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In vivo and *in vitro* analysis of *ptl1*, a yeast *ts* mutant with a membrane-associated defect in protein translocation

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Mutants defective in the ability to translocate proteins across the membrane of the endoplasmic reticulum were selected in *Trp*$^{-}$ *Saccharomyces cerevisiae* on the basis of their ability to retain a fusion protein in the cytosol. The fusion comprised the prepro region of prepro-α-factor (MFα1) N-terminal to phosphoribosyl anthranilate isomerase (TRP1). The first of the protein translocation mutations, called *ptl1*, results in temperature-sensitivity of growth and protein translocation. At the non-permissive temperature, precursors to several secretory proteins accumulate in the cytosol. Using this mutant, we demonstrate that the prepro-carboxypeptidase Y that had been accumulated in the cytosol at the non-permissive temperature could be post-translationally translocated into the endoplasmic reticulum when cells were returned to the permissive temperature. This result indicates that post-translational translocation of preproteins across endoplasmic reticulum membranes can occur *in vivo*. We have also determined that the temperature-sensitive component is membrane-associated in *ptl1*, and that the membranes derived from this strain show a reversible temperature-sensitive translocation phenotype *in vitro*.

Key words: protein translocation/*ptl1* yeast mutant/endoplasmic reticulum/secretion/intracellular protein transport

Introduction

Translocation into the endoplasmic reticulum (ER) is a common starting point for proteins destined for transport to organelles of the secretory pathway, lysosomes, plasma membrane and secretion. The heterologous cell-free translocation assay (Blobel and Dobberstein, 1975) has facilitated the analysis of translocation and resulted in the characterization of a number of participating proteins. These include the signal recognition particle (SRP) (Walter and Blobel, 1981a,b; Walter *et al.*, 1981), its receptor, the docking protein (Meyer *et al.*, 1982; Gilmore *et al.*, 1982) signal peptidase–purified as a complex–(Evans *et al.*, 1986), the putative signal sequence receptor (SSR; Wiedmann *et al.*, 1987) and a protein associated with the docking protein (Tajima *et al.*, 1986). The binding of ribosomes to the ER appears to depend on an unidentified membrane protein (Hortsch *et al.*, 1986). There may be other proteins in the ER that are involved in translocation, but, without successful reconstitution of translocation from fractionated proteins of the ER, it is difficult to identify them.

An alternative approach to identifying proteins required for translocation is to obtain translocation-defective mutants of the yeast *Saccharomyces cerevisiae*. This would overcome the limitations to identifying as yet unfractionable proteins in the ER membrane. The yeast cell-free translocation assay (Rothblatt and Meyer, 1986a; Hansen *et al.*, 1986; Waters and Blobel, 1986) can then be used to determine if and how the products of genes identified by yeast mutations are directly involved in translocation.

A proven procedure for the isolation of translocation mutants in microbial systems depends on the incorrect localization of an artificial fusion protein in the mutants. In the case of a translocation mutant, the fusion protein is transported out of the cytosol in the wild-type strain, but can be conveniently detected accumulating in the cytoplasm of mutant strains. Several export mutants have been selected in *Escherichia coli* using such an approach (for review, see Beckwith and Ferro-Novick, 1986; Ito, 1986). In yeast, defective translocation of a HIS4C-based gene fusion protein resulted in the survival of mutants on a suitable growth medium (Deshaies and Schekman, 1987). The *S.cerevisiae* TRP1 gene encodes phosphoribosyl anthranilate (PRA) isomerase, a cytoplasmic enzyme required for tryptophan biosynthesis. In this study, the N-terminal fusion of the prepro region of MFα1 to TRP1 caused this enzyme to be translocated out of the cytosol in a *trp1* yeast host strain. Mutants were selected that accumulate sufficient fusion protein in the cytosol, to reverse the *Trp*$^{-}$ phenotype. This mislocalized TRP1 activity was unable to support growth of the host strain in the absence of exogenous tryptophan. Mutants obtained in this way have been designated *ptl* for *p*rotein *t*rans*l*ocation-defective.

We describe here *in vivo*, and *in vitro* analysis of a novel temperature-sensitive mutant, *ptl1*. *In vivo*, intracellular protein transport was reduced substantially, with different secretory proteins accumulating in the cytosol at the non-permissive temperature. Shifting growth conditions to the permissive temperature restores transport and enabled the demonstration that protein translocation can occur post-translationally *in vivo*. Examination of the *ptl1* mutant in a cell-free system localized the site of the defect to the membranes (as opposed to the cytosol). Furthermore, isolated membranes from *ptl1* show a reversible *ts* translocation phenotype *in vitro*.

Results

The mutation *ptl1* was first isolated in the yeast strain J51, which survived the selection described in Materials and methods. The strain J51-5c, derived from crossing J51 with J30a, was used for all of the experiments described below (for strains, refer to Table I in Materials and methods). *ptl1* is recessive and is conditionally lethal at 37°C (data not shown).

