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Nonlethal *sec71-1* and *sec72-1* Mutations Eliminate Proteins Associated with the Sec63p-BiP Complex from *S. cerevisiae*

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The *sec71-1* and *sec72-1* mutations were identified by a genetic assay that monitored membrane protein integration into the endoplasmic reticulum (ER) membrane of the yeast *Saccharomyces cerevisiae*. The mutations inhibited integration of various chimeric membrane proteins and translocation of a subset of water soluble proteins. In this paper we show that *SEC71* encodes the 31.5-kDa transmembrane glycoprotein (p31.5) and *SEC72* encodes the 23-kDa protein (p23) of the Sec63p-BiP complex. *SEC71* is therefore identical to *SEC66* (*HSS1*), which was previously shown to encode p31.5. DNA sequence analyses reveal that *sec71-1* cells contain a nonsense mutation that removes approximately two-thirds of the cytoplasmic C-terminal domain of p31.5. The *sec72-1* mutation shifts the reading frame of the gene encoding p23. Unexpectedly, the *sec71-1* mutant lacks p31.5 and p23. Neither mutation is lethal, although *sec71-1* cells exhibit a growth defect at 37°C. These results show that p31.5 and p23 are important for the trafficking of a subset of proteins to the ER membrane.

INTRODUCTION

The use of gene fusions has aided studies of the biology of eukaryotic and prokaryotic cells (reviewed by Silhavy and Beckwith, 1985; Manoil *et al.*, 1990). In the yeast *Saccharomyces cerevisiae*, a selection assay employing a water soluble chimeric protein consisting of a signal peptide fused to histidinol dehydrogenase (HD) was used to study protein translocation across the endoplasmic reticulum (ER) membrane. Because HD was inactive in the ER lumen, selections for cells that utilized histidinol for growth identified *sec61*, *sec62*, and *sec63* mutants (Deshaies and Schekman, 1987; Rothblatt *et al.*, 1989).

SEC61, *SEC62*, and *SEC63* encode essential multispanning membrane proteins that are required for protein translocation (Deshaies and Schekman, 1989; Sadler *et al.*, 1989; Stirling *et al.*, 1992). Sec61p is a component of the translocation complex through which proteins traverse the membrane in yeast (Sanders *et al.*, 1992) and mammals (Görlich and Rapoport, 1993). A high-copy suppressor of a mutation in *SEC61*, termed *SSS1* (Esnault *et al.*, 1993), encodes a protein that shares homology with the γ-subunit of the mammalian Sec61p complex (Hartmann *et al.*, 1994). Sec63p, a DnaJ homologue (Sadler *et al.*, 1989), interacts with a 31.5-kDa glycoprotein (p31.5) and a 23-kDa protein (p23) in two distinct complexes containing either Kar2p or Sec62p. The Sec63p complex contains Sec63p, Sec62p, p31.5, and p23 (Deshaies *et al.*, 1991), and the Sec63p-BiP complex contains Sec63p, Kar2p, p31.5, and p23 (Brodsky and Schekman, 1993). Kar2p shares homology with mammalian BiP/GRP78 (Normington *et al.*, 1989; Rose *et al.*, 1989). The gene encoding p31.5, termed *SEC66* (also *HSS1*), was recently cloned and sequenced (Feldheim *et al.*, 1993; Kurihara and Silver, 1993). The encoded protein contains a single transmembrane segment and is glycosylated near the N-terminus. Components of the Sec63p complex can be cross-linked to Sec61p and precipitated as a multsubunit complex (Deshaies *et al.*, 1991).

Gene fusion methodologies have also been applied to studies of membrane protein integration in yeast. Searches for mutants inhibiting integration of a chimera consisting of HD and a fragment of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, a ER membrane protein, identified a *sec61* allele, *sec61-3*, that inhibits in-

tegration and translocation and a novel mutant, *sec65* (Stirling *et al.*, 1992). *SEC65* encodes the 19-kDa homologue of mammalian signal recognition particle (SRP), a ribonucleoprotein complex that is important for cotranslational targeting of membrane and soluble proteins to the ER membrane (Walter and Blobel, 1980, 1982; Gilmore *et al.*, 1982; Meyer *et al.*, 1982; Hann *et al.*, 1992; Stirling and Hewitt, 1992). *SEC65* is nonessential for growth, although, unexpectedly, the *sec65-1* mutant exhibits a conditional-lethal phenotype (Stirling and Hewitt, 1992). Genes encoding the 54-kDa and RNA homologues of mammalian SRP and the α-subunit homologue of the SRP receptor have also been isolated (Hann *et al.*, 1989; Amaya *et al.*, 1990; Hann and Walter, 1991; Ogg *et al.*, 1992). Null mutations in these genes are not lethal and inhibit translocation of only a subset of proteins (Hann and Walter, 1991; Ogg *et al.*, 1992).

Some preproteins can be translocated across the membrane in a posttranslational manner in yeast (Hansen *et al.*, 1986; Rothblatt and Meyer, 1986; Waters and Blobel, 1986). Posttranslational translocation of the precursor to α-factor is mediated by cytoplasmic heat-shock proteins (Chirico *et al.*, 1988; Deshaies *et al.*, 1988) and cytoplasmic Ydj1p, a DnaJ homologue (Caplan *et al.*, 1992). In addition, the precursor to carboxypeptidase Y can be posttranslationally translocated across the membrane (Hansen and Walter, 1988).

We previously described a genetic assay that utilized a series of chimeric membrane proteins to identify mutations in *SEC70*, *SEC71*, and *SEC72* (Green *et al.*, 1992; Green and Walter, 1992). A189invHD and A255invHD contain residues 1–189 and 1–255 of arginine permease, respectively, followed by a spacer sequence that is then fused to HD. In Sec$^+$ cells, the transmembrane segments of A189invHD and A255invHD are integrated into the membrane that results in transport of HD to the lumen. Mutations in *SEC70* inhibit translocation of the HD moiety of A255invHD but not A189invHD. The *sec71* and *sec72* mutations inhibit translocation of the HD moieties of A189invHD and A255invHD and translocation of a subset of native proteins. The molecular identity of these novel genes has not been reported. In this paper we show that *SEC71* and *SEC72* encode p31.5 and p23, respectively, of the Sec63p-BiP complex.

