

Signal Sequences Specify the Targeting Route to the Endoplasmic Reticulum Membrane

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Abstract. In the yeast *Saccharomyces cerevisiae*, only a subset of preproteins that are translocated across the ER membrane require the function of the signal recognition particle (SRP), suggesting that an alternative, SRP-independent pathway must exist (Hann, B.C., and P. Walter. 1991. *Cell*. 67:131–144). We have established that the two targeting pathways function in parallel. Mutant alleles of *SEC62* and *SEC63* were isolated that specifically impaired the translocation of SRP-indepen-

dent preproteins *in vivo* and *in vitro*, whereas SRP-dependent preproteins were unaffected. Based on this analysis, preproteins fall into three distinct classes: SRP dependent, SRP independent, and those that can use both pathways. Pathway specificity is conferred by the hydrophobic core of signal sequences. Our studies show a previously unrecognized diversity in ER-directed signal sequences, that carry structural information that serves to identify the route taken.

THE first steps of the eukaryotic secretory pathway include targeting and translocation of nascent proteins across the ER membrane. *In vitro* analyses using components largely derived from mammalian sources (for reviews see Walter and Johnson, 1994; Rapoport et al., 1996) have revealed that targeting is catalyzed by the signal recognition particle (SRP)¹ and its membrane-localized receptor. The translocation events are carried out by a multi-subunit membrane protein complex, the translocon or Sec61p complex (Görlich and Rapoport, 1993). Mechanistically, translocation is cotranslational: SRP binds signal sequences as they emerge from the ribosome, pauses translation, and targets the nascent chain/ribosome complexes to the ER membrane where they are docked onto the translocon.

A posttranslational mode of translocation has also been reported for mammalian systems, but neither the mechanism nor components involved have been characterized (Schlenstedt et al., 1990). These observations suggest that SRP-mediated targeting is not the only route through which proteins enter the secretory pathway.

Analyses of protein translocation across the ER membrane in budding yeast have reinforced the view that SRP, SRP receptor, and translocon components are conserved among all eukaryotic cells (Hann et al., 1989; Görlich et al., 1992; Ogg et al., 1992; Stirling et al., 1992; Brown et al., 1994). Surprisingly, however, yeast cells are viable in the

absence of SRP and SRP receptor. Although the loss of SRP function results in translocation defects for a number of ER-targeted proteins, the extent of the defect is variable from one protein to another: at one extreme, translocation of the vacuolar protease dipeptidyl aminopeptidase B (DPAP B) is severely affected; at the other extreme, carboxypeptidase Y (CPY) is unaffected (Hann and Walter, 1991). Furthermore, cells lacking SRP function “adapt” over time and thereby gain the ability to translocate all proteins that have been examined so far (Ogg et al., 1992). From these observations, it was hypothesized that an alternative or salvage pathway must exist to translocate proteins in the absence of SRP function.

In contrast to higher eukaryotes, components involved in a posttranslational mode of translocation have been identified in yeast whose functional equivalents in other organisms have yet to be characterized. These include Sec62p, Sec63p, Sec71p, and Sec72p (Deshaies and Schekman, 1989; Rothblatt et al., 1989; Green et al., 1992; Feldheim et al., 1993; Feldheim and Schekman, 1994) which form a complex (Sec62/63p complex) at the ER membrane. This complex, together with the Sec61p complex (the membrane translocon), and Kar2p (BiP) associated with the ER lumen mediate translocation. Using *in vitro* reconstitution assays, these were the only membrane constituents required to support a posttranslational mode of translocation into artificial proteoliposomes (Brodsky and Schekman, 1993; Panzner et al., 1995). Interestingly, some mutations in genes encoding these proteins, as with SRP mutants, cause variable defects in translocation efficiency depending on the substrate (Deshaies and Schekman, 1989; Feldheim and Schekman, 1994). The significance of these observations, however, was unclear. Because only a small number of substrates have been examined in these sys-

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1. *Abbreviations used in this paper:* CPY, carboxypeptidase Y; DPAP B, dipeptidyl aminopeptidase B; SRP, signal recognition particle.

tems, it was not known what role the Sec62/63p complex plays in the overall trafficking of secretory pathway proteins in yeast.

To examine the role of multiple translocation pathways (defined here to include both targeting and membrane translocation steps), we devised a genetic strategy to select for mutants specifically defective in an SRP-independent route to the ER. From this selection, we obtained mutants that severely impair the translocation of substrates unaffected in cells lacking SRP function while leaving SRP-mediated translocation intact. By combining genetic and biochemical approaches, we have dissected two pathways: one dependent and the other independent of SRP. Using this system to examine a wide variety of endogenous substrates, we show that both pathways are required to process the full complement of secretory pathway proteins. Analysis of recombinant chimeric substrates demonstrates that the hydrophobic core of signal sequences serves as an "address" for entry into either pathway. This is surprising since signal sequences show no primary structure conservation suggesting that they have little specific information content (von Heijne, 1985; Kaiser et al., 1987). Thus, our studies show a previously unrecognized diversity in ER-directed signal sequences, which carry specific structural information that serves to identify the route taken.

Materials and Methods

Strains Used in this Study

YJL183 (*MAT α* , *ura3 Δ 99*, *leu2 Δ 1*, *trp1 Δ 99*, *ade2-101^{ochre}*), YJL184 (*MAT α* , YJL183 background) from Dr. J. Li, University of California, San Francisco; W303 (*MAT α* , *leu2-3-112*, *his3-11*, *trp1-1*, *ura3-1*, *can1-100*, *ade2-1*); DNY53 (YJL183, pDN106), DNY54 (YJL184, pDN106), DNY66 (DNY54, *sec63-201*), DNY70 (DNY54, *sec62-101*), DNY116 (DNY54, *sec61-101*) from this study; SOY162 (W303, *MAT α* , *srp102::HIS3*) [SRP Δ] from Dr. S. Ogg, University of California, San Francisco; PS886 (*MAT α* , *trp1*, *leu2*, *ura3-52*, *pep4-3*, *prb*, *prc*, *bar1::LEU2/BAR1*) from Dr. P. Sorger, Massachusetts Institute of Technology; RSY458 (*MAT α* , *sec65-1*, *ura3-52*, *ade2-1*, *trp1- Δ 1*, *leu2-3-112*, *his3*) from Dr. R. Schekman, University of California, Berkeley.

Plasmids Used in This Study

Construction of pDN106 and pDN107. The reporter gene termed *CU* was constructed by fusing sequences encoding the amino-terminal 110 amino acids of *PRC1* (CPY) and its promoter in frame to *URA3* with glycine replacing the *Ura3p* initiator methionine as a linker. This recombinant gene was inserted in the centromeric vector pRS313 (Sikorski and Hieter, 1989) creating the plasmid pDN106. A variant of pDN106, pDN107, was created by replacing the codon for leucine₁₁ with that for aspartic acid. pDN106 was then transformed into YJL183, a strain that bears a complete deletion of *URA3* creating the strain DNY53.

