the translocation of precursor proteins in Figs. 4 and 5, A was ribosome dependent. These results also demonstrate that glycosylation was not required for nor did it affect the degree of translocation. Furthermore, the protease protection used to monitor translocation of pNV (data not shown), ppCPY and pNVΔ is not a result of altered conformation due to oligosaccharide addition. For pNV (data not shown), ppCPY and pNVΔ reduction in molecular weight due to signal peptide cleavage during translocation across the microsomal membrane was observed when glycosylation was inhibited.

Discussion

We have shown that, in addition to prepro-α-factor (ppαF), two other proteins, prepro-carboxypeptidase Y (ppCPY) and a truncated form of pre-invertase (pNVΔ), can be translocated across the yeast microsomal membrane posttranslationally in a homologous in vitro system. Thus the protein translocation machinery of the yeast ER, like those of the mitochondrion, the chloroplast, and the bacterial plasma membrane, is generally capable of accepting substrates from a ribosome-free, soluble pool.

However, not every fully synthesized pre-protein can use this pathway. Rather some proteins, as shown here for full-length pre-invertase (pNV), have to be targeted to the membrane co-translationally. We can interpret these data readily if we assume that proteins need to be translocated in an unfolded state. Fully synthesized proteins can fold into structures that may be more or less resistant to subsequent unfolding, depending on their particular tertiary or quaternary structures. Note, in particular, that a dimeric form of invertase exists in the cytoplasm of yeast cells (Gascon and Lampen, 1968; Trimble and Maley, 1977). Thus pNV has the potential to assume an enzymatically active and presumably stable dimeric structure in the reducing environment of the cytoplasm. This interpretation is consistent with the finding that removal of the carboxy-terminal half of pNV (which would certainly prevent it from folding into a structure resembling the native enzyme) rendered the truncated protein (pNVΔ) translocation competent in the posttranslational assay. Similar evidence consistent with an inverse correlation between formation of stable tertiary structure and the translocation competence of precursors has been obtained for protein translocation across the prokaryotic plasma membrane (Randall and Hardy, 1986) and the mitochondrial envelope (Eilers and Schatz, 1986).

We have determined that two distinct classes of full-length ppCPY and pNVΔ molecules were present in our translocation extracts, when the translocation assays were performed. The first class of molecules had been properly terminated and released from the ribosome, and thus acted as substrates for a truly posttranslational translocation reaction. The second class of molecules were still ribosome-associated, i.e., trapped before termination of protein synthesis, and thus were targeted to the membrane as ribosome-bound peptidyl-tRNAs (as shown by their susceptibility to puromycin release). Properly, the translocation of the latter class should not be considered “posttranslational,” since termination is regarded as part of the translational process. Furthermore, the ribosome may play an active role in the targeting reaction which consequently may display distinct molecular requirements from the truly posttranslational reaction. For example, studies with mammalian microsomal membranes show that targeting and translocation of non-terminated proteins uses the same molecular machinery that is required for the bona fide co-translational process (e.g., SRP, SRP receptor, GTP) (Connolly and Gilmore, 1986; Garcia and Walter, 1988 [see page 1043, this issue] Garcia, P., V. Siegel and P. Walter, unpublished data), and that it can be distinguished from that used by a few, exceptionally small proteins that can cross the mammalian membrane posttranslationally (e.g., no ribosome, no SRP and no SRP receptor dependence) (Muller and Zimmermann, 1987; Schlenstedt and Zimmermann, 1987; Perera et al., 1986). We speculate that the SRP and SRP receptor system evolved to enhance the rate of targeting (with respect to that of folding of proteins which would eventually obtain structures incompatible with translocation) of pre-proteins to the ER membrane where transmembrane movement occurs via similar mechanisms co- or posttranslationally.

While the fraction of full length polypeptide chains that are still nonterminated is comparatively small (as judged by their behavior upon sedimentation velocity analysis in gradients of glycerol), these chains contribute ~80% of the protein that can be translocated after elongation has been inhibited. Hence the ribosome associated chains appear to be far more efficient translocation substrates than the released proteins. If the ribosome itself functions as a ligand in the targeting reaction as discussed above, then this could account for the increased efficiency. Alternatively, yet not mutually exclusive, retention on the ribosome may serve to maintain the polypeptide chain in an unfolded state compatible with translocation. These effects may of course be more or less severe for different proteins, the extreme cases being ppαF which translocates rather efficiently posttranslationally and pNV, the posttranslational translocation of which is not detectable. We cannot rule out, however, that even pNV may be translocated posttranslationally if the experimental conditions are optimized appropriately.

What yeast components participate in the respective reactions remains to be determined. A soluble factor(s) known to stimulate the posttranslational translocation of ppαF (Waters et al., 1986) may keep the signal sequences accessible for interaction with a signal sequence receptor in the membrane and/or facilitate unfolding to keep or render the substrate translocation competent. In the membrane a signal sequence receptor may bind the pre-protein as the first step in the translocation reaction. Such a receptor has recently been identified in mammalian microsomal membranes and binds to signal sequences of nascent secretory proteins after they have been targeted to the membrane by SRP/SRP receptor (Wiedmann et al., 1987).

No SRP analogue has yet been positively identified in S. cerevisiae (we have however good evidence for an analogous particle in Schizosaccharomyces pombe and in Yarrowia lipolytica; Poritz et al., 1988). It is therefore not clear to what degree posttranslational targeting to the membrane represents a bypass of an also existing SRP-dependent targeting reaction, which, in analogy to the mammalian situation, would provide for coupling between translation and translo-
cation (Walter, 1987). Since pINV could only be translocated co-translationally, it may prove to be a suitable substrate to assay for components (such as a putative yeast SRP) that are specifically required for ribosome-dependent targeting.

A major question remaining regards the relative importance of posttranslational translocation in vivo. Since the in vitro translation systems have been experimentally deprived of ER membranes, we may be able to uncouple translocation from translation in vitro even though posttranslational translocation may never occur in living cells where ER membranes are always present during synthesis. To date, no cytoplasmic pools of pre-secretory proteins have been convincingly demonstrated in vivo in wild-type yeast cells. However, under conditions where translocation was perturbed by introducing mutations either into the signal sequence (Balchly-Dyson and Stevens, 1987) or into components that may be part of the translocation machinery itself (sec61; Deshaies and Shekman, 1987) cytoplasmic pools of proCPY and ppCPF accumulated in sec61 mutant cells occurs with slow kinetics (Deshaies, R., and R. Schekman, personal communication). In contrast, and in agreement with our in vitro data, mutants in the signal sequence of pINV also led to the accumulation of cytoplasmic pINV, but in this case no subsequent translocation could be detected (Ngsee, J., and M. Smith, personal communication). Thus translocation of some, but not all fully elongated pre-proteins can also occur in vivo. It remains to be determined what fraction of molecules were translocated in a truly post-translational fashion and whether this pathway contributes significantly under normal physiological conditions.

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