MATERIALS AND METHODS

Derivation of Strains

Haploid *sec71-1* mutant strains NGY52 and NGY50 were derived from a cross between strains NGY27 and IH27-1 (Green *et al.*, 1992). Strains NGY04 (*sec72-1*) and NGY03 (*sec70-3*) were derived from a cross between strain JC1-3C and strains NGY32 and NGY30, respectively (Green *et al.*, 1992). Strains NGY80 (Sec$^+$), NGY81 (*sec65-1*), NGY82 (*sec72-1*), and NGY83 (*sec65-1 sec72-1*) were derived from a single tetrad that was obtained from a cross between strains CSa59 (*sec65-1*) and NGY04 (*sec72-1*). Diploid strains NGY52-70, NGY52-66, NGY22-66, NGY27-66, NGY28-66, NGY1A-72, and NGY1A-71 were derived from crosses between strains NGY52 and NGY03, NGY52 and 10-4D, NGY22 and 10-4D, NGY27 and 10-4D, NGY28 and 10-4D, 1A and NGY04, and 1A and NGY50, respectively. Genotypes of yeast strains are listed in Table 1.

Radiolabeling of Cells, Immunoprecipitation of Proteins, Western Blot Analysis, and Quantitation of Translocation Defects

Procedures for pulse labeling of cells have been described previously (Green and Walter, 1992). Briefly, strains listed in Table 2 were grown at 23°C in YPD (rich) medium (1 ml) to OD_{600} = 1, shifted to methionine-depleted media for 1 h, and then shifted to 37°C for 1 h before pulse labeling with [^{35}S]-methionine. Other strains used in this study were grown at 30°C, shifted to methionine-depleted media, and then pulse labeled. After a 5 min pulse, cells were broken by vortex mixing with glass beads in 0.2 ml of 10% trichoroacetic acid (TCA). Proteins were sedimented in an eppendorf centrifuge then dissolved in 20 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and boiled for 5 min. The mixture was diluted into a solution containing phosphate-buffered saline + 1% Triton X-100 (0.7 ml) then sedimented for 2 min in an eppendorf centrifuge. Proteins were precipitated from the supernatant with 1 μl anti-carboxypeptidase (CPY) antibodies or 0.5 μl anti-Kar2p antibodies (added to 0.7 ml supernatant). Precipitated proteins were analyzed by SDS-PAGE on a 7% polyacrylamide gel followed by autoradiography. Autoradiograms were analyzed on a model DU 70 spectrophotometer (480 nm wavelength) using a gel scanner adaptor (Beckman Instruments, Fullerton, CA). A plot of optical density versus position along the autoradiogram was obtained. The areas under the peaks corresponding to bands on the autoradiogram were measured by programming provided by the manufacturer. The translocation defect in cells was recorded as "% inhibition" by measuring the areas under the peaks corresponding to precursor and translocated proteins. Antibodies directed against CPY (Dr. Randy Schekman, University of California, Berkeley) and Kar2p (Dr. Mark Rose, Princeton University) were kindly provided. Western blot analyses were performed by methods described previously (Green *et al.*, 1992). Briefly, cells were grown to OD_{600} = 1 then broken by vortex mixing in 10% TCA (above). Proteins were sedimented, mixed with SDS-PAGE sample buffer, boiled for 5 min, and then analyzed on a 12% polyacrylamide gel by Western blotting with anti-p23 antibodies (diluted 1:100) (Brodsky and Schekman, 1993; Feldheim and Schekman, 1994).

Sequencing of sec71-1 and sec72-1 genes

Chromosomal DNA from strain NGY52 (*sec71-1*) was used as a template in a polymerase chain reaction to amplify the *SEC66* gene. The sequences of the oligonucleotides that were used as primers in the reaction are TCTGATAAGCATACTTCACT and GTAGTGAGCAAGAAGAAGGG. The amplified DNA fragment was restricted with *Eco*RI and *Hin*dIII. This restriction fragment (830 base pairs [bp]), which contains the protein-coding sequence (Feldheim *et al.*, 1993; Kurihara and Silver, 1993), was inserted into M13mp18 and M13mp19 cloning vectors then sequenced by the dideoxy method using a protocol provided by the supplier (United States Biochemical, Cleveland, OH). The *sec72-1* gene was similarly amplified using oligonucleotide primers AGCAAGCTTCGGCC and TCTTCGGTTATGCACCTTA and DNA template from strain NGY04 (*sec72-1*). The amplified fragment (600 bp) was restricted with *Hin*dIII that cuts 13 bp upstream of the initiation codon and at codon 97 (Feldheim and Schekman, 1994). The *Hin*dIII restriction fragment was inserted into M13mp18 in both orientations then sequenced as described above.

RESULTS

SEC71 Is Identical to SEC66

We previously identified two mutations, *sec71-1* and *sec72-1*, that inhibited translocation of a similar subset