Construction of Plasmids Encoding Chimeric Reporter Proteins. The expression vector, pDN201, was constructed by inserting the strong constitutive GPD (glyceraldehyde-3-phosphate dehydrogenase) promoter and the actin terminator into the centromeric vector YCp50. Coding sequences of recombinant genes to be expressed were inserted into unique *BamHI* and *XbaI* sites contained between promoter and terminator sequences. Coding sequences of reporter proteins were constructed as follows: (a) D_N reporter protein: Using the wild-type *DAP2* (DPAP B) gene as a template, a *BamHI* site was introduced upstream of the initiator ATG and an *NcoI* site upstream of the termination codon by PCR amplification. An *NcoI/XbaI* fragment containing the HA epitope tag was ligated in frame with the cleaved PCR product into pDN201 generating the plasmid pDN317. (b) CD_N reporter protein: Using *DAP2* as a template, a 5' PCR primer encoding the hydrophobic core of the CPY signal sequence (first 18 amino acids) fused to Lysine₄₆ of DPAP B (sequence complementary through Histidine₅₁) was used to amplify the amino-proximal region. A *BamHI/*

Clal fragment of the PCR product encoding the amino-terminal portion of the fusion protein was subcloned into pDN317 to create pDN321. (c) α -F reporter protein: Using the *MfaI* gene as the template, a fragment bearing a *BamHI* site upstream of the initiator codon and an *NcoI* site upstream of the terminator was generated by PCR. Along with an HA epitope tag bearing *NcoI/XbaI* fragment, it was inserted into pDN201 generating the plasmid pDN229. pDN229 encodes prepro- α -factor with a carboxyl-terminal HA epitope tag. (d) D_N- α -F reporter protein: Using *DAP2* as the template, the first PCR step used the D_N 5' primer and a 3' primer encoding the designed DPAP B/ α -F fusion junction (5'-GAATTTGTGC-CDGTTTCTATACTTTTGTAGCAAC-3') to generate the 5' megaprimer for the second step. The PCR second step and subsequent cloning was performed as for the α -F construct but using the megaprimer as the 5' primer. The resulting expression vector was designated pDN280. (e) D_{HC}- α -F reporter protein: Using the D_N- α -F coding sequences as a template, a 5' PCR primer encoding the first two residues of prepro- α -factor fused to hydrophobic core sequences of DPAP B was used to amplify the gene. The amplified fragment inserted into pDN201 generated the plasmid pDN314.

For in vitro studies, coding sequences were inserted into *BamHI* and *XbaI* sites in pSP65 generating plasmids pJD75 (D_N- α -F) and pJD82 (D_{HC}- α -F). Myc epitope-tagged *PHO8* was inserted into pSP65 as a *BamHI-PstI* fragment from pTPPP creating plasmid pJD76. *PRC1* coding sequences were amplified by PCR from yeast genomic DNA and inserted between the *SmaI* and *HindIII* sites of pSP65 creating plasmid pJD77.

Genetic Selection

DNY53 cells were mutagenized with UV light irradiation at rates resulting in 30 and 57% lethality. 1.4×10^7 cells irradiated to 30% lethality and 3.4×10^7 cells irradiated to 57% lethality were plated out in various densities on SC media lacking uracil and incubated at 22°C. Over the course of 5 d after plating, a total of 240 *Ura*⁺ colonies were picked and restreaked onto selective media. Only the larger colonies were picked; many colonies that grew slowly because they were sick or only slightly *Ura*⁺ were not included in this first group. Of the 240 isolates, 59 were eliminated due to slow growth on SC-uracil media. The rest were crossed to a wild-type strain (W303) to form diploids. The dominant or recessive nature of the mutants was determined by assessing the ability to confer uracil prototrophy in the heterozygous diploids. The growth of diploid cells on selective media indicated three mutant categories: (1) dominant: growth indistinguishable from haploid mutant; (2) semi-dominant: some growth but worse than the haploid mutant; (3) recessive: growth identical to wild-type cells carrying the reporter. 95 mutants fell in the first two categories and were not examined further. The remaining 86 mutants were then tested for the ability of centromeric plasmids carrying previously cloned genes involved in translocation to complement the mutant phenotype. Plasmids carrying *SEC61*, *SEC62*, *SEC63*, *SEC71*, *SEC72*, and *KAR2* were introduced into each isolate by transformation and then tested for growth on media lacking uracil. *Ura*⁻ indicated that the introduced plasmid complemented the mutant defect.

Isotopic Labeling and Immunoprecipitation

Metabolic labeling and immunoprecipitation assays were performed exactly as described (Ng and Walter, 1996). All yeast cultures were grown and labeled at 30°C except for *sec65-1* mutant cells which were grown to log phase at 22°C and shifted to 37°C 25 min before labeling at 37°C. Monospecific polyclonal antisera were used for immunoprecipitation of endogenous proteins. Och1p and Pho8p were expressed as Myc epitope-tagged proteins from plasmids pUOCHM (Chapman and Munro, 1994) and pTPPP (both gifts of Dr. R. Chapman, University of Cambridge, England). These proteins were immunoprecipitated with the anti-Myc 9E10 monoclonal antibody.

In Vitro Protein Translocation Assay

Yeast translation extract was prepared from strain PS886 as described (Deshaies and Schekman, 1989) except that cells from 9l YPD at OD₆₀₀ = 2.5–3.5 (35–45 g) were lysed in liquid nitrogen (Brown et al., 1994) and buffer A (Deshaies and Schekman, 1989) was added (0.5 ml/g) to the cell lysate after thawing. YTE was depleted of SRP by immunoaffinity chromatography on a column of anti-Sec65p antibodies (Brown, J.D., and P. Walter, unpublished results). yRM were isolated essentially as described (Brodsky et al., 1993) with the following modifications: Cells from 6 l of

YPD at $OD_{600} = 2.5\text{--}3.5$ were spheroplasted by digestion with 15 μg zymolyase 100-T for 40 min in 200 ml 50 mM KPi, pH 7.5, 1.4 M sorbitol, 10 mM DTT; spheroplasts were resuspended in 35 ml lysis buffer and homogenized with 15 slow strokes of a motorized homogenizer (Potter; B. Braun, Germany); the cell lysate was centrifuged sequentially 7,000 rpm for 15 min, at 12,000 rpm for 15 min, and then cleared at 17,000 rpm for 30 min in a Sorval SS-34 rotor, the pellet from the last spin contained translocation competent microsomes; after washing, the membranes were resuspended in membrane storage buffer (MSB, 250 mM sorbitol, 50 mM KOAc, 1 mM DTT, 20 mM Hepes-KOH, pH 7.4), at $OD_{280} = 40$ (determined in 1% SDS), frozen in liquid nitrogen, and stored at -80°C . Before use, yRM were treated sequentially with 20 mM Hepes, pH 7.4, 250 mM sucrose, 25 mM KOAc, 25 mM EDTA, pH 8.0, 1 mM DTT (15 min on ice) and 20 mM Hepes, pH 7.4, 250 mM sucrose, 500 mM KOAc, 1 mM DTT (60 min on ice). After this treatment membranes were washed twice with MSB and resuspended to the original volume in MSB. mRNA was synthesized from linearized plasmids using the SP6 Megascript kit (Ambion Inc., Austin, TX) as recommended by the manufacturer.

Translation reactions (33% YTE, 10% yRM, 170 mM KOAc, 31 mM Hepes-KOH, pH 7.4, 2.7 mM $\text{Mg}(\text{OAc})_2$, 2.7 mM DTT, 83 mM sorbitol, 25 mM creatine phosphate, 0.75 mM ATP, 0.1 mM GTP, 0.04 $\mu\text{g}/\text{ml}$ yeast tRNA, 40 mM amino acids without methionine, 5 μCi [^{35}S]methionine, 2.1 μg creatine kinase, and an amount of mRNA determined empirically for each message as required for maximal efficiency translation), were carried out for 1 h at 25°C .