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ptl1 IS TEMPERATURE - SENSITIVE FOR TRANSLOCATION

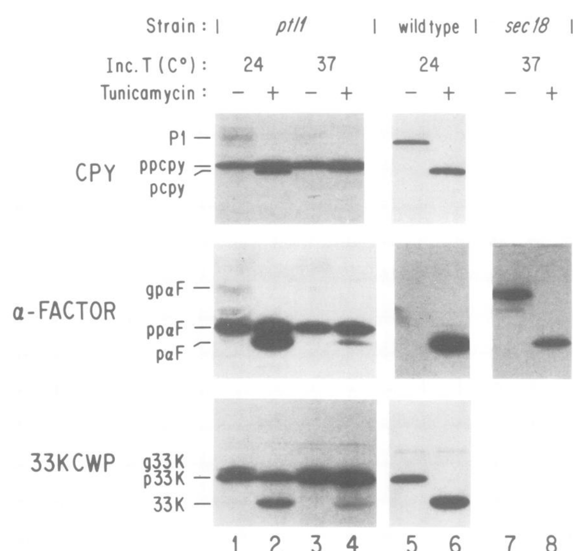

Fig. 1. *ptl1* is temperature-sensitive and pleiotropic for pre-secretory protein accumulation. Appearance of pre-secretory proteins increases dramatically in *ptl1* cells grown at 37°C. In this experiment, glycosylated (processed) and unprocessed forms of preproteins are discriminated by a change in mobility caused by the presence of tunicamycin (**lanes 2** and **4**). At 37°C, however, the amounts of unprocessed forms are significantly increased (**lanes 3** and **4**). Strain J51-5c (*ptl1*) in log phase was preincubated for 1 h at 24°C (**lanes 1** and **2**) or 37°C (**lanes 3** and **4**) and then labelled for 5 min at the same temperature with [^{35}S]sulfate. Tunicamycin at 15 μg/ml was added 30 min before labelling (**lanes 2** and **4**). Cell contents were solubilized using 1% SDS and vortexing with glass beads. Immunoprecipitates of CPY, ppαF and the 33kCWP were prepared and separated by SDS−PAGE on 10%, 18%/4 M urea and 13% acrylamide gels respectively. The proteins were visualized fluorographically. The same proteins immunoprecipitated from the parental strain GY65α[YlpαT] at 24°C are shown (**lanes 5** and **6**) and from *sec18* at 37°C (**lanes 7** and **8**) for ppαF only.

ptl1 exhibits a temperature-sensitive, pleiotropic accumulation of pre-secretory proteins

In order to establish the pleiotropy of the mutant *ptl1*, we pulse labelled cells at the permissive and non-permissive temperatures for 5 min in the presence and absence of tunicamycin. Treatment with tunicamycin results in the accumulation of unglycosylated secretory proteins in the endoplasmic reticulum thereby blocking their further intracellular transport. This enables the visualization of all material that had been translocated into the ER as unglycosylated, processed (signal peptide cleavage) forms, and its direct comparison with unprocessed forms. We chose three proteins to study; each ones precursor enters the secretory pathway at the level of the ER, but has a different ultimate destination. These include the vacuolar protein carboxypeptidase Y (CPY), α-factor which is secreted into the medium, and a cell wall glycoprotein of 33 kd apparent mol. wt (33kCWP, Sanz *et al.*, 1987). As can be seen in Figure 1, the unprocessed forms of all three of these proteins represent ~50% of the total labelled material in *ptl1* cells grown at 24°C (compare lanes 1 and 2 with 5 and 6). At the non-permissive temperature of 37°C, however, unprocessed material represents >95% of the total (lanes 3 and 4). It seems most likely, in light of these results, that the bulk of cellular pre-secretory proteins are not translocated into the ER at the non-permissive temperature in *ptl1*.

Pre-secretory proteins accumulate in the cytosol in *ptl1*

A determination of the sub-cellular localization of the accumulated precursors was undertaken. Untranslocated proteins, located in the cytosol, should be accessible to exogenously-added proteases and degraded after cells have been gently lysed. Cells were incubated at the non-permissive temperature for a period where both precursor and processed forms could be observed, pulse-labelled, and spheroplasts were prepared and lysed and the resulting extracts subjected to proteolysis with proteinase K. Immunoprecipitation was carried out to examine the various forms of CPY and α-factor that remained. As can be seen in Figure 2, panel A, the precursor (ppCPY), but not the glycosylated (ER) form of CPY (p1) was degraded. The same was observed in the case of α-factor (panel B, lanes 1−5), where accumulated prepro-α-factor was degraded. By comparison, the (glycosylated) ER form, accumulated in the mutant *sec18* at a non-permissive temperature, was completely protected under the same conditions (lanes 6−10). From these data we conclude that the precursors accumulating at the non-permissive temperature in *ptl1* are localized in the cytosol. It must be pointed out, however, that this type of experiment cannot allow one to distinguish between precursors that are soluble in the cytosol, and ones that may be specifically bound to the ER membrane, but not translocated.

Translocation, and not subsequent transport steps are blocked in *ptl1*

As a first step in verifying that the *ptl1* mutation specifically affects translocation, and does not produce a defect in transport processes in general, we chose to follow the transport of pulse-labelled prepro-CPY to the vacuole. In this study, wild-type and *ptl1* cells were pulsed and chased at the non-permissive temperature. Treatment with endoglycosidase H was used to reveal the intracellular location of the various forms of CPY. As can be seen in Figure 3, the mutant accumulates an endo H-resistant form of CPY, not seen in wild-type, that corresponds to prepro-CPY (ppCPY). It is converted very slowly to the ER and Golgi forms (p1,p2) and to the mature form (mCPY) and still represents a major species at the end of the chase period (60 min). In contrast, p1, p2 is chased to the mature form with similar kinetics in mutant and wild-type cells (10 min on average). This implies that translocation is the rate-limiting step, and that transport out of the ER and as far as the processing step in the vacuole is unaffected. From these data one can also calculate that in *ptl1*, at the non-permissive temperature, the appearance of mature CPY takes 6−10 times longer than in the wild-type at the same temperature.

