

# 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- $\alpha$ -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.

**P**ROTEIN translocation across the endoplasmic reticulum in mammalian cells is a co-translational process in which the nascent polypeptide chain is vectorially translocated as it is extruded from the ribosome. This tight coupling between translation and translocation is achieved by an elaborate targeting machinery that recognizes signal sequences on the nascent proteins by interaction with the signal recognition particle (SRP)<sup>1</sup> in the cytoplasm as soon as they emerge from the ribosome (Walter and Blobel, 1981). Interaction of SRP with the SRP receptor assures that the functional attachment of the ribosome to the endoplasmic reticulum membrane occurs early in the synthesis of the nascent protein (Gilmore, et al., 1982; Meyer and Dobberstein, 1982; Connolly and Gilmore, 1986). There the signal sequences are handed over to another recently identified integral membrane protein, the signal sequence receptor (Wiedmann et al., 1987) and translocation of the protein initiates. Since the protein crosses the membrane as it is being made, no folding can occur on the cytoplasmic side of the membrane which could then impede translocation.

In contrast, several laboratories have recently reported that translocation of the precursor of the yeast mating pheromone  $\alpha$ -factor, prepro- $\alpha$ -factor (pp $\alpha$ F), across the endoplasmic reticulum of yeast cells need not be tightly coupled to translation (Hansen et al., 1986; Rothblatt and Meyer, 1986b; Waters and Blobel, 1986). In particular, *in vitro* analysis of the translocation of the pp $\alpha$ F indicated that this protein could be located translocationally after being released from the ribosome (Hansen et al., 1986; Waters and Blobel, 1986).

This mechanism was shown to require membrane proteins in the yeast microsomal vesicles (Hansen, et al., 1986) an energy source supplied as ATP (Hansen et al., 1986; Rothblatt and Meyer, 1986b; Waters and Blobel, 1986) and was stimulated by a soluble factor(s) (Waters et al., 1986).

The requirements for the translocation of pp $\alpha$ F thus resemble those found for bacterial secretory proteins, which can in general be translocated posttranslationally (Date and Wickner, 1981; Koshland and Botstein, 1982; Chen and Tai, 1985; Randall and Hardy, 1987), as well as those described for posttranslational import of mitochondrial protein precursors (Gasser et al., 1982; Pfanner and Neupert, 1985), and hints that the mechanism of transmembrane movement in the different systems may actually be rather similar. In this report we show that posttranslational translocation across yeast microsomal vesicles *in vitro* is not unique to pp $\alpha$ F, although the efficiency with which other proteins use this mode of translocation appears to vary. Fully elongated, yet still ribosome-associated precursor proteins were found to be translocated more efficiently than the proteins which were terminated and released from the ribosome. In contrast to the co-translational mode of translocation, it appears that limitations exist with respect to the folding of protein substrates which do not allow all yeast secretory proteins to be translocated after their synthesis is complete.

## 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

1. *Abbreviations used in this paper:* ER, endoplasmic reticulum; SRP, signal recognition particle.

Stevens, University of Oregon, Eugene, Oregon) which encodes prepro-carboxypeptidase Y, was linearized by digestion at the Pvu I site located beyond the 3' end of the PRC1 coding region (Valls et al., 1987) and the cohesive ends were removed with mung bean nuclease. The resulting plasmid was ligated to Eco RI 8-mer linkers, and digested with excess Eco RI which generated a 2.3-kb fragment containing the PRC1 gene with Eco RI cohesive ends. The 2.3-kb fragment was purified by gel electrophoresis in 1% low melting point agarose then ligated into a SP6 transcription vector pSP64 (Promega Biotec, Madison, WI) which had previously been digested with Eco RI and calf intestine alkaline phosphatase. Plasmid DNA which contained the PRC1 gene in the sense orientation with respect to the SP6 promoter was isolated and digested with Sal I. The resulting fragments were circularized with DNA ligase and digested with Sst II before transformation of *Escherichia coli*. This resulted in the deletion of the majority of the sequences 5' of the coding region of the PRC1 gene including all out-of-frame ATG codons between the SP6 promoter and the PRC1 initiation codon. The major product produced when RNA transcripts derived from this vector were translated in the yeast in vitro translation extract could be precipitated by anti-yeast carboxypeptidase Y antiserum (a gift of Drs. G. Payne and R. Schekman, University of California, Berkeley, CA), confirming that the major translation product was carboxypeptidase Y.