Results

To obtain mutants that affect the SRP-independent protein translocation pathway, we devised a genetic selection based on principles similar to those used previously (Manoil and Beckwith, 1985; Deshaies and Schekman, 1987). The strategy uses a chimeric reporter consisting of a normally cytosolic enzyme fused to a targeting domain that directs translocation of the enzyme into the ER lumen, where it is inactive. Mutations that impair proper translocation of the reporter result in its "mislocalization" to the cytosol, where it is active. Thus, translocation mutants can be selected by subjecting cells bearing the reporter to conditions under which growth requires the functional enzyme (Fig. 1 A).

Our reporter gene, designated *CU*, encodes the amino-terminal 110 amino acids of prepro-CPY fused to sequences encoding orotidine-5'-phosphate decarboxylase, Ura3p (Fig. 1 A). As a targeting signal, we chose the amino-terminal portion of prepro-CPY, because its translocation across the ER membrane is insensitive to the loss of SRP and SRP receptor function (Hann and Walter, 1991; Ogg et al., 1992), and because its signal sequence is required for translocation (Blachly-Dyson and Stevens, 1987). The reporter enzyme, Ura3p, functions in the biosynthetic pathway of uracil and is required for cell growth in the absence of exogenously supplied uracil. Ura3p is inactive when targeted to mitochondria, suggesting that its localization in the cytosol is required for activity (Maarse et al., 1992).

The *CU* reporter gene was placed under control of a constitutive promoter in plasmid pDN106, which was transformed into *URA3*-deleted cells. The growth of the resulting strain, DNY53, was indistinguishable from wild-type cells on rich media (not shown) or on medium selecting for the plasmid (Fig. 1 B). In contrast, on medium lacking uracil, DNY53 cells failed to grow, indicating that the reporter enzyme was inactive (Fig. 1 B), presumably because of its localization to the ER lumen. This phenotype was indeed due to the presence of a functional signal

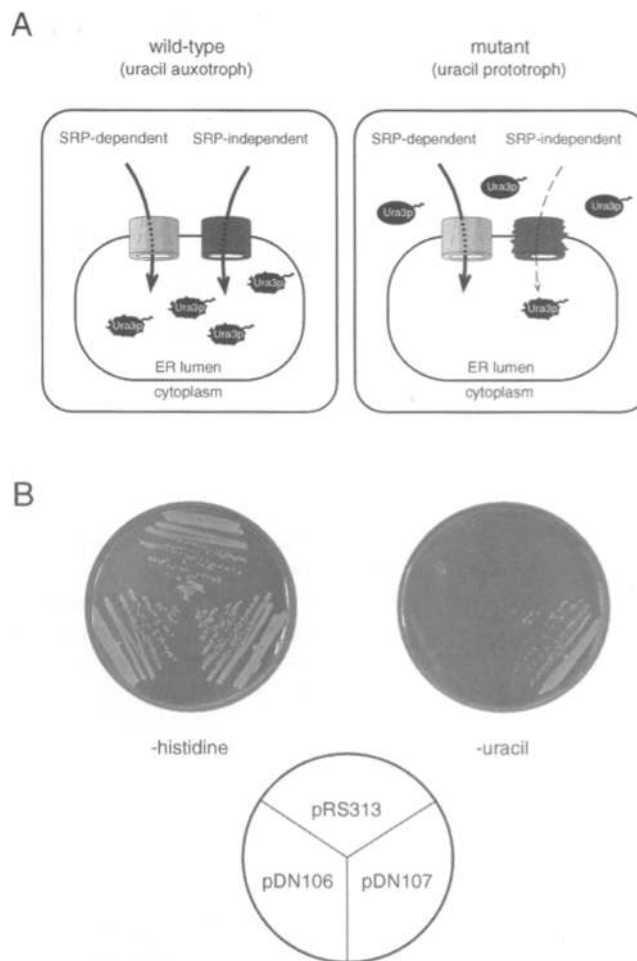


Figure 1. Selection for mutants of the SRP-independent pathway. (A) Schematic representation of selection strategy. In (*ura3 Δ*) wild-type cells the CPY-Ura3p (CU) reporter protein localizes to the ER lumen where it is inactive (crinkled Ura3p). These cells are uracil auxotrophs. In mutants of the SRP-independent pathway CU is mislocalized to the cytosol where it is active (smooth Ura3p). These cells are uracil prototrophs and grow on medium lacking uracil. (B) Growth of reporter strain under selective conditions. Wild-type YJL183 cells (*ura3 Δ*) transformed with pRS313 (vector control), pDN106 (pRS313 carrying the *CU* reporter), and pDN107 (pRS313 carrying the *CU* reporter with a defective signal sequence) streaked onto synthetic media lacking histidine ($-\text{histidine}$, selecting for the vector) or lacking uracil ($-\text{uracil}$, selecting for the reporter).

sequence as cells expressing the *CU* protein with a mutated and therefore inactive signal sequence grew on media lacking uracil (Fig. 1 B, cells bearing plasmid pDN107).

To ascertain whether the *CU*-encoded reporter enzyme specifically monitors SRP-independent translocation, plasmid pDN106 was transformed into the SRP mutant strains *sec65-1* and *srp54- Δ 1* (Hann and Walter, 1991; Stirling et al., 1992). In both cases, the reporter failed to confer growth in the absence of uracil, suggesting that ER translocation of the reporter enzyme was not (or not sufficiently) perturbed in these strains. Thus as designed, the *CU* reporter gene fulfilled the necessary requirements to monitor SRP-independent protein translocation and was

unlikely to detect mutations that impair SRP or SRP receptor function.

Mutants Specific for SRP-independent Translocation

We selected 86 mutants of strain DNY53, all of which were shown to be recessive (see Materials and Methods). These mutants fell into four complementation groups affecting previously known genes each encoding a protein involved in translocation: *sec61* (33 isolates), *sec62* (21 isolates), *sec63* (24 isolates), and *kar2* (8 isolates). As discussed below, many of the *sec61* mutants showed pleiotropic translocation defects, whereas all characterized *sec62* and *sec63* mutants consistently showed translocation defects for a specific subset of proteins (see below). This latter phenotype would be expected for mutants that selectively block an SRP-independent pathway. Two representative alleles, *sec62-101* and *sec63-201*, were analyzed in more detail contrasted with the previously described conditional SRP mutant *sec65-1* (Stirling et al., 1992). The *sec65-1* mutant was chosen because SRP function in these cells is rapidly lost at the restrictive temperature. Protein translocation defects are therefore not diminished due to adaptation as seen in SRP-disrupted strains (Ogg et al., 1992).

Mutants were analyzed directly for translocation defects of endogenous substrates. The proteins chosen for analysis were prepro- α -factor and prepro-CPY (both SRP independent), preKar2p (partially SRP dependent), and DPAP B (SRP dependent). In addition, a number of other proteins were examined whose dependence on SRP activity for translocation had not previously been assessed. Cells were pulse-labeled with [³⁵S]methionine, the various forms of translocation substrate proteins collected by immunoprecipitation with specific antibodies, and the immunoprecipitated proteins analyzed by SDS-PAGE. Defects were monitored as the accumulation of precursor proteins lacking glycosylation and/or signal sequence cleavage, both of which occur in the ER lumen (Hann and Walter, 1991).