Post-translational translocation can occur *in vivo*

Numerous reports have appeared recently describing the ability, especially in yeast, of full-length preproteins to cross ER-derived membranes in the absence of protein synthesis (for review, see Zimmermann and Meyer, 1986). Although a small amount of post-translational translocation has been seen in the case of mutant preproteins *in vivo* (Blachly-Dyson and Stevens, 1987), efficient post-translational translocation has been observed only *in vitro*. These findings have always raised the question as to whether translocation can occur post-translationally *in vivo*. The fact that *ptl1* efficiently accumulates precursors in the cytosol at the non-permissive

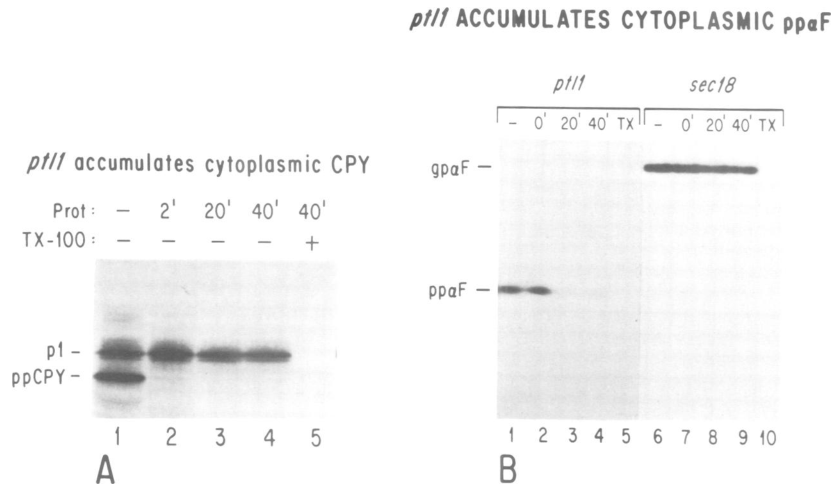

Fig. 2. *ptl1* accumulates CPY and prepro-α-factor in the cytosol. (**A**) CPY. In pulse-labelled extracts of *ptl1* in which the p1 (ER) form and the cytoplasmic precursor (ppCPY) were detected, ppCPY was sensitive to protease whereas p1 was protected. Thus ppCPY is not sequestered within membrane vesicles. Strain J51-5c (1.25 OD_{600} units) was labelled with [^{35}S]sulfate and cell extracts prepared by spheroplasting and lysis. The cell extract was divided into 50-μl aliquots. To four of the aliquots (**lanes 2–5**), proteinase K was added to a concentration of 100 μg/ml. Incubation was at room temperature for the times indicated. Triton X-100 was present at a concentration of 0.5% in one of the two aliquots that were incubated for 40 min with proteinase K (**lane 5**). The reaction was stopped with 1 mg/ml PMSF on ice for 5min. CPY was immunoprecipitated, separated by SDS–PAGE and detected by fluorography. (**B**) ppαF. Radiolabelled extracts of *ptl1* and *sec18* contain cytoplasmic ppαF (**lane 1**) and ER gpαF (**lane 6**) respectively. The cytoplasmic form is sensitive (**lanes 3** and **4**) to protease whereas the ER form is sensitive (**lane 10**) but protected (**lanes 8** and **9**). Thus ppαF is not sequestered within membranes in *ptl1*. Strains J51-5c (*ptl1*) and RH288 (*sec18*) in log phase on synthetic medium were preincubated for 15 min at 37°C and [^{35}S]sulfate was then added for 15 min. Cell extracts were made by spheroplasting and lysis. The extracts were divided into 50 μl aliquots and proteinase K, 0.1 mg/ml, was added to four of them (**lanes 2–5** and **7–10**). One aliquot also had Triton X-100, 0.5% (**lanes 5** and **10**). Incubation was at room temperature for the times indicated. The digestion was stopped on ice by 5 min with 1 mg/ml PMSF. Protein was immunoprecipitated with anti-ppαF, separated by SDS–PAGE and fluorographed.