Table 1. Yeast strains used in this study

Strain	Genotype	Source
10-4D	*MATα sec71::LEU2 suc2 ura3-52 ade2-101 trp1Δ1 his3Δ200 leu2Δ1*	Randy Schekman, University of California, Berkeley
1A	*MATa ura3-52 ade2-101 lys2-801 his3Δ200 leu2Δ1 trp1-Δ63 sec72::HIS3*	Randy Schekman
CSa59	*MATa sec65-1 his4-401 leu2-3, -112 ura3-52 HOL1-1 trp1-1*	Randy Schekman
NGY22	*MATa ade5-1 trp1-1 his4-401 ura3-52 leu2-1 HOL1-1 sec70-3*	(Green *et al.*, 1992)
NGY27	*MATa ade5-1 trp1-1 his4-401 ura 3-52 leu2-1 HOL1-1 sec 71-1*	(Green *et al.*, 1992)
NGY28	*MATa ade5-1 trp1-1 his4-401 ura3-52 leu2-1 HOL1-1 sec72-1*	(Green *et al.*, 1992)
MS177	*MATα ura3-52 ade2-101 kar2-159*	Mark Rose, Princeton University
NGY03	*MATα ade5-1 trp1-1 ura3-52 sec70-3 his4-401 HOL1-1*	This study
NGY04	*MATα his4-401 ura3-52 trp1-1 leu2-1 ade5-1 sec72-1 HOLI-1*	This study
NGY52	*MATa ade5-1 trp1-1 leu2-1 ura3-52 sec71-1 HOL1-1 his4-401*	This study
NGY50	*MATα ade5-1 trp1-1 leu2-1 ura3-52 sec71-1 HOL1-1 his4-401*	This study
NGY52-70	*MATa/α ade5-1/ade5-1 trp1-1/trp1-1 ura3-52/ura3-52 SEC71/sec71-1 SEC70/sec70-3 LEU2/leu2-1 HOL1-1/HOL1-1 his4-401/his4-401*	This study
NGY52-66	*MATa/α ADE5/ade5-1 ura3-52/ura3-52 leu2Δ1/leu2-1 sec71-1/sec71::LEU2 HOL1-1/HOL1 ADE2/ade2-101 trp1Δ1/trp1-1 HIS3/his3Δ200 HIS4/his4-401*	This study
NGY22-66	*MATa/α ADE5/ade5-1 ura3-52/ura3-52 leu2Δ1/leu2-1 SEC71/sec71::LEU2 HOL1-1/HOL1 ADE2/ade2-101 trp1Δ1/trp1-1 HIS3/his3Δ200 SEC70/sec70-3 HIS4/his4-401*	This study
NGY27-66	*MATa/α ADE5/ade5-1 ura3-52/ura3-52 leu2Δ1/leu2-1 sec71-1/sec71::LEU2 HOL1-1/HOL1 ADE2/ade2-101 trp1Δ1/trp1-1 HIS3/his3Δ200 HIS4/his4-401*	This study
NGY28-66	*MATa/α ADE5/ade5-1 ura3-52/ura3-52 leu2Δ1/leu2-1 SEC71/sec71::LEU2 HOL1-1/HOL1 ADE2/ade2-101 trp1Δ1/trp1-1 HIS3/his3Δ200 SEC72/sec72-1 HIS4/his4-401*	This study
NGY1A-71	*MATa/α ura3-52/ura3-52 ADE2/ade2-101 LYS2/lys2-801 HIS3/his3Δ200 leu2Δ1/leu2-1 trp1-1/trp1-Δ63 SEC72/sec72::LEU2 SEC71/sec71-1 HOL1-1/HOL1 ADE5/ade5-1 HIS4/his4-401*	This study
NGY1A-72	*MATa/α ura3-52/ura3-52 ADE2/ade2-101 LYS2/lys2-801 HIS3/his3Δ200 leu2Δ1/leu2-1 trp1-1/trp1-Δ63 sec72-1 sec72::LEU2 HOL1-1/HOL1 ADE5/ade5-1 HIS4/his4-401*	This study
NGY80	*MATα his4-401 ura3-52 trp1-1 leu2-1 ade5-1 HOL1-1*	This study
NGY81	*MATa his4-401 ura3-52 trp1-1 leu2-1 sec65-1 ade5-1 HOL1-1*	This study
NGY82	*MATa his4-401 ura3-52 trp1-1 leu2-1 sec72-1 HOL1-1*	This study
NGY83	*MATα his4-401 ura3-52 trp1-1 leu2-1 sec72-1 sec65-1 HOL1-1*	This study

of soluble proteins (Green *et al.*, 1992). Recently, it was reported (Feldheim *et al.*, 1993) that a null mutation in *SEC66* encoding the 31.5-kDa glycoprotein (p31.5) of the Sec63p-BiP complex inhibited translocation of a subset of proteins that was similar to the substrate profile affected by our mutations in *SEC71* and *SEC72*. Because it seemed plausible that *SEC66* may be identical to *SEC71* or *SEC72*, we searched for a genetic complementation assay with which to compare the corresponding mutants. Strains 10-4D (*Δsec66*), NGY52 (*sec71-1*), and NGY04 (*sec72-1*) were placed on agar plates then incubated at 37°C, a nonpermissive temperature for the *Δsec66* (*Δhss1*) mutant (Feldheim *et al.*, 1993; Kurihara and Silver, 1993). Although the *sec72-1* mutant did not exhibit a strong growth defect (shown below), the *sec71-1* mutant formed small colonies on agar plates. This growth defect of the *sec71* mutant was recessive as diploid strain NGY52-70 (*sec71* × *sec70*) (MATERIALS AND METHODS) formed large colonies at 37°C (Figure 1). With this genetic assay in hand, a diploid strain heterozygous for *sec66* and *sec71* mutations was constructed by crossing strains NGY52 (*sec71*) and 10-4D (*Δsec66*). Cells of diploid strain NGY52-66 (*sec71* × *sec66*) were then placed at 37°C. As shown in Figure 1, strain NGY52-66 grew in a similar manner as the *sec71* mutant, indicating the *sec66* and *sec71* haploid mutants did not complement each other.

We next utilized a biochemical assay to substantiate these genetic data. Haploid strains NGY52 (*sec71*), 10-4D (*sec66*), and three diploid strains [NGY52-66 (*sec66* × *sec71*), NGY22-66 (*sec66* × *sec70*), and NGY28-66 (*sec66* × *sec72*)] (MATERIALS AND METHODS) were grown at 30°C to OD_{600} = 1 then shifted to 37°C for 1 h. Cells were pulse-labeled for 5 min with [^{35}S]-methi-

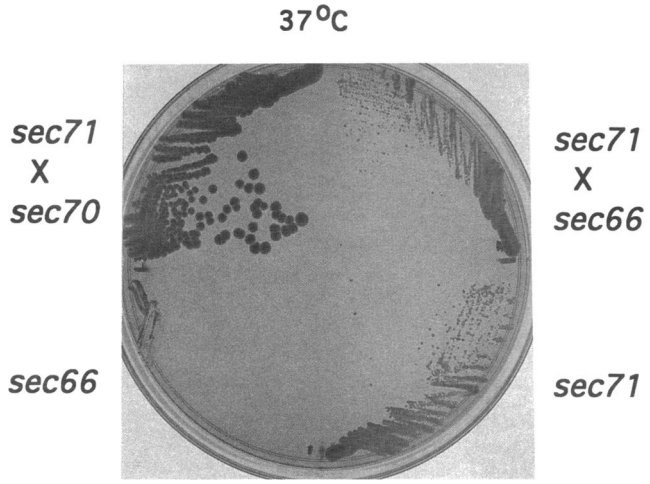

Figure 1. The *sec66* mutant does not complement the growth defect of the *sec71* mutant. Cells of haploid strains NGY52 (*sec71*) and 10-4D (*sec66*) and diploid strains NGY52-70 (*sec71* × *sec70*) and NGY52-66 (*sec71* × *sec66*) were placed on an agar plate containing YPD medium (MATERIALS AND METHODS) then incubated at 37°C for 4 d. Genotypes of yeast strains are described in Table 1. The derivation of these strains is described in MATERIALS AND METHODS.