As shown in Fig. 2, *sec62-101* and *sec63-201* mutants displayed virtually complete translocation blocks of prepro-CPY (Fig. 2 A, lanes 2 and 3, ppCPY). Only minor traces of the translocated form of CPY (gpCPY) were visible on fluorographs after prolonged exposures. We observed very similar translocation defects for α -factor, Gas1p (a glycosylated phospholipid-anchored surface protein) (Nuoffer et al., 1991), and PDI (the ER-luminal protein disulfide isomerase) (LaMantia et al., 1991). In each case, virtually all the labeled protein species in the mutant cells migrated as unglycosylated precursor (Fig. 2, A–D, lanes 2 and 3), indicative of a severe translocation block. The translocation of these proteins was only slightly affected, if at all, in *sec65-1* cells after shift to the nonpermissive temperature (Fig. 2, lane 4) where SRP-dependent protein translocation is blocked.

In striking contrast, the translocation of the SRP-dependent protein DPAP B and another, previously uncharacterized, translocation substrate Pho8p (both type II integral membrane proteins) was not affected in the *sec62-101* and *sec63-201* mutant cells (Fig. 2, G–H, compare lanes 2 and 3 with lane 1). Translocation of both of these proteins was, however, severely affected in *sec65-1* cells shifted to the nonpermissive temperature.

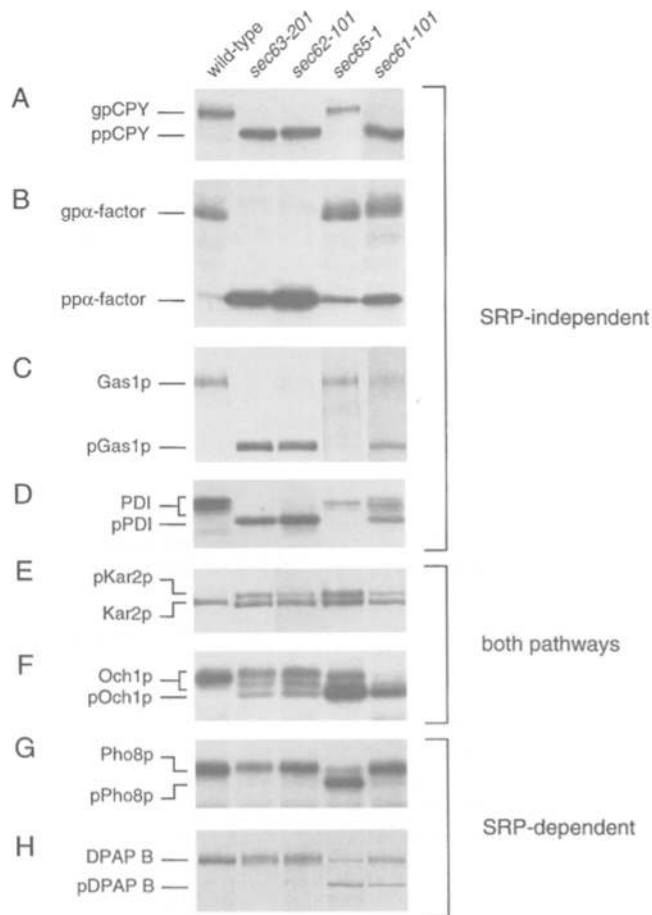


Figure 2. In vivo translocation assays. Cells as indicated were pulse-labeled with [³⁵S]methionine for 5 min before lysis and labeled proteins were immunoprecipitated from the extracts with specific antisera. Labeling was performed at 30°C except for *sec65-1* cells which were labeled at 37°C. Relevant portions of each autoradiogram are shown. (A) CPY: gpCPY, glycosylated pro-CPY (luminal); ppCPY, prepro-CPY (cytoplasmic). (B) α factor pheromone: gp α -F, glycosylated pro- α -factor (luminal); pp α -F, prepro- α -factor (cytoplasmic). (C) GPI-anchored cell surface protein: Gas1p (luminal); pGas1p, pre-Gas1p (cytoplasmic). (D) Protein disulfide isomerase: PDI (luminal); pPDI, pre-PDI (cytoplasmic). (E) Kar2p (BiP): Kar2p (luminal); pKar2p, pre-kar2p (cytoplasmic). (F) α -1,6-mannosyltransferase: Och1p (luminal); pOch1p, pre-Och1p (cytoplasmic). (G) Alkaline phosphatase: Pho8p (luminal); pPho8p, pre-pho8p (cytoplasmic). (H) Dipeptidyl aminopeptidase B: DPAP B (luminal); pDPAP B, pre-DPAP B (cytoplasmic).

Two further translocation substrates, Kar2p and Och1p (α 1,6-mannosyltransferase, a Golgi resident type II integral membrane protein), showed partial translocation defects in *sec62-101* and *sec63-201* mutant cells. A significant fraction, but not all of the labeled Kar2p was recovered as the slower migrating precursor form (Fig. 2 E, compare lanes 2 and 3 with lane 1; Kar2p is not glycosylated and the slower mobility of the precursor form represents the presence of an uncleaved signal sequence). Similarly, a significant fraction but not all of the labeled Och1p was recovered as the faster migrating, unglycosylated precursor form (Fig. 2 F, compare lanes 2 and 3 with lane 1). Just as in the *sec62-101* and *sec63-201* mutant strains, Kar2p and Och1p

showed partial translocation blocks in the SRP-deficient cells (Fig. 2, E–F, compare lane 1 with lane 4).

Taken together, these results provide strong evidence that the SRP-independent pathway requires functions of Sec62p and Sec63p that are not required for SRP-dependent translocation. Moreover, the results show that translocation substrates can belong to one of three distinct classes: (1) those that use the SRP-independent pathway exclusively, (2) those that require both pathways for efficient translocation, and (3) those whose efficient translocation is dependent on SRP.

In contrast to the selective phenotype displayed by all mutations that we isolated in *SEC62* and *SEC63*, most cells bearing *sec61* mutations showed pleiotropic translocation defects. The *sec61-101* allele, for example, displayed translocation defects for all translocation substrates belonging to each of the three classes (Fig. 2, A–H, lane 5). Interestingly, the *sec61-101* mutant only slightly affected Pho8p translocation indicating that different proteins may have different requirements at the translocon per se. As the Sec61 complex is required for posttranslational translocation in yeast (Panzner et al., 1995) and cotranslational translocation in mammals (Görlich and Rapoport, 1993), this observation supports the view that Sec61p is a component of the translocation pore that serves both pathways. A yeast homologue of Sec61p, Ssh1p, was recently shown to be part of a novel ER membrane complex involved in protein translocation (Finke et al., 1996). In light of the existence of two distinct pathways to the ER membrane, it seemed reasonable that different, albeit related, translocon complexes function specifically for each pathway. However, in a strain deleted of *SSH1* no translocation defects were observed for any of the substrates examined in Fig. 2 including Pho8p (Ng, D.T.W., and P. Walter, unpublished results). This observation supports further the notion that Sec61p is the major translocon constituent for both pathways.

Dissection of the Two Translocation Pathways In Vitro

To dissect the two translocation pathways indicated by the above mutants in more detail, we established an in vitro translocation system. We examined the translocation of two substrates, CPY and Pho8p, that use the SRP-independent and SRP-dependent pathway, respectively, under conditions predicted to disable either pathway or both. To this end, we combined microsomal membrane fractions isolated from wild-type or mutant cells with SRP-containing or SRP-depleted cytosol fractions (see Materials and Methods).