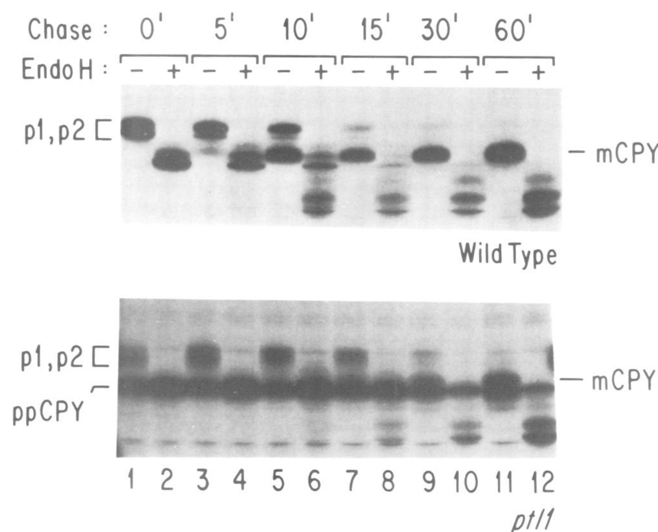

Fig. 3. CPY translocation, but not subsequent transport, is markedly reduced in *ptl1*. A pulse–chase experiment of CPY shows the biogenesis of vacuolar CPY (mCPY) via ER and Golgi (p1,p2). All these forms are glycosylated and endoglycosidase H digestions permit visualization of mCPY. The *ptl1* mutant accumulates a form having a unique mobility and endo H insensitivity that corresponds to preproCPY (ppCPY). This form is chased very slowly to p1,p2, but with normal kinetics to mCPY. Strains GY65b (**upper panel**) and J51-5c (**lower panel**) were grown on synthetic medium containing 2% glucose as the carbon source. They were preincubated for 15 min at 37°C and labelled for 5 min with [^{35}S]sulfate. Labelling was stopped with 5 mM ammonium sulfate and 20 mg/l of L-methionine and L-cysteine. Samples of 0.5 OD_{600} units of cells were taken at the times indicated. Cell extracts were prepared with SDS and glass beads. CPY was immuno-isolated and incubated prior to electrophoresis either in the absence (**odd numbered lanes**) or the presence of endo H (**even numbered lanes**).

temperature enables one to do the experiment needed to answer this question. To assist in the visualization and quantitation of translocation, experiments were carried out in a *ptl1–pep4* strain. In *pep4* strains CPY is not processed to its mature form, which would otherwise migrate similarly to the prepro-CPY on acrylamide gels. When *ptl1* cells were pulse-labelled at the non-permissive temperature, prepro-CPY accumulated (Figure 4, lanes 1–3). Based on

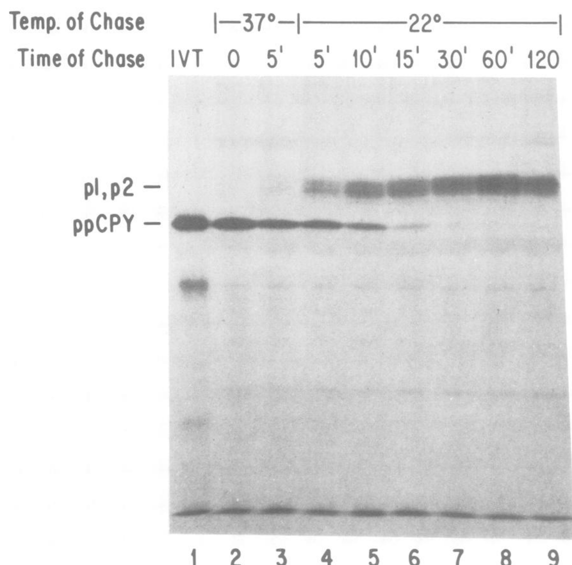

Fig. 4. Post-translational translocation can occur *in vivo*. The cytoplasmic precursor of CPY was allowed to accumulate in *ptl1 in vivo* at 37°C. After protein synthesis was blocked, the accumulated CPY translocated into the ER and became glycosylated when cells were shifted to 22°C. A derivative of J51-5c in which PEP4 gene had been deleted was used for this experiment. The absence of PEP4 prevents cleavage of p1,p2-CPY to the mature form thus simplifying the number of CPY species observed. Cells were preincubated at 37°C for 75 min and then pulse-labelled with [^{35}S]sulfate at the same temperature for 5 min. Further labelling and protein translation was stopped by adding chase mix (5 mM ammonium sulfate, 20 mg/ml L-methionine and L-cysteine final concentrations) and cycloheximide to 0.1 mg/ml. Samples were taken for immunoprecipitation of CPY at 0 and 5 min of chase at 37°C. The chase was continued at 22°C and samples were taken for immunoprecipitation of CPY at 5, 10, 15, 30, 60 and 120 min. Immunoprecipitates were separated by SDS−PAGE and detected by fluorography. **Lane 1** shows the *in vitro* translation (IVT) product of a yeast cell-free synthesis system programmed with synthetic mRNA encoding ppCPY.

the data shown in Figure 2, this represents cytosolic prepro-CPY. Cells were then chased in medium containing high amounts of unlabelled sulfate, methionine and cysteine, together with a sufficient concentration of cycloheximide to inhibit protein synthesis completely. At 5 min into the chase, cells were shifted to the permissive temperature and CPY transport was followed. With increasing times of chase, the cytosolic forms disappeared in conjunction with the appearance of the ER and Golgi forms, p1 and p2 (lanes 4−9). These data clearly show that the post-translational translocation of a protein (at least) as large as 50−60 kd occurs efficiently *in vivo*.

Membranes from ptl1 have a thermoreversible translocation defect that can be demonstrated in vitro

A great advantage in being able to study translocation in an homologous yeast cell-free system is being able to discriminate the subcellular localization of a particular defect. Moreover, one is also in a position to assess whether a mutation has a direct, and hence a rapidly appearing−and possibly reversible−effect on translocation. To this end, we isolated cytosolic and microsomal fractions from both wild-type and *ptl1* cells. Preliminary studies demonstrated that prepro-α-factor, synthesized in lysates from either type of cell, was translocated and glycosylated to normal levels only into wild-type membranes (data not shown). This result suggested that the defect in *ptl1* cells is membrane-associated, and not present in the cytosol. As it is not possible to carry out the traditional lysate-based translocation assay at the non-permissive temperature (A.R.Hibbs and D.I.Meyer, unpublished observations), we made use of a lysate-free assay using affinity-purified preproteins that allows translocation at 37°C *in vitro*.