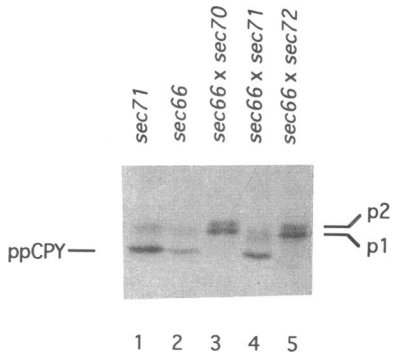

Figure 2. The *sec71* mutant does not complement the translocation defect in the *sec66* mutant. Cells of haploid strains NGY52 (*sec71*) (lane 1) and 10-4D (*sec66*) (lane 2) and diploid strains NGY22-66 (*sec66* × *sec70*) (lane 3), NGY27-66 (*sec66* × *sec71*) (lane 4), and NGY28-66 (*sec66* × *sec72*) (lane 5) were grown at 30°C in YPD medium to OD_{600} = 1 then shifted to methionine-depleted medium for 1 h, and then to 37°C for 1 h. Cells were pulse labeled for 5 min with [^{35}S]-methionine. Proteins were precipitated from cell (1 OD_{600} equivalent) extracts with antibodies directed against CPY, then fractionated on a 7% SDS-polyacrylamide gel, and visualized by autoradiography (Green and Walter, 1992). The positions of ppCPY, p1, and p2 are indicated. The derivation of these strains is described in MATERIALS AND METHODS.

onine then broken by vortex mixing with glass beads (MATERIALS AND METHODS). Proteins were precipitated from cell extracts with antibodies directed against CPY, a vacuolar protein, and analyzed by SDS-PAGE. Three distinct precursor forms of CPY were detected: ppCPY (cytoplasmic precursor form of CPY), p1 (core glycosylated), and p2 (Golgi form) (Figure 2). As expected, strains NGY52 (*sec71*) (lane 1) and 10-4D (*sec66*) (lane 2) accumulated ppCPY. Diploid strains NGY22-66 (*sec70* × *sec66*) (lane 3) and NGY28-66 (*sec72* × *sec66*) (lane 5) did not accumulate ppCPY, whereas diploid strain NGY52-66 (*sec71* × *sec66*) (lane 4) clearly inhibited translocation of this protein to a similar degree as *sec71* and *sec66* haploid mutants (lanes 1 and 2). These results demonstrated that the *sec71* and *sec66* haploid mutants inhibited translocation of ppCPY to similar degrees, and the *sec70* and *sec72* mutants, but not the *sec71* mutant, complemented the translocation defect in the *sec66* mutant. Taken together with the genetic evidence, the data show that *SEC71* and *SEC66* are in the same complementation group.

To determine whether the *SEC66* gene was mutationally altered in the *sec71* mutant, we amplified the *SEC66* gene from the chromosome of strain NGY52 (*sec71-1*) (MATERIALS AND METHODS). The amplified DNA fragment was inserted into appropriate M13 vectors and sequenced. The deduced sequence did not deviate from published data (Feldheim *et al.*, 1993; Kurihara and Silver, 1993) except at codon #97 (TTG) (Figure 3). In the *sec71* mutant, this codon was converted to a translational stop signal (TAG). The *sec71-1* mutation thus truncated the protein encoded by *SEC66*, p31.5, in the cytoplasmic, C-terminal domain.

The sec71-1 Mutant Exhibits a Defect in Translocation of preKar2p at Higher Temperatures

We previously reported that the *sec71-1* mutation inhibited translocation of the precursor to CPY but not Kar2p and that mutant cells did not show a strong growth defect at 30°C (Green *et al.*, 1992). Because as described above the *sec71-1* mutant grew poorly at

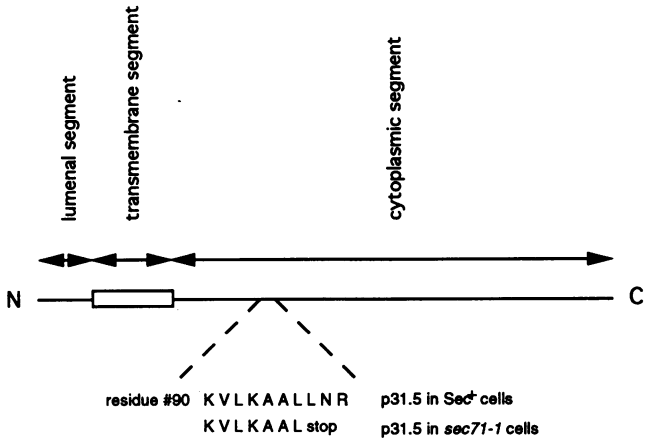

Figure 3. The *sec71-1* mutant contains a nonsense mutation in the gene encoding p31.5. The diagram depicts three topologically distinct domains of p31.5: a lumenal domain at the N-terminus (residues 1–27), a transmembrane domain (□) (residues 28–51), and a cytoplasmic domain (residues 52–206) (Feldheim *et al.*, 1993; Kurihara and Silver, 1993). Approximate lengths are given. Beginning with residue 90 and ending with residue 99, the sequence of p31.5 is shown. The corresponding sequence in the *sec71-1* mutant is also shown.

higher temperatures, we reasoned that it may exhibit a more severe translocation defect. Two distinct *sec71-1* mutant strains, NGY52 and NGY50 (MATERIALS AND METHODS), were grown at 23°C to OD_{600} = 1 then shifted to 37°C for 1 h. Cells were subsequently incubated with [^{35}S]-methionine for 5 min and broken by vortex mixing with glass beads. Cell extracts were divided into two fractions, and proteins were precipitated with anti-Kar2p or anti-CPY antibodies (MATERIALS AND METHODS). As shown in Figure 4A, both mutant strains strongly inhibited translocation of ppCPY (lanes 5 and 6). Indeed, most of ppCPY that was synthesized during a 5-min pulse was retained in the cytoplasm (~85%) (Table 2). However, neither mutant accumulated preKar2p (lanes 2 and 3). Importantly, because preKar2p and ppCPY were precipitated in parallel from the same cell extract, these results demonstrated that the *sec71-1* mutant had a more immediate effect on the translocation of ppCPY. For comparison, we performed a similar analysis with strain CSa59, which contained the conditional *sec65-1* mutation in a subunit of yeast SRP (Hann *et al.*, 1992). Results depicted in Figure 4A show that in contrast to *sec71-1*, the *sec65-1* mutation inhibited translocation of preKar2p, but not ppCPY at 37°C (lanes 1 and 4).