**Invertase.** The vector pS5 containing the entire yeast SUC2 gene, which codes for invertase, inserted behind an SP6 promoter was provided by Drs. J. Ngsee and M. Smith (University of British Columbia, Vancouver, British Columbia, Canada) (Ngsee, J., and M. Smith, unpublished results). A plasmid which contained the first 789 nucleotides of the SUC2 coding sequence followed by an in-frame termination codon was constructed to produce an mRNA encoding the amino-terminal one half of the invertase protein. The plasmid pS5 was digested with Bam HI and Xba I endonucleases which cut the SUC2 gene 786 and 828 nucleotides into the coding sequence, respectively. The resulting recessed ends were filled in using the Klenow fragment of DNA polymerase. Circularization of this vector with T4 DNA ligase created a termination codon at the Bam HI site in frame with the invertase initiating methionine.

**RNA Transcription and Translation In Vitro.** Transcription of all plasmids with SP6 polymerase was carried out as described previously (Hansen, et al., 1986) except that the resulting RNA transcripts were frozen at  $-80^{\circ}\text{C}$  in aliquots immediately after transcription without phenol extraction or ethanol precipitation. The transcripts were translated in the yeast translation system as described previously (Hansen, et al., 1986). Immunoprecipitation with antiserum recognizing carboxypeptidase Y or invertase (a gift of Drs. J. Ngsee and M. Smith, University of British Columbia, Vancouver, B.C. and Dr. R. Schekman, University of California, Berkeley, CA) was carried out as described previously (Hansen, et al., 1986).

**Inhibition of Glycosylation with a Synthetic Peptide Acceptor Molecule.** A synthetic peptide, N-acetyl-asparaginyl-tyrosyl-threonyl-amide (NYT), which is an inhibitor of N-linked core oligosaccharide addition by virtue of its ability to be glycosylated by core oligosaccharide transferase (Lau et al., 1983) was synthesized at the Biomolecular Resource Center at the University of California in San Francisco. To inhibit core glycosylation of glycoprotein precursors the synthetic peptide was included in translocation reactions at a final concentration of 2 mM.

**Preparation of Yeast Post-ribosomal Supernatant Fraction.** After translation was arrested by addition of emetine to a final concentration of 0.1 mM the translation reactions were centrifuged for 30 min at  $4^{\circ}\text{C}$  in a Beckman A-110 rotor in the Beckman Airfuge at 30 PSI.

**Termination of Polypeptide Chains with Puromycin.** To cause polypeptide termination translation reactions were incubated with emetine for 5 min at a concentration of 0.1 mM followed by addition of 1 mM puromycin. After incubation for 15 min microsomal membranes were added. Post-ribosomal supernatant fractions were treated similarly.

**Protease Protection.** At the end of the incubation of in vitro synthesized proteins in the presence or absence of microsomal membranes the reactions were chilled to 0 in an ice bath, then adjusted to 10 mM calcium chloride. After 10 min of incubation equal aliquots of this mixture were removed and incubated with no further additions, with the addition of TPCK-treated trypsin to a concentration of 750  $\mu\text{g/ml}$ , or with the addition of the nonionic detergent Nikkol and trypsin to 1% and 750  $\mu\text{g/ml}$ , respectively. Before use, trypsin was incubated in 10 mM calcium chloride and 10 mM Tris-HCl for 15 min at  $37^{\circ}\text{C}$ . After incubation of these mixtures for 1 h at  $0^{\circ}\text{C}$  the trypsin inhibitor aprotinin was added to 820  $\mu\text{g/ml}$ . After a 10-min incubation at  $0^{\circ}\text{C}$ , three volumes of 15% (wt/vol) trichloroacetic acid containing 2 mM freshly dissolved PMSF were added and the samples were prepared for immunoprecipitation and SDS-electrophoresis as described previously (Hansen, et al., 1986). Radioactive proteins were quantified by densitometric scanning of autoradiograms on a Bio Rad VD 620 Video Densitometer.