When affinity-purified proOmpA is rapidly diluted out of 8 M urea, it is efficiently translocated across yeast microsomes and processed without the addition of any cytosolic factors other than ATP (Sanz and Meyer, 1988). This assay works equally well at 24 or 37°C. Making use of this assay, we have determined that the defect in *ptl1* membranes is both temperature sensitive and reversible *in*

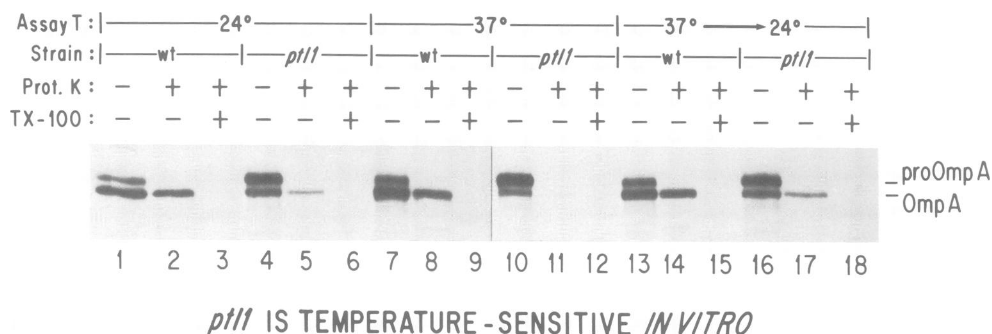

Fig. 5. Cell-free translocation into *ptl1* membranes is reversibly inhibited by temperature. Affinity-purified bacterial ProOmpA can translocate into yeast membrane preparations in the absence of cytosol, when diluted out of 8 M urea. Translocation is accompanied by the processing of proOmpA to OmpA, as indicated. Translocation was confirmed by protection of the mature OmpA from proteinase K, added where indicated. Translocation by *ptl1* membranes *in vitro*, is reduced compared to wild-type in assays conducted at 24°C (compare **lanes 4−6** with **lanes 1−3**). At 37°C, *ptl1* membranes are translocation-incompetent (**lanes 10−12**), whereas wild-type membranes show no change at this temperature (**lanes 7−9**). When *ptl1* membranes−inactivated by incubation at 37°C−were shifted back to 24°C, translocation was fully restored to pre-incubation levels (compare **lanes 16−18** with **lanes 4−6**). Membranes from J51-5c (*ptl1*) or ABYS1 (*PTL*) were preincubated for 5 min at either 24 or 37°C (**lanes 1−12**). proOmpA was then added at the same temperature for 10 min. To test if the inhibition of translocation at 37°C is reversible, one aliquot of membranes that had been preincubated for 5 min at 37°C was first shifted to 24°C for 10 min before proOmpA was added at the same temperature for another 10 min (**lanes 13−18**). Each reaction was aliquoted three ways for addition of Proteinase K and Triton X-100 as indicated. Proteins were separated by SDS−PAGE and the gel prepared for fluorography.

vitro. The data depicting these findings are shown in Figure 5. When proOmpA was diluted out of urea into wild-type microsomes, it was translocated and processed to OmpA at assay temperatures of either 24°C (lanes 1–3) or 37°C (lanes 7–9). Membranes from *ptl1* have a reduced ability to translocate proOmpA *in vitro*, compared to wild-type at 24°C (lanes 4–6), which is similar to that seen *in vivo* (Figure 1). These membranes become virtually translocation-incompetent when assayed at 37°C (lanes 10–12). This block was reversed upon shifting the assay temperature back to 24°C (compare lanes 16–18 with 10–12 and 4–6). It also appears as though the processed proOmpA is more sensitive to protease in protection assays performed on mutant membranes compared to wild-type. This could reflect either a greater fragility of *ptl1* membranes, or that one characteristic of the mutant phenotype is the insertion and processing, but not complete translocation, of some of the proOmpA. Further experiments are required to discriminate between these possibilities. Nonetheless, these data strongly support the idea that the product of the *PTL1* gene is a membrane component directly involved in translocation.

ptl1 is a new translocation mutant

We have carried out a genetic analysis to see if *ptl1* is allelic to either of the translocation mutations previously described, *sec61* or *sec62* (Deshaies and Schekman, 1987). The mutant *sec61* was found to complement *ptl1* for accumulation of cytoplasmic prepro-CPY (the diploid has no mutant phenotype). Furthermore, 35% of the spores resulting from the *sec61* × *ptl1* cross were wild-type with regards to precursor accumulation, indicating that the genes are non-allelic. Another mutation, *sec62*, is known to be tightly linked to the gene *PEP4*, and hence on chromosome XVI (R.Deshaies, personal communication). Using the method devised by Wakem and Sherman (unpublished data), we have localized *ptl1* to chromosome XV. Subsequent linkage studies place *ptl1* about 25 cM distal to the *HIS3* locus (data not shown). From these data we concluded that *ptl1* is not allelic to either *sec61* or *sec62*.

Discussion

The results of this study describe a new mutant, *ptl1*, which is defective in the ability to translocate nascent secretory proteins across the membrane of the rough ER. The mutant shows a temperature-sensitive membrane defect that is exhibited both *in vivo* and *in vitro*. Taking advantage of the mutant's capacity to accumulate full-length precursors at the non-permissive temperature, we have demonstrated that post-translational translocation can occur *in vivo*. Since the defect in *ptl1* is membrane-associated, the genetic approach has given us a handle on a membrane component that might not have been easily identifiable via biochemical means.