We next analyzed the *sec71-1* mutant at a higher temperature. Log-phase cells of strains NGY50 and NGY52 were preincubated for 1 h at 39°C before addition of label then analyzed as described above. As expected, translocation of ppCPY was strongly inhibited in both strains analyzed by pulse labeling at this elevated temperature (Figure 4B, lanes 3 and 4). It was apparent, however, that a small fraction of preKar2p (~10%) accumulated in *sec71-1* mutant cells at 39°C (lanes 1 and 2). Together with our previous results (Green *et al.*, 1992), the data in Figure 4 show that the *sec71-1* mutation strongly inhibits translocation of ppCPY at all tested temperatures, whereas translocation of a small amount of preKar2p is inhibited at higher temperatures.

Table 2. Inhibition of protein translocation in a selected group of *sec* mutants

Strain	Relevant mutation	% inhibition ppCPY	% inhibition preKar2p
CSa59	*sec65-1*	0	43
NGY52	*sec71-1*	85	0
NGY81	*sec65-1*	0	38
NGY82	*sec72-1*	28	0
NGY83	*sec65-1 sec72-1*	53	66

Translocation defects of strains listed above were measured after a 5-min pulse at 37°C (MATERIALS AND METHODS). The average value from two independent trials is presented. Strain derivations are described in MATERIALS AND METHODS and complete genotypes in Table 1. Translocation of ~10% of preKar2p is inhibited in strain NGY52 at 39°C (Figure 4).

SEC72 Encodes p23

We previously demonstrated that the *sec71-1* and *sec72-1* mutations partially blocked the trafficking of a similar subset of water soluble and membrane proteins at 30°C (Green *et al.*, 1992), suggesting *SEC71* and *SEC72* gene products may be functionally related. We therefore reasoned that because *SEC71* encodes p31.5 (above), *SEC72* may also encode a protein of the Sec63p-BiP complex. Recently, protein sequence data were used to isolate the gene encoding the 23-kDa protein (p23) of the Sec63p-BiP complex (Feldheim and Schekman, 1994). A null mutation was constructed, revealing the gene was non-

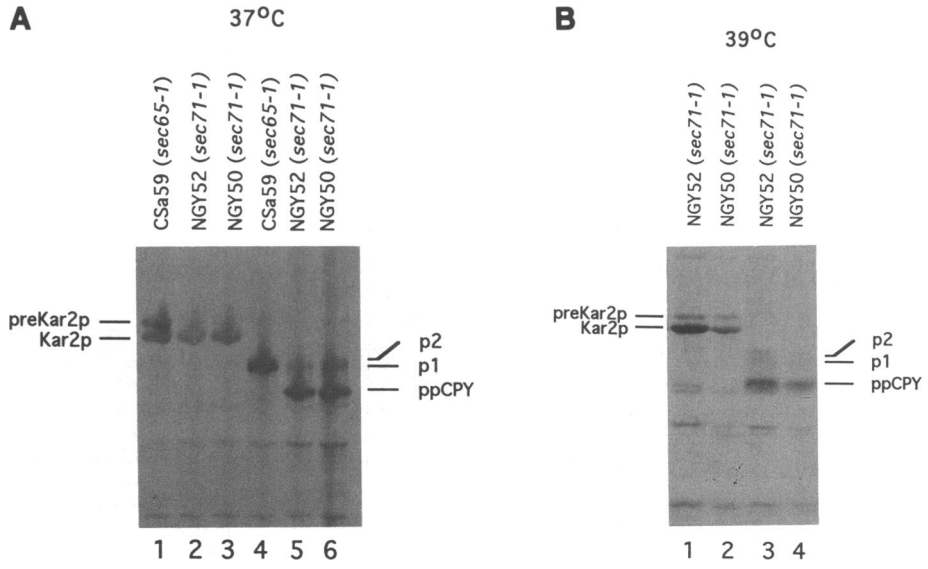

Figure 4. Differential effects of *sec71-1* and *sec65-1* mutations. Strains NGY52 (*sec71-1*) (lanes 2 and 5), NGY50 (*sec71-1*) (lanes 3 and 6), and CSa59 (*sec65-1*) (lanes 1 and 4) were grown in YPD medium at 23°C then analyzed as described in Figure 2. Cells were subjected to a 5-min pulse at 37 (A) or 39°C (B). Proteins were precipitated from cell extracts with anti-CPY (A, lanes 4–6) (B, lanes 3 and 4), or anti-Kar2p (A, lanes 1–3) (B, lanes 1 and 2) antibodies and analyzed on a 7% SDS polyacrylamide gel followed by autoradiography. The positions of p1, p2, ppCPY, Kar2p, and preKar2p are shown.

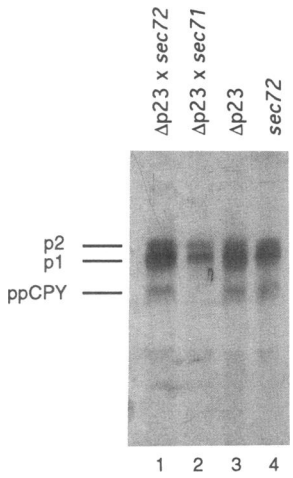

Figure 5. The *sec72* mutant does not complement the translocation defect in cells bearing a null mutation in the gene encoding p23. Haploid strains NGY04 (*sec72*) (lane 4) and 1A (Δp23) (lane 3), and diploid strains NGY1A-71 (Δp23 × *sec71*) (lane 2) and NGY1A-72 (Δp23 × *sec72*) (lane 1) were grown in YPD medium at 30°C to OD_{600} = 1, then pulse labeled for 5 min with [^{35}S]-methionine. Proteins were precipitated from cell (1 OD_{600} equivalent) extracts with anti-CPY antibodies than analyzed as described in Figure 2. The positions of ppCPY, p1, and p2 are indicated. Strain derivations are described in MATERIALS AND METHODS.