When CPY and Pho8p were synthesized in extracts programmed with synthetic mRNAs, full-length proteins were obtained in the absence of microsomal membranes (Fig. 3, A and B, lane 1). In the presence of wild-type membranes, slower migrating species were generated (Fig. 3, A and B, lane 2). Endoglycosidase H digestion and protease protection assays confirmed that the slower migrating forms corresponded to the translocated, glycosylated forms of CPY and Pho8 (data not shown). When the same reactions were performed with microsomal membranes prepared from *sec63-201* or *sec62-101* cells, translocation of CPY was blocked (Fig. 3 A, lane 3), whereas translocation of Pho8p

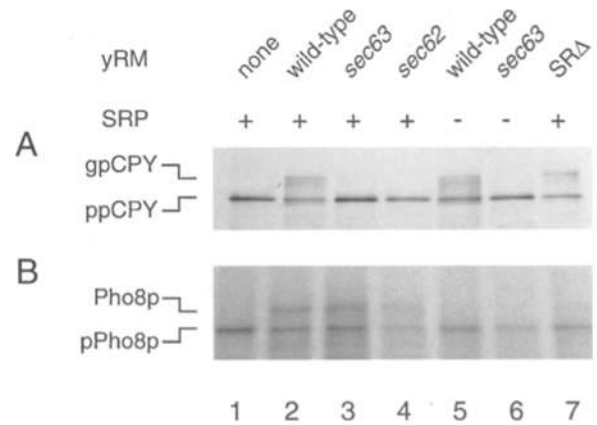


Figure 3. In vitro dissection of translocation pathways. In vitro translation reactions were performed using synthetic mRNAs encoding either preproCPY (A) or prePho8p (B) yeast cytosolic translation extract (YTE) with or without microsomal membranes (yRM) from wild-type or mutant cells as indicated. YTE either contained (lanes 1–4 and 7) or was immunodepleted of (lanes 5 and 6) SRP. Translation products were immunoprecipitated with either 9E10 (anti-Myc) monoclonal (Pho8p) or anti-CPY polyclonal antibodies as described (Hansen et al., 1986). Untranslocated (pPho8p and ppCPY) and processed, translocated (Pho8p and gpCPY) proteins are indicated. A small amount of residual Pho8p that was translocated in the absence of SRP was apparent after long exposures of the gel and is eliminated when wild-type membranes are replaced with *sec63-201* membranes (lane 6). In lanes 2, 5, and 7, the two bands indicated as gpCPY correspond to signal sequence–cleaved and uncleaved glycosylated forms and the minor band below ppCPY corresponds to a translocated, signal sequence–cleaved, unglycosylated form.

was unaffected (Fig. 3 B, lane 3). In contrast, translation of CPY and Pho8p mRNAs in SRP-depleted extracts in the presence of wild-type membranes allowed efficient translocation of CPY (Fig. 3 A, compare lanes 2 and 5), whereas translocation of Pho8p was strongly inhibited (Fig. 3 B, compare lanes 2 and 5). Identical results to those using SRP-depleted cytosol were obtained when SRP-dependent translocation was eliminated by using microsomal membranes prepared from cells lacking SRP receptor (Fig. 3, A and B, compare lanes 5 and 7). These results mirror those obtained from the in vivo experiments where the *sec63* and *sec62* mutations only affected translocation of SRP-independent substrates.

Signal Sequences Determine Translocation Pathway Use

The results described so far provide strong evidence for two distinct physiologically relevant translocation pathways leading to the ER membrane. The information that specifies the entry of a translocation substrate into either one of the two pathways could reside in the signal sequence, in the main body of the protein, or in both. To test this directly, we carried out two experiments. First, we asked if the CPY signal sequence alone (rather than the amino terminal 110 amino acids used in our selection) could direct a protein into the SRP-independent pathway. To this end we replaced sequences encoding the amino-terminal portion of DPAP B including the transmem-

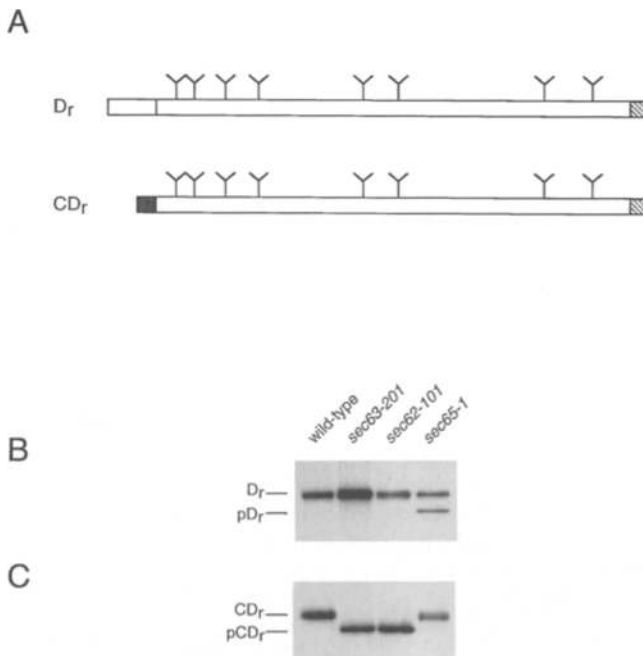


Figure 4. The CPY signal sequence can direct an SRP-dependent protein into the SRP-independent pathway. (A) Schematic representation of constructs. DPAP B sequences are in white, the CPY signal sequence as a stippled box, and the HA epitope-tag as hatched. Ys mark the sites for N-linked glycosylation used to monitor translocation. D_r (B) and CD_r (C) proteins were expressed in wild-type and mutant cells and assayed for translocation as described in Fig. 2. Positions of the cytoplasmic precursor (pD_r and pCD_r) and processed luminal (D_r and CD_r) forms of the proteins are shown. Designations were confirmed by endoglycosidase H digestion (not shown). Proteins were immunoprecipitated using anti-DPAP B antisera as it was more efficient than anti-HA antibodies for these proteins. Because the recombinant proteins are expressed in great excess over endogenous DPAP B, they are the only clearly visible species.

brane/signal sequence region with those encoding the CPY signal sequence (Fig. 4 A). Both the CPY signal sequence-DPAP B fusion (designated CD_r) and overproduced recombinant DPAP B (designated D_r) were efficiently translocated when expressed from plasmids transformed into wild-type cells (Fig. 4, B and C, lane 1). However, unlike endogenous DPAP B and D_r which were both strongly SRP-dependent (Fig. 2 H and Fig. 4 B), translocation of CD_r was not affected in *sec65-1* cells but was strongly inhibited in *sec63-201* and *sec62-101* cells. Thus, CD_r required the SRP-independent pathway (Fig. 4 C, lanes 2–4), and the CPY signal sequence was sufficient to commit a normally SRP-dependent protein to the SRP-independent pathway.

In a reciprocal experiment, we investigated the ability of the amino-terminal regions of DPAP B to direct a protein into the SRP-dependent translocation pathway. We replaced the sequences encoding the prepro- α -factor signal sequence with sequences encoding the amino-terminal amino acids (including the transmembrane/signal sequence region) of DPAP B, creating the chimeric protein D_N - α -F (Fig. 5 A).