Several components of the translocation machinery have been isolated and characterized from mammalian sources (for review see Hortsch and Meyer, 1986; Walter and Lingappa, 1986). As the yeast system seems to show some inherent mechanistic differences, such as a greater ability to translocate proteins post-translationally (Rothblatt and Meyer, 1986b; Hansen *et al.*, 1986; Waters and Blobel, 1986), it is worthwhile to isolate and characterize the molecules that mediate this process. Indeed in the short period of time since translocation research began using yeast, both genetics and biochemistry have provided insights. Cytosolic proteins, such as hsc70, have been implicated in maintaining structural features of preproteins necessary for translocation (Deshaies *et al.*, 1988; Chirico *et al.*, 1988). The same authors report that additional cytosolic factors are required to fully reconstitute translocation. Signal sequence-mediated recognition clearly occurs in yeast, but putative receptors have not been identified. As the studies described here show, membrane components are clearly required. This is backed up by biochemical evidence demonstrating that microsomes treated with the proteolytic enzyme papain lose their ability to translocate both proOmpA and prepro-α-factor (Sanz and Meyer, 1988). Additionally, a gene encoding the yeast signal peptidase, *SEC11*, has been cloned and analysed (Bohni *et al.*, 1988). Two other translocation mutants have been isolated, *sec61* and *sec62* (Deshaies and Schekman, 1987), but the subcellular location of their defects has yet to be reported.

Strong evidence for the direct participation of the product of the *PTL1* gene in translocation lies in the fact that the membranes derived from *ptl1* are reversibly temperature-sensitive for translocation *in vitro*. The rapid conversion from translocation-competent to incompetent and back speaks against a defect that indirectly affects translocation through synthesis of membrane components needed in this reaction. To the best of our knowledge, this represents the first case of a reversible temperature-sensitive translocation defect exhibited *in vitro*. The closest one has come to isolating such a mutant is the example of *secY* in *E.coli* where it was shown that membranes isolated either from cells grown at the non-permissive temperature or at the permissive temperature and subsequently shifted to a non-permissive one were translocation-defective *in vitro* (Bacallao, *et al.*, 1986; Fandl and Tai, 1987). In these instances however, the defect could not be reversed *in vitro* by shifting to a permissive temperature as was seen for membranes from *ptl1*.

In vivo studies carried out with *ptl1* allowed us to demonstrate that post-translational translocation can occur *in vivo* in a eukaryotic organism. It has been possible to show that the translocation of preproteins from several sources across both mammalian and yeast microsomes can occur post-translationally *in vitro*. The common theme has been the necessity to preserve a translocation-competent conformation of the preprotein. This is accomplished by cytosolic factors such as heat shock-like proteins in the case of yeast (Deshaies *et al.*, 1988; Chirico *et al.*, 1988) and reticulocyte lysates (Zimmermann *et al.*, 1988), and by SRP and the ribosome (Ainger and Meyer, 1986; Siegel and Walter, 1988) or by SRP alone (Crooke *et al.*, 1988; Sanz and Meyer, 1988) in heterologous systems. Prepro-α-factor can be efficiently translocated across yeast microsomes *in vitro*, and this reaction has been studied in great detail both from the point of view of the requirements of the prepro-α-factor and of the translocation system (Rothblatt and Meyer, 1986b; Hansen *et al.*, 1986; Waters and Blobel, 1986; Waters *et al.*, 1986; Rothblatt *et al.*, 1987; Garcia and Walter, 1988). Although some but not all yeast preproteins have been shown to be capable of being translocated post-translationally *in vitro*, the consensus has shown prepro-α-factor to be exceptional in its efficiency. The results presented here show that a protein that is post-translationally translocated with low efficiency (Hansen and Walter, 1988) or not at all (Rothblatt *et al.*, 1987) in a yeast cell-free system, namely prepro-

CPY, can be quantitatively shunted out of the cytosol and into the secretory pathway as a full-length precursor.

The long-term significance of *ptl1* resides in the fact that the temperature-sensitive translocation (and hence, growth) phenotype will enable the isolation of the wild-type allele by complementation. In this way the biochemical studies can commence that will result in the characterization of the role played by the Ptl1p in translocation.

Materials and methods

Growth conditions

In liquid culture, yeast was grown on either YPD (0.5% yeast extract, 1% bactopeptone and 2% glucose) or on synthetic medium. The salts and vitamins in this synthetic medium were the same composition as the salts and vitamins in the yeast nitrogen base (Difco), except that all sulfates were replaced by chlorides so that [^{35}S]sulfate could be used for labelling. The cultures were swirled vigorously in conical flasks or glass culture tubes at 24°C. For labelling with [^{35}S]sulfate, cells were swirled vigorously in 15 ml disposable polypropylene screw-cap tubes. On plates, yeast was grown on either YPD containing 2% agar (from Difco) or minimal medium prepared from yeast nitrogen base, 2% glucose and amino acid supplements depending on the strain. A list of yeast strains used in this study is given in Table I.

SDS–PAGE of proteins

Immunoprecipitated carboxypeptidase Y was separated on 10% polyacrylamide, 33-kd cell wall protein on 13% polyacrylamide and prepro-α-factor on either 18% polyacrylamide containing 4 M urea (Figure 1) or on 13% polyacrylamide (Figure 2B). Immunoblotting was carried out according to Hortsch *et al.* (1985).