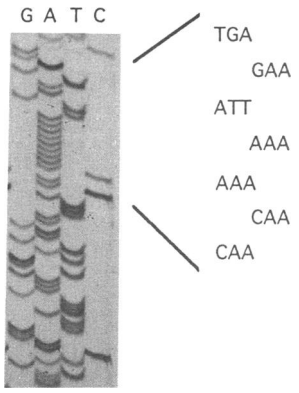

Figure 6. The *sec72-1* mutant contains a frame-shift mutation in the gene encoding p23. The gene encoding p23 was amplified from the chromosome of strain NGY04 (*sec72-1*) (MATERIALS AND METHODS). The sequence of a portion of the amplified DNA fragment containing the frame-shift mutation is depicted.

essential for growth. Furthermore, the Δp23 mutation selectively inhibited translocation of ppCPY. To compare these mutants, haploid strains NGY04 (*sec72-1*) and 1A (Δp23) and diploid strains NGY1A-72 (*sec72* × Δp23) and NGY1A-71 (*sec71* × Δp23) (MATERIALS AND METHODS) were grown at 30°C then pulse labeled with [^{35}S]-methionine as described above. Proteins were precipitated from cell extracts with anti-CPY antibodies and analyzed by SDS-PAGE (Figure 5). Diploid strain NGY1A-71 (*sec71* × Δp23) did not show a translocation defect (lane 2), indicating that the *sec71* mutant complemented the Δp23 mutant. In contrast, the Δp23 and *sec72-1* haploid mutants (lanes 3 and 4) and diploid strain NGY1A-72 (*sec72* × Δp23) (lane 1) accumulated similar amounts of ppCPY, indicating that the *sec72* and Δp23 mutants did not complement each other.

To confirm that *SEC72* encodes p23, we sequenced the corresponding gene in the *sec72-1* mutant. Oligonucleotide primers that were identical to sequences at the 5′- and 3′-ends of the coding sequence were prepared and then used to amplify the gene from the chromosome of strain NGY04 (*sec72-1*) (MATERIALS AND METHODS). A *Hin*dIII fragment (300 bp) encoding the first 97 residues of p23 (194 total residues) was inserted in both orientations into a M13 sequencing vector. The deduced sequence of this fragment differed from the sequence of the gene encoding p23 at two positions. First, a CTT codon (#33) was changed to GTT. This change resulted in the conservative substitution of valine for leucine. Second, the sequence CAA CAA AAA AAA TTG AAT GAG (codons 70–76) was converted to CAA CAA AAA AAA ATT GAA TGA (Figure 6). To control for the fact that these apparent changes could result from an error in amplification of the *SEC72* gene, we synthesized a DNA fragment by a second polymerase chain reaction. Sequence analysis revealed that these two mutations were present in this independent analysis. We therefore conclude that the *sec72-1* mutant contains a frame-shift mutation in the gene encoding p23.

The sec71-1 and sec72-1 Mutants Lack p23

We next asked whether p23 was physically altered in the *sec72-1* mutant as would be expected if this mutant contains a frameshift mutation. Cell extracts from strains NGY04 (*sec72-1*), NGY03 (*sec70-3*), NGY52 (*sec71-1*), and control strain 1A (Δp23) were analyzed by Western blotting with anti-p23 antibodies. The results depicted in Figure 7 show that p23 was missing in the Δp23 mutant (lane 1) and *sec72* mutant (lane 4) but present in the *sec70* mutant (lane 2). Surprisingly, p23 was missing in the *sec71* mutant (lane 3) that contained a nonsense mutation in the gene encoding p31.5 (Figure 3). The results in Figure 7 thus show that p23 is missing in the *sec71-1* and *sec72-1* mutants.

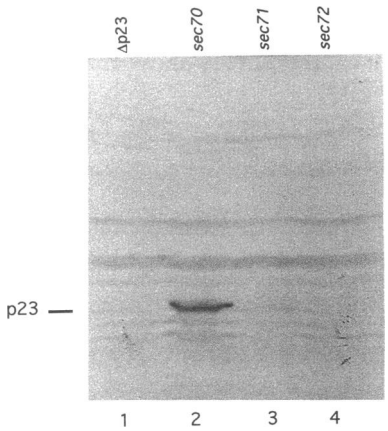

Figure 7. The *sec71* and *sec72* mutants lack p23. Haploid strains 1A (Δp23) (lane 1), NGY03 (*sec70*) (lane 2), NGY52 (*sec71*) (lane 3), and NGY04 (*sec72*) (lane 4) were grown at 30°C in YPD medium to OD_{600} = 1. Cells (1 OD_{600} equivalent) were broken by vortex mixing with glass beads in 10% TCA (Green and Walter, 1992). Proteins were boiled in SDS-PAGE sample buffer then analyzed by Western blotting with antibodies directed against p23 (MATERIALS AND METHODS). The position of p23 is indicated. Proteins are displayed on a 12% polyacrylamide gel and visualized with alkaline phosphatase conjugated to goat anti-rabbit antibodies.