**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). Posttranslational 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  $\mu\text{M}$ , 20 mM and 0.2 mg/ml, respectively.

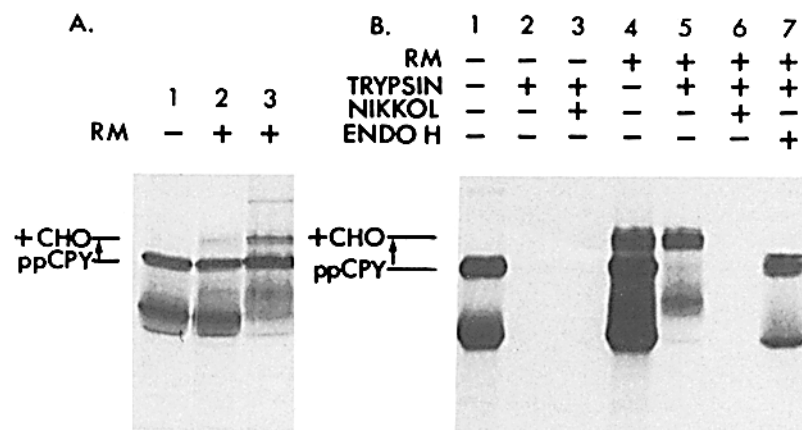
## Results

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

We wondered whether the posttranslational translocation of pp $\alpha$ F 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  $\sim 10\%$ ) 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 pp $\alpha$ F. One possible explanation for this observed difference was the potential for disulfide bond formation in the case of ppCPY which contains 13 cysteines, while pp $\alpha$ F contains no cysteines (Valls et al., 1987; Kurjan and Herskowitz, 1982). Increasing the dithiothreitol concentration to



**Figure 1.** 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).

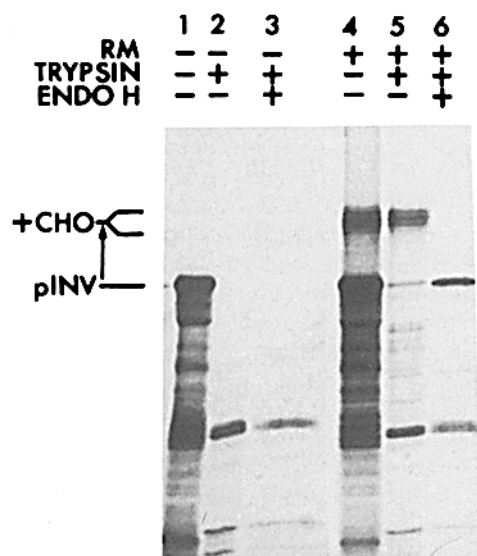
#### Fully Synthesized Yeast Invertase Cannot be Translocated *In Vitro*

The observation that full length ppCPY could be translocated indicated that ppaF is not unique in this respect. To

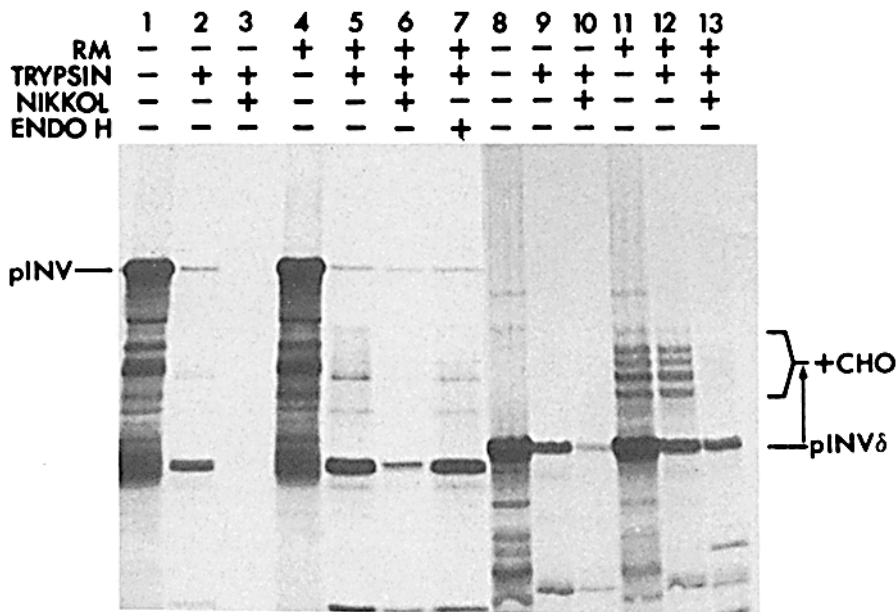
further evaluate the generality of this type of translocation reaction, we examined the behavior of pre-invertase (pINV), the precursor of another yeast secretory protein. pINV is nearly identical in molecular weight to ppCPY and, like CPY, invertase is glycosylated upon its translocation into the ER.