As expected, translocation of prepro- α -factor in this sys-

tem required the SRP-independent pathway (Fig. 5 B). When the translocation of D_N - α -F was monitored in *sec63-201* and *sec62-101* cells, however, nearly all of the labeled protein was detected as the glycosylated form indicating that replacement of the prepro- α -factor signal sequence with the DPAP B amino-terminal region was sufficient to bypass the translocation block imposed by the mutations (Fig. 5 C, lanes 2 and 3). In contrast to the efficient translocation in *sec62-201* and *sec63-201*, a significant translocation defect was detected for D_N - α -F in *sec65-1* cells, indicating that it uses the SRP pathway (Fig. 5 C, lane 4).

D_N - α -F contains more than just the signal/anchor sequence of the DPAP B. To investigate whether, as with SRP-independent proteins, the signal sequence of an SRP-dependent protein is sufficient to direct a protein into the SRP-dependent pathway, we constructed D_{HC} - α -F, in which sequences encoding the hydrophobic core of prepro- α -factor were replaced with sequences encoding the 16 amino acid-long hydrophobic core of the DPAP B signal sequence (Fig. 5 A). Like D_N - α -F, D_{HC} - α -F was also efficiently translocated in wild-type, *sec62-201* and *sec63-201* cells, and its translocation was impaired in *sec65-1* cells (Fig. 5 D). Thus, as with SRP-independent proteins, the signal sequence alone is sufficient to specify the translocation pathway that is used by a protein. We obtained similar results using the *in vitro* translocation assay described above. The translocation of both D_N - α -F and D_{HC} - α -F was inhibited when assays were performed with SRP-depleted translation extracts (Fig. 5 E, compare lanes 5 and 8 with lanes 6 and 9). As expected, translocation of prepro- α -factor was insensitive to SRP depletion (Fig. 5 E, left), and microsomal membranes prepared from *sec63-201* cells translocated both D_N - α -F and D_{HC} - α -F but not prepro- α -factor (Fig. 5 F).

Pathway Use Corresponds to Signal Sequence Hydrophobicity

The analysis above indicates that signal sequences are the sole or a major determining factor in translocation proteins into either pathway. We therefore examined the signal sequences of the substrate proteins tested in Fig. 2 for differences between those that used the SRP-dependent and SRP-independent pathway. Analysis of the relative hydrophobicities of the hydrophobic cores of signal sequences indicated that SRP-dependent substrates and those requiring both pathways carry signal sequences with significantly greater hydrophobicity than those of SRP-independent substrates (Fig. 6). Hydrophobicity plots show that signal sequences of SRP-independent substrates have peaks that do not exceed +2.0 U (as defined by Kyte and Doolittle [1982]), whereas those that use SRP have peaks approaching +3.0 U (Fig. 6 A, shaded areas). Calculating the average hydrophobicity of residues in the hydrophobic core of the different signal sequences yields a similar picture (Fig. 6 B). Using window sizes of 12 and 16 residues (because the minimal size of the signal sequences is not known) and a hydrophobicity index derived from analyses of α -helical peptides—the structure predicted for signal sequences (Cornette et al., 1987)—a striking correlation becomes apparent between average hydrophobicity and pathway use (Fig. 6 B).

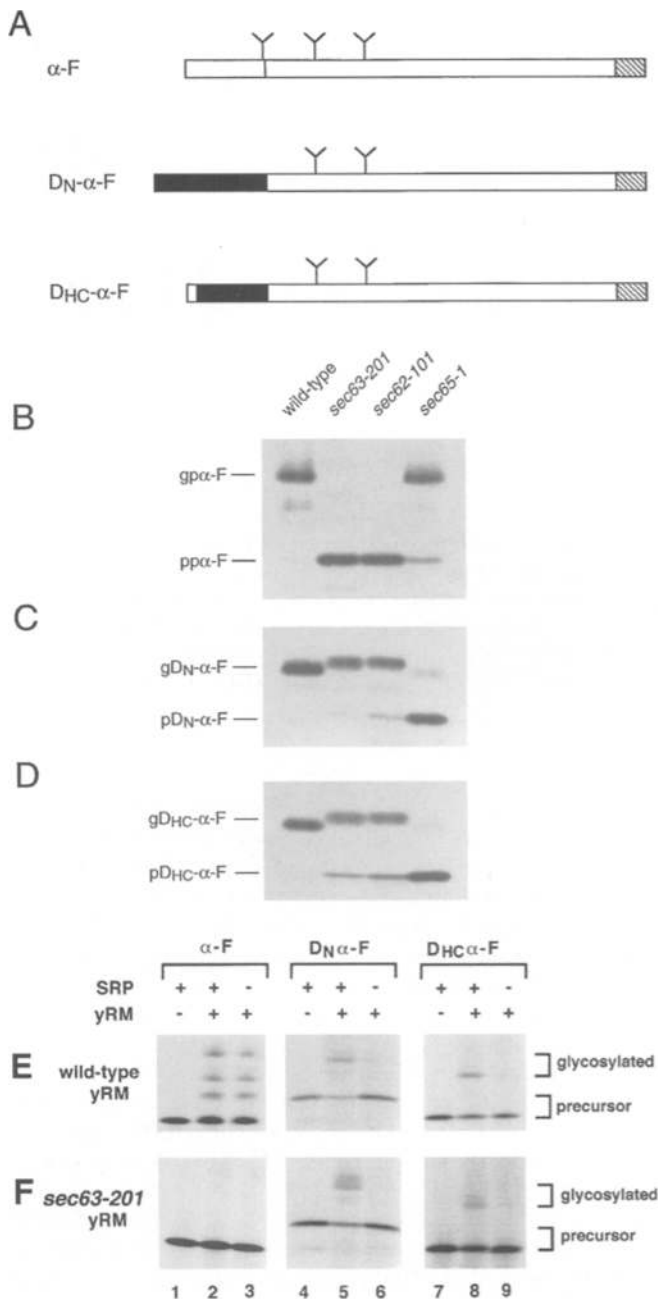


Figure 5. The DPAP B signal sequence can direct an SRP-independent protein into the SRP-dependent pathway. (A) Schematic representation of chimeric proteins. α -factor sequences are shown in white, DPAP B sequences in black, and the HA epitope tag as hatched. Ys represent N-linked glycosylation sites. α -F, wild-type prepro- α -factor containing a COOH-terminal HA epitope tag; DN- α -F, α -F modified by replacing the signal sequence with NH₂-terminal targeting domain of DPAP B (47 amino acids); DHC- α -F, α -F signal sequence replaced with the hydrophobic core of the DPAP B signal/anchor domain. (B–D) Translocation of chimeric proteins. Wild-type and mutant cells expressing α -F, DN- α -F, and DHC- α -F were assayed for translocation as described in Fig. 2. Proteins were immunoprecipitated using the 12CA5 anti-HA antibody. In each panel, the luminal glycosylated forms (gp or g prefix) and cytoplasmic preforms (pp or p prefix) of each protein are indicated. Note: the difference in electrophoretic mobility between the glycosylated forms of DN- α -F in wild-type and mutant cells is due to differences in core carbohydrate modifica-

The differences in hydrophobicity in Fig. 6, A and B result from a remarkably different amino acid composition of the hydrophobic core: whereas SRP-dependent signal sequences are rich in amino acids with hydrophobic aliphatic side chains (e.g., leucine, isoleucine, valine), signal sequences of SRP-independent proteins are rich in amino acids with small side chains and hydroxylated side chains (e.g., alanine, serine, threonine). Although neither analysis allows us to distinguish signal sequences that are entirely SRP dependent from those that require both pathways, these analyses may have strong predictive value in distinguishing SRP-independent proteins from those that are at least partially SRP dependent. To test this directly, we plotted the relative hydrophobicities of the signal sequences of three additional substrates, Sec71p, Kex2p, and Ost1p, before analysis. We predicted that Kex2p would be SRP independent (Kyte-Doolittle hydrophobicity peaking at less than +1.5) and that both Sec71p and Ost1p would be at least partially dependent on SRP (Kyte-Doolittle hydrophobicity peaking at approximately +3.0 and +2.5, respectively). Subsequent *in vivo* analysis indeed showed that translocation of Kex2p is SRP independent, translocation of Sec71p is SRP dependent, and translocation of Ost1p requires both pathways (data not shown).