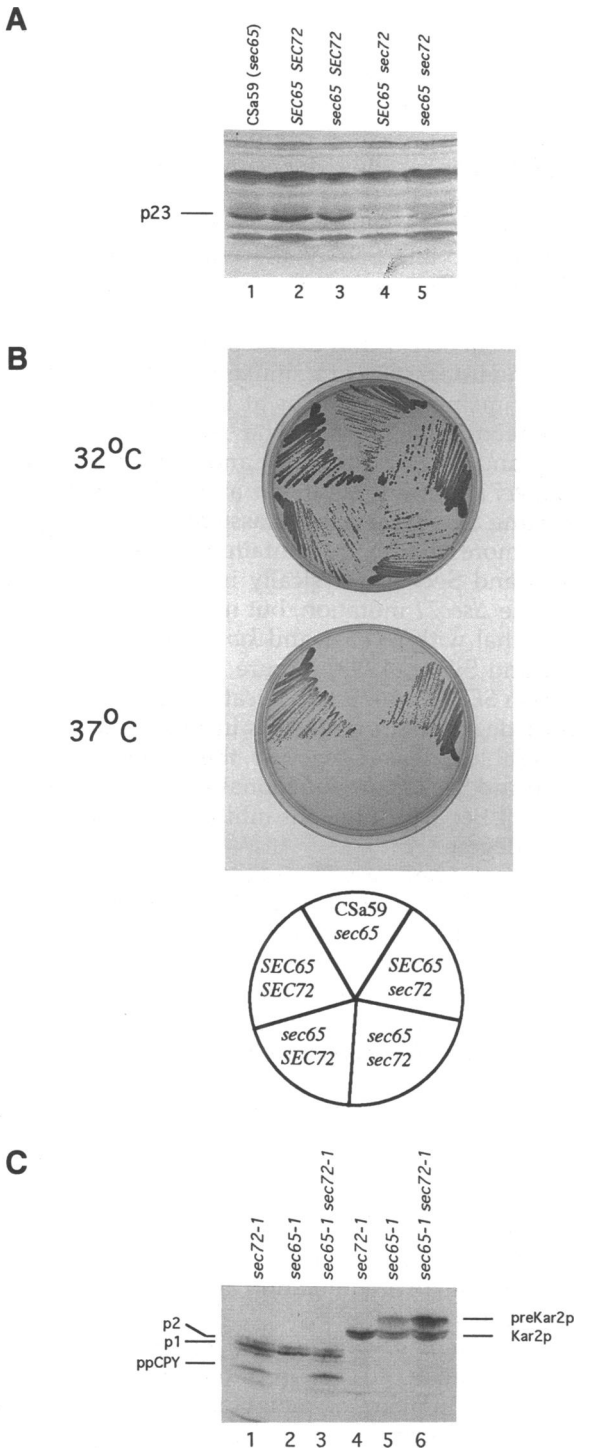

Figure 8. The *sec72-1* mutation is not synthetically lethal with the *sec65-1* mutation. (A) Haploid cells derived from a cross between strains CSa59 (*sec65-1*) and NGY04 (*sec72-1*) were grown at 23°C in YPD medium to OD_{600} = 1. Proteins were analyzed by Western blotting with anti-p23 antibodies as described in Figure 7. Haploid strains NGY80 (*SEC65 SEC72*), NGY81 (*sec65 SEC72*), NGY82 (*SEC65 sec72*), and NGY83 (*sec65 sec72*) that were derived from a single tetrad are depicted in lanes 2–5. Western blot analysis of control strain CSa59 (*sec65*) is also shown (lane 1). The position of p23 is indicated. (B) Cells derived from the tetrad depicted in A and strain CSa59 were placed on agar plates containing YPD then incubated at 32 or 37°C for 3 d. (C) Strains NGY81 (lanes 2 and 5), NGY82 (lanes 1 and 4), and NGY83 (lanes 3 and 6) were grown at 23°C in YPD medium then shifted to 37°C as described in Figure 2. Cells were subjected to a 5-min pulse with radiolabeled methionine (MATERIALS AND METHODS). Proteins were precipitated from cell extracts with anti-Kar2p (lanes 4–6) or anti-CPY (lanes 1–3) antibodies and analyzed by autoradiography as described in Figure 4. The positions of preKar2p, Kar2p, ppCPY, p1, and p2 are indicated.

The sec72-1 Mutation Is Not Synthetically Lethal with the sec65-1 or kar2-159 Mutations

The Δ*hss1* (Δ*sec71*) mutation was previously shown to be synthetically lethal with the conditional *sec65-1* mutation in a protein subunit of SRP (Kurihara and Silver, 1993). That is, the double mutant was inviable at temperatures where both single mutants were viable. To construct the corresponding strain containing mutations in *SEC72* and *SEC65*, strain NGY04 (*sec72-1*) was crossed with strain CSa59 (*sec65-1*). Diploid cells were sporulated, and asci containing four haploid spores were dissected and then incubated at 23°C. After several days of incubation, four viable progeny were detected in most of the tetrads (8/10). All the progeny from eight dissected tetrads grew at 32°C, the highest end of the permissive temperature range for the *sec65* mutant, whereas only two progeny from each tetrad grew at 37°C. This indicated that cells that were inviable at 37°C contained the *sec65-1* mutation. To identify cells containing *sec72-1*, haploid progeny from a randomly chosen tetrad were grown at 23°C to OD_{600} = 1 then examined by Western blotting with anti-p23 antibodies (MATERIALS AND METHODS). Two progeny (Figure 8A, lanes 2 and 3) and control strain CSa59 (*sec65*) (lane 1) expressed p23, whereas two progeny did not express p23 (lanes 4 and 5). Cells of strain NGY83 (*sec72-1 sec65-1*) depicted in lane 5 not only lacked p23 but were temperature sensitive for growth (Figure 8B), indicating the desired double mutant had been constructed. As shown by the growth patterns at 32 and 37°C (Figure 8B), the double mutant grew in a similar manner as the *sec65* mutant. Thus, the *sec65-1* and *sec72-1* mutations were not synthetically lethal.

We next wished to examine the translocation defect in this double mutant. Strain NGY83 (*sec72-1 sec65-1*) and control strains obtained from the tetrad depicted in Figure 8B, NGY82 (*sec72-1*) and NGY81 (*sec65-1*), were grown at 23°C to OD_{600} = 1 then shifted to 37°C for 1 h (MATERIALS AND METHODS). Cells were pulse labeled for 5 min with [^{35}S]-methionine and broken by vortex mixing with glass beads. Extracts from each strain were separated into two fractions, and proteins were precipitated with anti-CPY or anti-Kar2p antibodies. As expected, the *sec72-1* mutation inhibited translocation of ppCPY (Figure 8C, lane 1) but not

preKar2p (lane 4), whereas the *sec65-1* mutant selectively accumulated preKar2p (lanes 2 and 5). The double mutant, however, only partially blocked translocation of both precursor proteins (lanes 3 and 6). Although the double mutant accumulated ~30% more preKar2p than the *sec65* mutant and ~25% more ppCPY than the *sec72* mutant (Table 2), these data clearly demonstrated that ppCPY and preKar2p were directed to and across the ER membrane in cells deficient in both p23 and SRP.

The Δ*sec71* mutation is also synthetically lethal with *kar2-159*, a mutation that pleiotropically inhibits protein translocation (Vogel *et al.*, 1990; Kurihara and Silver, 1993). To analyze cells containing *sec72-1* and *kar2-159*, we crossed strains MS177 (*kar2-159*) and NGY28 (*sec72-1*). Diploid cells were sporulated, and ten asci were dissected. All the progeny from ten dissected tetrads grew at 23 and 30°C, whereas only two progeny from each tetrad grew at 32 and 37°C. Strain MS177 (*kar2-159*) was also inviable at 32 and 37°C. One tetrad was examined by Western blotting with anti-p23 antibodies. A nonparental ditype distribution of progeny was detected (i.e., the double mutant was represented twice among the four progeny). The growth properties of the double mutant were therefore indistinguishable from the conditional *kar2* mutant.

DISCUSSION

We have argued here that the *SEC66* and *SEC71* genes are identical because the corresponding mutants do not complement each other (Figures 1 and 2) and the *sec71-1* mutant contains an amber mutation that removes most of the cytoplasmic domain of p31.5 (Figure 3). In addition, we isolated a plasmid that complemented the *sec71-1* defect in integration of chimeric membrane proteins (Fang and Green, unpublished data). The plasmid contained *SEC66* and *SMY2*, thus providing further evidence that *SEC71* and *SEC66* are identical. *SMY2* that encodes a 87-kDa protein is closely linked (~200 bp) to *SEC66* (Kurihara and Silver, 1993). *SMY2* is a high-copy suppressor of a mutation in unconventional myosin (*myo2-66*) (Johnston *et al.*, 1991; Lillie and Brown, 1994). It also suppresses the growth defects of various conditional strains containing mutations in genes required for numerous cellular events (Fang, unpublished data) (Ferro-Novick, Poon, Weil, personal communication), including mutations in genes (*SEC12*, *SEC16*, *SEC18*, and *SEC22*) required for vesicular transport from the ER to the Golgi membranes (Kaiser and Schekman, 1990).