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-translational 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-translational 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.



**Figure 2.** Co-translational translocation of full-length pINV. mRNA encoding pINV was translated in the absence (lanes 1–3) or in the presence (lanes 4–6) of yeast microsomal vesicles. Reactions shown in lanes 2, 3, 5, and 6 were further incubated in the presence of trypsin. After inactivation of the trypsin the reactions shown in lanes 3 and 6 were incubated with endoglycosidase H. Proteins immunoprecipitated by antibody recognizing invertase were then subjected to SDS-PAGE and visualized by autoradiography.



**Figure 3.** pINV $\delta$ , but not pINV can be translocated after having been fully-elongated. Translation reactions were programmed with pINV mRNA (lanes 1-7) or pINV $\delta$  mRNA (lanes 8-13). After 60 min of translation cycloheximide was added and reactions shown in lanes 1-3 and 8-10 were incubated for 60 min in the absence of microsomal vesicles, while reactions shown in lanes 4-7 and 11-13 were incubated in the presence of microsomal vesicles. The reactions shown in lanes 2, 3, 5-7, 9, 10, 12, and 13 were then treated with trypsin. The detergent Nikkol was also included in the reactions shown in lanes 3, 6, 10, and 13. After inactivation of the trypsin the reaction shown in lane 7 was incubated with endoglycosidase H. Proteins immunoprecipitated by antibody recognizing invertase were then subjected to SDS-PAGE and visualized by autoradiography.

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 pp $\alpha$ F 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 pp $\alpha$ F and ppCPY translocation incompetent.

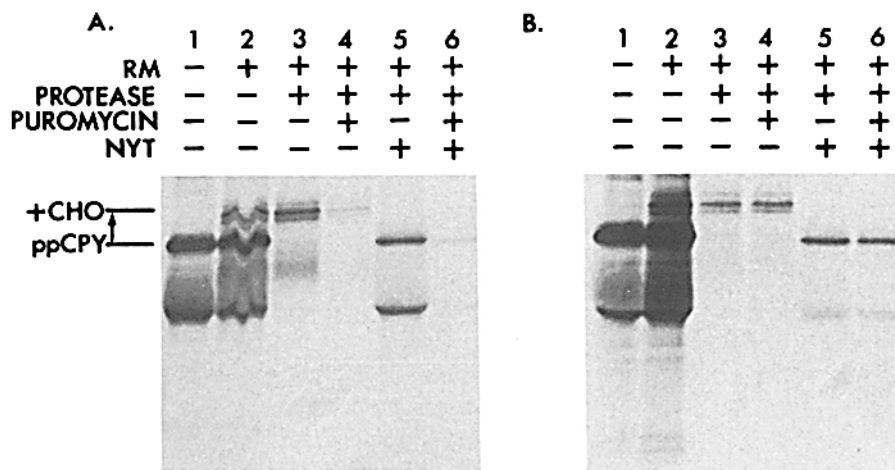
To distinguish between these two possibilities, we decided to alter the structure of pINV by inserting a termination codon into the middle of the coding sequence. When mRNA encoding this truncated pINV was translated in vitro, a protein of the predicted molecular weight was synthesized (Fig. 3, lane 8). In contrast to the complete pINV, some of the truncated product (pINV $\delta$ ) 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 pp $\alpha$ F (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 pINV $\delta$  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 11).