Discussion

We describe the genetic and biochemical dissection of two distinct translocation pathways to the yeast ER membrane translocon. Using novel mutant alleles of *SEC62* and *SEC63* specific for SRP-independent translocation, we show that translocation substrates most affected by these mutations are those least affected by loss of SRP function, and vice versa. Characteristics of the signal sequence of a protein determine which of the two pathways is used.

Our results are consistent with a model in which signal sequences are subject to sequential recognition events (Fig. 7). SRP first examines nascent chains for the presence of a signal sequence as they emerge from the ribosome. The first recognition event is obligatorily cotranslational (Ogg and Walter, 1995). If the signal sequence binds to SRP with sufficient affinity, SRP targets the nascent chain/ribosome complex to the ER membrane cotranslationally (Fig. 7, pathway A). If, on the other hand, no productive interaction is formed with SRP, then translation proceeds, and the synthesized preprotein becomes targeted to the translocon through Sec63p and Sec62p using a posttranslational mechanism described previously (Fig. 7, pathway B) (Brodsky and Schekman, 1993; Panzner et al., 1995). Proteins that require both pathways contain signal

tion, because removal of the carbohydrate moieties using endoglycosidase H yielded polypeptides of identical mobility (data not shown). Minor species (faster migrating than gp and g forms) representing singly and doubly glycosylated forms are also evident. (E and F) *In vitro* translation reactions were carried out as in Fig. 3 using synthetic mRNAs encoding α -F, DN- α -F, or DHC- α -F as indicated. Translation extract contained or was immunodepleted of SRP and membranes (wild-type in E, sec63-201 in F) were added to reactions as indicated. Whole translation reactions were analyzed.

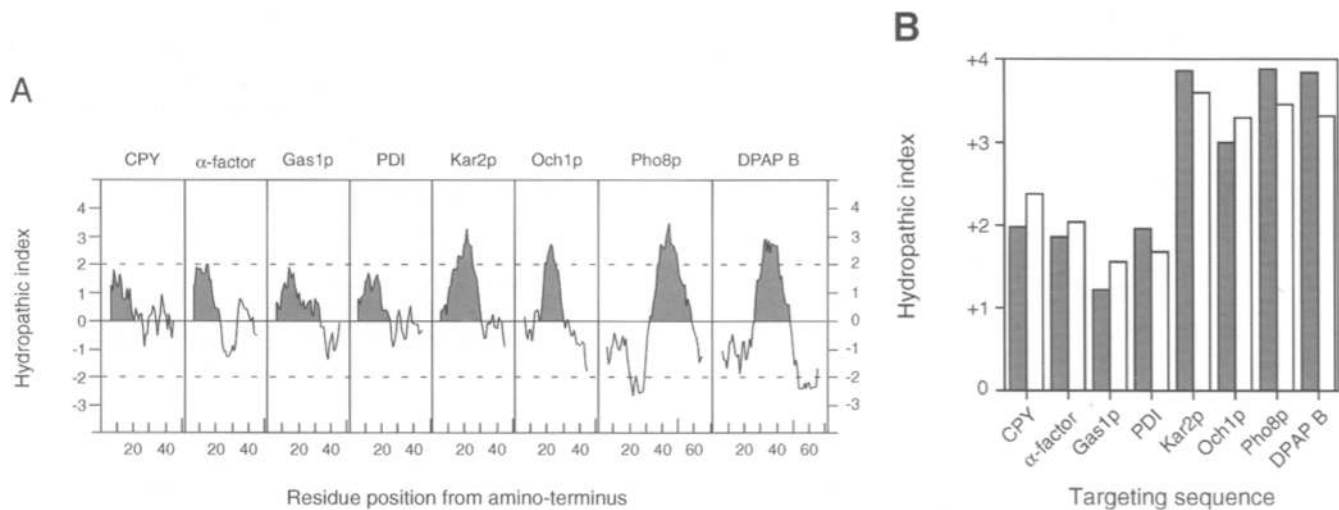


Figure 6. Analysis of signal sequence hydrophobicity. (A) Using the algorithm developed by Kyte and Doolittle with a window size of 11 amino acids (Kyte and Doolittle, 1982), the amino-terminal regions containing the signal sequence of the various proteins assayed for translocation in Fig. 2 were analyzed. (B) Using the hydrophobicity index developed by Cornette et al. (1987), the average hydrophobicity of hydrophobic core sequences from the proteins analyzed in A were calculated. Here, the window is defined as the amino acids that immediately follow the last positively charged residue of the n-region that precedes the hydrophobic core (von Heijne, 1990). For Och1p, the window = 16 score was based on 14 residues since the 15th is charged and considered excluded from the hydrophobic core. ■, window = 16; □, window = 12.

sequences that are suboptimal for binding by SRP, and the fraction of the protein chains that does not interact productively with SRP uses the posttranslational route. Both pathways converge on a common translocon composed of the Sec61p complex.

The *sec62-101* and *sec63-201* mutants discussed here show severe translocation defects for SRP-independent translocation substrates under all conditions tested; no conditional phenotype was demanded in our selection. The observed precursor forms in Fig. 2 are therefore the result of a translocation defect at steady state. Because the SRP-dependent pathway is intact in these cells, it follows directly that proteins that use the SRP-independent pathway cannot be targeted by SRP. In contrast, defects of SRP-dependent substrates are largely alleviated over time following the loss of SRP function by adaptation (Ogg et al., 1992). Thus, SRP-dependent substrates use an SRP-independent pathway (one described here or another yet uncharacterized pathway) if SRP function is impaired or saturated. The SRP pathway, therefore, poses a more stringent requirement on the information contained in a signal sequence, while SRP-independent translocation accommodates a broader range of signal sequences. In fact, as cells are viable in the absence of SRP, every secretory pathway protein that is essential for cell growth must be able to use an SRP-independent route.

SEC62 and *SEC63* are essential genes, yet the mutants characterized here show only minor growth defects. Although the translocation block of many proteins analyzed appears severe, it is not complete, and even the most affected protein CPY is translocated with ~5% efficiency (data not shown). As expected, however, the combination of *sec63-201* and SRP mutants shows compounded growth and translocation defects and is lethal for spore germination (data not shown), indicating that both pathways play

important physiological roles. Beyond the hydrophobicity of the signal sequence, we have found no obvious characteristics that distinguish SRP-dependent from SRP-independent proteins. Because many membrane proteins have highly hydrophobic signal sequences which also serve as transmembrane anchors, we consider it likely that they will be targeted by SRP. Our results with Kex2p, a type I integral membrane protein using the SRP-independent pathway indicate, however, that not all membrane proteins are targeted by SRP.