^{35}S labelling of yeast proteins in vivo

A 2-ml preculture of yeast was grown on YPD to stationary phase. Synthetic medium, 10–20 ml, containing 50 μM ammonium sulfate and amino acid supplements, was then inoculated with 1/100 volume of the YPD preculture. The yeast was incubated at 24°C to a density of OD_{600} = 1–2, harvested by centrifugation and washed twice in distilled water. The cells were resuspended in the same synthetic medium lacking sulfate at a cell density of OD_{600} = 5.0. After 15–75 min preincubation at the desired temperature, 100 μCi of [^{35}S]sulfate was added per 50 μl of culture. For pulse–chase experiments, a 1/10 volume of 50 mM ammonium sulfate, 0.2 g/l L-methionine, 0.2 g/l L-cysteine was added 5 min after the [^{35}S]-sulfate. For experiments in which labelled cell extract was to be prepared in the absence of detergent, cell metabolism was stopped by 0.1% sodium azide at 0°C for 5 min.

Table I. Yeast strains used in this study

Name	Genotype	Source
ABYS1	MATα *pra1 prb1 prc1 cps1 ade*	D.Wolf, Freiburg
GY65a	MATa *ura3-52 lys2-801 ade2-101* Δ*trp1-901*	G.Cesareni, EMBL, Heidelberg
GY65α	MATα *ura3-52 lys2-801 ade2-101* Δ*trp1-901*	G.Cesareni EMBL, Heidelberg
J30a	MATa *ura3-52* Δ*trp1-901 leu2-3,112 his4*	This study
J51 [YlpαT,URA3]	MATα *ura3-52 lys2-801 ade2-101* Δ*trp1-901 ptl1-1*	This study
J51-5c	MATα *ura3-52 ade2-101* Δ*trp1-901 leu2-3,112 his4 ptl1-1*	This study
RDM7-4B	MATα *ura3-52 leu2-3,112 his4 trp1 sec61-1*	R.Deshaies & R.Schekman, Berkeley
RH228	MATα *his4 sec18*	H.Riezman, Biozentrum, Basel

Preparation of ^{35}S-labelled cell extracts

After labelling, preparation of cell extract for proteinase K treatment was done by spheroplasting followed by lysis without detergent. Labelled cells were resuspended in 100 mM Tris sulfate, pH 9.4, 10 mM DTT at a density of OD_{600} = 1. They were swirled at room temperature for 5 min. The cells were again collected by centrifugation and resuspended at a density of OD_{600} = 1 for cell wall digestion in 1.2 M sorbitol, 20 mM potassium phosphate, pH 7.5, 10 mM DTT containing 1000 U/ml of oxalyticase. The cells were incubated with gentle mixing for a further 15 min at room temperature. The resulting spheroplasts were collected by centrifugation for 2 min at 1000 *g* and resuspended in 0.5 M sorbitol, 20 mM Hepes, pH 7.4, 50 mM potassium acetate, 2 mM EDTA, 1 mM DTT at a density equivalent to OD_{600} = 5.0 of the original cells. Lysis was assisted by 15 strokes in a tight-fitting glass homogenizer. Unlysed cells and spheroplasts were removed by centifugation for 2 min at 1000 *g*. Extracts were aliquoted before treatment with proteinase K, as detailed in the figure legends. After proteinase K treatment, two volumes of extraction buffer (see below) were added and samples heated to 95°C for 5 min before adding dilution buffer (see below) for immunoisolation of proteins. For experiments not involving addition of proteinase K, cell extract was prepared by adding 150 μl of extraction buffer (1% SDS, 20 mM Tris, pH 7.5, 5 mM EDTA, 10 mM DTT, 1 mg/ml PMSF) per 50 μl labelling and vortexing at top speed for 2 min with glass beads (type V, 450–500 microns, Sigma).

Immunoprecipitation of ^{35}S-labelled proteins

Four volumes of dilution buffer (1.25% Triton X-100, 60 mM Tris, pH 7.5, 190 mM NaCl, 6 mM EDTA, 12.5 mM IAA) were added to cell extracts and insoluble material was removed by centrifugation for 5 min at 16 000 *g* in a bench top centrifuge. One half of the new volume of 20% calf serum containing 1 μl of antiserum per original 0.25 OD_{600} unit of yeast was then added. Incubation from 4 to 20 h at 4°C was followed by 30 min mixing with 20 μl of a 1:1 suspension of protein A Sepharose CL-4B beads in 10 mM Tris, pH 7.5. The beads were washed four times with 1 ml of 0.1% Triton X-100, 0.02% SDS, 50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA. They were boiled in sample buffer to release protein for SDS–PAGE. Digestion with endoglycosidase H was carried out as described (Rothblatt and Meyer, 1986a).

Antisera

The antisera used were all rabbit polyclonal. Anti-prepro-α-factor was raised against a *lacZ* fusion protein (Rothblatt and Meyer, 1986a). Anti-carboxypeptidase Y was a generous gift from Dr G.Payne (UCLA). Anti-33kCWP was supplied by Dr P.Sanz (Sanz *et al.*, 1987).