The protein species produced were protected from digestion by exogenous protease in the absence of added detergent (Fig. 3, lane 12), but were digested if detergent was added with the protease (Fig. 3, lane 13). A small amount of pINV $\delta$  remained undigested by protease in lanes 9 and 12, however we can be certain that some pINV $\delta$  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 pINV $\delta$ Can Occur Posttranslationally***

The observation that ppCPY and pINV $\delta$  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 pINV $\delta$  (Fig. 5 A and 5 B) as translocation substrates. Polypeptide elongation directed by the respective mRNAs was inhibited by the elon-



**Figure 4.** (A) Emetine was added to a 60- $\mu$ L translation reaction synthesizing ppCPY to a final concentration of 100  $\mu$ M (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

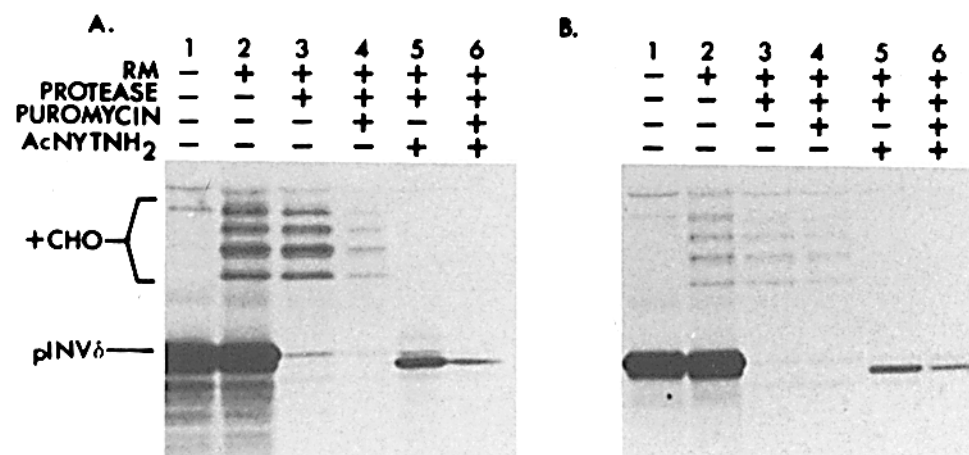
$\mu$ g/ml, respectively) of reactions shown in lanes 3–6. Proteins immunoprecipitated by antibody recognizing carboxypeptidase Y were then subjected to SDS-PAGE and visualized by autoradiography. Puromycin was found to inhibit 90% of the translocation detected. Puromycin also effectively inhibited the 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.

gation inhibitor emetine (Figs. 4 A and 5 A, lane 1). As above, subsequent incubation with yeast microsomal vesicles resulted in the production of glycosylated and protease protected (i.e., properly translocated) pCPY and INV $\delta$  (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 pINV $\delta$  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 pINV $\delta$  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 pINV $\delta$  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



**Figure 5.** The experiment shown in Fig. 6 is identical in all respects to that shown in Fig. 5 except that the messenger RNA used to program the translation reactions encoded pINV $\delta$  and the antibodies used were those recognizing invertase. In A puromycin was found to inhibit 70% of the translocation detected. We attribute the slightly reduced band intensity in B lane 6 as compared with lane 5 to losses during the immunoprecipitation (note that the intensity of the glycosylated species in lanes 3 and 4 are equal).

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 pINV (data not shown), ppCPY and pINV $\delta$  is not a result of altered conformation due to oligosaccharide addition. For pINV (data not shown), ppCPY and pINV $\delta$  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- $\alpha$ -factor (pp $\alpha$ F), two other proteins, prepro-carboxypeptidase Y (ppCPY) and a truncated form of pre-invertase (pINV $\delta$ ), 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 (pINV), 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 pINV 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 pINV (which would certainly prevent it from folding into a structure resembling the native enzyme) rendered the truncated protein (pINV $\delta$ ) 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 pINV $\delta$  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; Perara 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 pp $\alpha$ F which translocates rather efficiently posttranslationally and pINV, the posttranslational translocation of which is not detectable. We cannot rule out, however, that even pINV 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 pp $\alpha$ F (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 (Balchy-Dyson and Stevens, 1987) or into components that may be part of the translocation machinery itself (*sec61*; Deshaies and Schekman, 1987) cytoplasmic pools of proCPY and ppαF 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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