Sec62p and Sec63p are part of a protein complex that also contains Sec71p and Sec72p and luminally associated Kar2p (Deshaies and Schekman, 1990; Brodsky and Schekman, 1993; Feldheim et al., 1993). Reconstitution studies have shown that this complex in association with the Sec61p complex can promote posttranslational protein translocation (Panzner et al., 1995). Although the novel *sec62* and *sec63* mutants described here specifically impair an SRP-independent translocation pathway, our observations do not rule out a role for the Sec62/63p complex in the SRP-dependent route. A previously isolated mutant allele of *SEC63*, *sec63-1*, displayed minor defects in DPAP B translocation at the nonpermissive temperature (Stirling et al., 1992) suggesting that this complex may also be involved in the SRP-dependent translocation. However, membrane-bound ribosomes, suggestive of cotranslational translocation complexes, were found only associated with Sec61 proteins not associated with the Sec62/63p complex (Panzner et al., 1995). Thus the role of the Sec62/63p complex in the SRP-dependent pathway remains unclear and awaits the establishment of an in vitro SRP-dependent translocation assay using purified components.

For the SRP-independent route, it is unknown whether any of proteins comprising the Sec62/63p complex or a yet to be identified cytosolic factor provides the necessary sig-

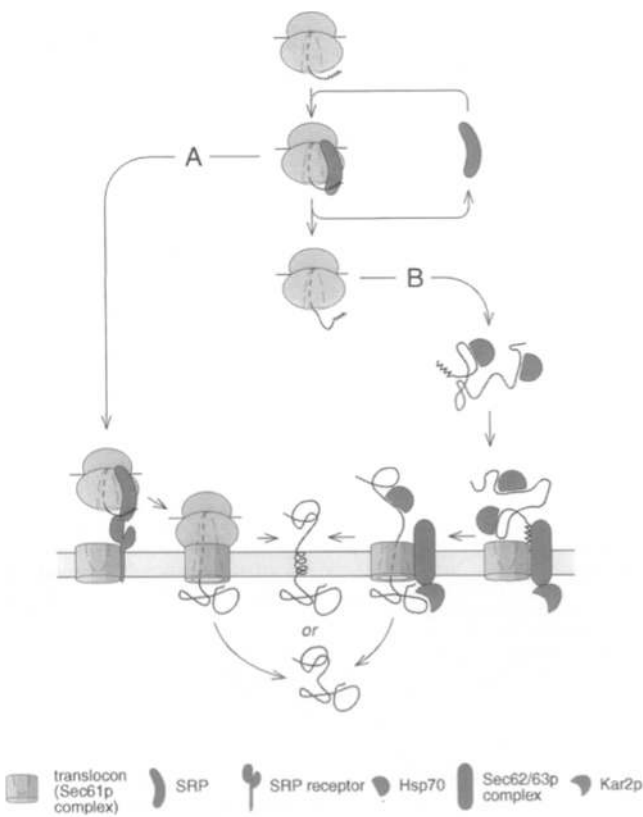


Figure 7. Model for protein translocation across the yeast ER. After the emergence of the signal sequence, the nascent chain ribosome complex is scanned by SRP. If there is productive binding by SRP, the entire complex is targeted to the ER membrane (A). Interaction of SRP and its receptor mediates release and transfer of the nascent chain to the translocon where it translocates to the lumen cotranslationally. However, if the signal sequence is specific for the SRP-independent pathway (B), no productive encounter is achieved with SRP and translation continues on free ribosomes in the cytoplasm. Interaction with cytosolic chaperones maintains the nascent protein in an unfolded conformation. The complex targets to the Sec62/63p complex and the protein translocates across the membrane posttranslationally.

nal recognition function. Previously, it was shown that strains deleted for *SEC72* displayed a partial defect in CPY translocation, but none for invertase and that these differences could be traced to their signal sequences suggesting a possible recognition function (Feldheim and Schekman, 1994). It seemed possible that the translocation phenotypes observed for *sec62-101* and *sec63-201* cells were caused by a disruption of the Sec72p interaction with the complex. This question was tested directly by examination of immunoprecipitated Sec62/63p complexes from *sec63-201* and wild-type cells (Ng and Walter, 1996). No disruption was detected as Sec72p (and Sec71p) was found associated with Sec63-201p similar to wild-type Sec63p. In addition, neither Sec71p nor Sec72p are essential for the SRP-independent pathway described here. Cells missing either or both components are viable and show only partial defects for SRP-independent substrates (Feldheim and Schekman, 1994; Ng and Walter, 1996; Ng, D.T.W., and P. Walter, un-

published data). Other translocation components proposed to interact with signal sequences include the bacterial and mammalian homologues of Sec61p (Derman et al., 1993; Jungnickel and Rapoport, 1995). However, these data are consistent with the view that Sec61p complex is the convergence point of the two translocation pathways, as most *sec61* mutants affect substrates targeted via either route. Therefore, if Sec61p recognizes signal sequences, it imposes a different selection criteria than SRP.

The existence of multiple translocation pathways to the ER may be common to all organisms. Posttranslational translocation has been reported for a few exceptional proteins in higher eukaryotic systems but required components remain unknown (Schlenstedt et al., 1990). Recently, putative homologues of *SEC62* and *SEC63* have been identified in higher eukaryotes (Noel and Cartwright, 1994; Brightman et al., 1995), suggesting that a common mechanism for SRP-independent translocation may exist. The SRP-independent substrate CPY, however, is not translocated in mammalian cells (Bird et al., 1987), indicating that substrates and targeting/translocation machineries are not freely interchangeable. Similarly, prepro- α -factor is targeted efficiently by mammalian SRP, yet is not targeted by SRP in yeast. This phenomenon may explain the difficulties commonly encountered when expressing heterologous proteins in yeast. As the biogenesis of many mammalian proteins may require cotranslational translocation, the prepro- α -factor signal sequence commonly used to effect foreign protein secretion from yeast may, in fact, be suboptimal, and it may be preferable to use an SRP-directed signal sequence.

The poor growth of SRP-deficient yeast and the extreme evolutionary conservation of SRP and SRP receptor from bacteria to mammalian cells suggest that cotranslational translocation is important (Walter and Johnson, 1994; Wolin, 1994). Structural features of some preproteins may preclude efficient translocation using a posttranslational route. Polytopic membrane proteins, for example, are highly hydrophobic and may have a tendency to misfold and/or aggregate if fully synthesized before translocation. Other proteins, such as secreted hydrolases, may need to be shunted into a cotranslational pathway as to prevent their synthesis in cytosol where they may be harmful to the cell. Furthermore, for cotranslational translocation there are no constraints imposed by either structure or function of the proteins to be translocated as they are never synthesized in the cytosol. Proteins that do not impose such restrictions, however, can have evolved signal sequences with a lowered affinity for SRP and consequently be targeted posttranslationally. Translocation via two different pathways may also offer cells additional means of control. SRP can influence the rate of translation elongation, for example, and could therefore provide the cell with a convenient on/off switch with which to adapt secretory protein synthesis to the physiological needs of the cell. The molecular characterization of the components that catalyze protein translocation by either route is the first step in deciphering their respective physiological roles.

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