Results described here demonstrate that *SEC72* encodes p23 of the Sec63p-BiP complex as the *sec72-1* mutant does not complement a Δp23 mutant (Figure 5), lacks p23 (Figure 7), and contains a frameshift mutation in the gene encoding p23 (Figure 6). Because it was proposed that p31.5 and p23 be named Sec71p and Sec72p, respectively (Feldheim and Schekman, 1994), we will use this terminology.

Accumulating evidence reveal similarities and specific differences between Sec71p and Sec72p. Both proteins associate in two distinct complexes with Sec63p and yeast BiP (Kar2p) (Brodsky and Schekman, 1993) or Sec63p and Sec62p (Deshaies *et al.*, 1991). Sec71p and Sec72p can be crosslinked to Sec61p (Deshaies *et al.*, 1991). Sec71p and Sec72p are nonessential, although Sec71p is important for growth at 37°C (Feldheim *et al.*, 1993; Kurihara and Silver, 1993) (Figures 1 and 8B). Mutations in *SEC71* and *SEC72* partially block translocation and integration of a similar subset of soluble and chimeric membrane proteins at 30°C (Figures 4A and 8C) (Green *et al.*, 1992). The *sec71-1* mutant lacks Sec71p and Sec72p (Figure 7), and Sec72p is degraded in a Δ*sec71* mutant (Feldheim and Schekman, 1994), suggesting Sec71p facilitates assembly of Sec72p into one or more complexes containing these proteins or Sec71p and Sec72p physically interact. On the other hand, the Δ*sec71* mutation, but not *sec72-1*, is synthetically lethal with *sec65-1* and *kar2-159* mutations (Kurihara and Silver, 1993) (Figure 8). Furthermore, mutations in *SEC72* inhibit translocation and integration to a lesser degree than mutations in *SEC71* (Green *et al.*, 1992) (Table 2). Taken together, these results argue that Sec71p and Sec72p are functionally related but that depletion of Sec71p from cells inhibits translocation to a greater degree.

The *sec71-1* and Δ*sec71* mutants grow relatively well at 25 and 30°C (Green *et al.*, 1992; Feldheim *et al.*, 1993; Kurihara and Silver, 1993) (Figure 1). Neither mutant exhibits an apparent defect in translocation of preKar2p at 25 (Feldheim *et al.*, 1993) or 30°C (Fang and Green, unpublished data), suggesting Sec71p is nonessential but is important for translocation of a subset of precursor proteins. Although it is unclear why Δ*sec71* and *sec71-1* mutations inhibit growth at elevated temperatures, we believe that removal of Sec71p from the ER membrane may have an indirect effect on the stability of one or more essential proteins associated with Sec71p in the Sec63p and Sec63p-BiP complexes or removal may affect the translocation complex (Sec61p), thus leading to a small accumulation of preKar2p at elevated temperatures.

In vitro studies with purified components derived from mammalian cells and in vivo studies in yeast point to different numbers of membrane proteins as being required for translocation. For example, preprolactin is targeted to the translocation complex, consisting of three membrane protein subunits, by cytoplasmic SRP and the SRP receptor, consisting of two membrane protein subunits (Görlich and Rapoport, 1993). Based on the fact that yeast contain a homologue to the α-subunit of the SRP receptor (Ogg *et al.*, 1992) and the homologues to at least two subunits of the mammalian Sec61p complex (Stirling *et al.*, 1992; Esnault *et al.*, 1993), what is

the role of Sec71p and Sec72p? It is possible that additional membrane-bound components are required for translocation of precursors that are not efficiently recognized by SRP. Distinct membrane-bound components may accept precursor proteins from cytoplasmic heat shock proteins then posttranslationally mediate their transfer to Sec61p. Because Sec71p is predominately exposed to the cytoplasmic side of the membrane, this topology is consistent with it functioning as a membrane-bound receptor for a subset of precursor proteins or with it mediating the transfer of such proteins to the translocation complex.

The precursors to α-factor and CPY, but not invertase, can be posttranslationally translocated across the membrane (Hansen *et al.*, 1986; Rothblatt and Meyer, 1986; Waters and Blobel, 1986; Hansen and Walter, 1988). In this regard, we have previously demonstrated that the *sec71-1* and *sec72-1* mutants accumulate ppCPY but not preinvertase (Green *et al.*, 1992). Translocation of prepro-α-factor is inhibited in Δ*sec71* cells at permissive and nonpermissive temperatures (Feldheim *et al.*, 1993), and in SRP mutant cells (Hann and Walter, 1991), suggesting prepro-α-factor utilizes SRP, Sec71p, and cytoplasmic heat shock proteins for its transport to the membrane. In summary, the data suggest that Sec71p and Sec72p function in the posttranslational transport of a subset of precursor proteins to the translocation complex, with Sec71p playing a more important role. However, the possibility that Sec71p and Sec72p are directly involved in the translocation of proteins across the membrane cannot be excluded.

A genetic assay utilizing A189invHD and A255invHD identified *sec71-1* and *sec72-1* mutations (Green *et al.*, 1992). Despite the fact that numerous genetic studies have successfully utilized chimeric proteins containing the HD reporter enzyme, our use of arginine permease for targeting HD to the membrane is the only genetic assay that identified *SEC71* and *SEC72*. Because Shr3p, a membrane protein that is structurally related to Sec62p, is important for integration of amino-acid permeases (Ljungdahl *et al.*, 1992), the integration of arginine permease may have a specific requirement for Sec71p and Sec72p. However, our selection, which did not identify *sec61*, *sec62*, or *sec63* mutations, did not require that mutant cells exhibit a conditional-lethal phenotype. We may therefore have devised an assay that preferentially identified nonessential genes. Only eight mutants were isolated by this assay. Six of these mutants were in the *SEC70* complementation group. Unlike *sec71* and *sec72*, *sec70* mutations inhibit translocation of the HD moiety of A255invHD, but not A189invHD. Because residues 189–255 of arginine permease putatively contain two transmembrane segments (Green and Walter, 1992), characterization of *SEC70* will address the requirements for integration of arginine permease and may also explain why our selection assay identified mutations in novel genes.

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