Phosphoribosyl anthranilate (PRA) isomerase assay

This assay is based on a procedure communicated to us by Dr K.Kirschner and K.Lüger (Biozentrum, Basel, Switzerland). Preparation of cell extracts (carried out on ice): yeast cells were harvested from a log phase culture at 2–6 × 10^7 cells per ml and 0.3 ml of cell pellet was mixed with 1 ml of 0.5% Triton X-100, 100 mM triethanolamine, pH 8.6. Then 0.5 ml of glass beads (450–500 microns, Sigma) were added and the mixture was vortexed for 2 min. The cell homogenate was removed from the beads and centrifuged for 2 min at 16 000 *g* in a bench top centrifuge at 4°C. The clear extract was kept on ice or frozen at −80°C without loss of PRA isomerase activity. Assay: the reaction was monitored by the change in absorbance at 350 nm. Conversion of PRA to CdRP [1-(*o*-carboxyphenylamino)-1-deoxyribulose-5-phosphate] is accompanied by an absorbance increase at 350 nm of 1.8 per mM per cm. The substrate, PRA, was prepared by the method of Creighton (1968). In a double beam spectrophotometer 0.9 ml of 100 mM triethanolamine, pH 8.0, was added to each cuvette with 5–200 μl of extract. When the absorbance became stationary, 100 μl of PRA, ~0.25 mM final assay concentration, was added to one of the cuvettes. A minimum of 1 unit per assay can be detected this way.

Chemical mutagenesis of yeast

Yeast from a YPD plate was resuspended in 100 mM sodium phosphate, pH 7.0, to a concentration of OD_{600} = 5.0. Ethyl methanesulfonate (EMS) was added to a concentration of 3% and mixed in vigorously. The cells were mixed at 30°C for 1 h. The EMS was inactivated by adding 50 ml of 5% sodium thiosulfate. The cells were collected by centrifugation, washed once with distilled water and spread onto selective plates at 1 × 10^6 cells per plate.

Cell-free translocation of proOmpA

ABYS1 (2 μl) or 2 μl of *ptl1* membranes (40 units/ml) (for membrane

preparation see Rothblatt and Meyer, 1986a) were mixed with 2 μl of energy mix (30 mM ATP, 1.8 M creatine phosphate, 2.4 mg/ml creatine phosphokinase) and 37 μl of buffer A [40 mM Hepes−KOH pH 7.4, 162 mM KOAc, 5 mM $Mg(OAc)_2$] and were preincubated at the corresponding temperature (25 or 37°C) for 5 min. Then 3 μl of [^{35}S]proOmpA (in 8 M urea, 30 000 c.p.m./μl), preincubated at the requisite temperature, were added to the samples and these were further incubated at 25 or 37°C for 10 min. The samples were then cooled at 0°C and treated with proteinase K (0.4 mg/ml final concentration) for 30 min. PMSF (1 mM final concentration) was added to the samples and the mixtures were left at 0°C at 5 min more. The samples were then mixed with electrophoresis sample buffer, heated to 95°C for 5 min and analysed by SDS−PAGE and fluorography.

Selection of mutants defective for protein translocation

See the Introduction for an explanation of the principle of the mutant-selection procedure. A gene fusion (called MFα1TRP1) between the prepro region of prepro-α-factor (ppαF) as far as the *Hin*dIII site and the entire TRP1 gene from the *Ava*II site was made. The fusion protein was confirmed to have the expected mobility of 38 kd by cell-free translation, and could be translocated and glycosylated by yeast membranes *in vitro* (for methods see Rothblatt and Meyer, 1986a). The fusion protein was expressed in yeast using a derivative of the shuttle-vector pEMBLyex4, putting its expression under the control of the GAL10 promoter (making the vector YIpαT). The derivative of pEMBLyex4 used lacks 2 μ sequences necessary for autonomous replication in yeast and can consequently be stably integrated into the LEU2 locus. It was confirmed by pulse-labelling that the fusion protein is efficiently translocated and glycosylated by the ER *in vivo*. A PRA isomerase assay (see Materials and methods) was used to confirm that the fusion protein is enzymatically active and is expressed under the control of the GAL10 promoter. The phenotype in the absence of tryptophan of *trp1* yeast containing the fusion protein was growth in the presence of 2% galactose and no growth in the presence of 2% glucose or mixtures of glucose and galactose. Because an identical system containing the non-secretory TRP1 gene under the control of the same GAL10 promoter allows growth of *trp1* yeast in the absence of galactose and tryptophan, it was presumed that expression from the GAL10 promoter is just very low in the absence of galactose; too low for detection by the PRA isomerase assay employed here but high enough to sustain growth in the absence of tryptophan. Therefore, knowing that the fusion protein MFα1TRP1 is efficiently secreted, at least part of the reason that *trp1* yeast containing this fusion protein do not grow in the absence of galactose (2% glucose) is its efficient removal from the cytoplasm. In the presence of galactose its expression is extremely high and sufficient enzymic activity for growth apparently remains in the cytoplasm, even in wild-type cells. Conditions for selection of translocation-defective mutations were chosen; strain GY65α with the integrated expression vector was chemically mutagenized with EMS to increase the incidence of mutations. Survivors were selected on minimal medium plates at 22°C for growth on 2% glucose in the absence of tryptophan. The survival rate is ~1 in 100 000. Of these, 5% were also temperature-sensitive for growth on YPD at 37°C. About 10% of the temperature-sensitive survivors also have translocation phenotypes. One of these, strain J51, contains the mutation *ptl1*, which is described in the results. Analysis of 32 tetrads resulting from a cross of J51-5c with GY65a showed 2:2 cosegregation of ppCPY accumulation and temperature sensitivity of growth at 37°C on YPD.

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