Mutants in Three Novel Complementation Groups Inhibit Membrane Protein Insertion into and Soluble Protein Translocation across the Endoplasmic Reticulum Membrane of *Saccharomyces cerevisiae*

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Abstract. We have isolated mutants that inhibit membrane protein insertion into the ER membrane of *Saccharomyces cerevisiae*. The mutants were contained in three complementation groups, which we have named SEC70, SEC71, and SEC72. The mutants also inhibited the translocation of soluble proteins into the lumen of the ER, indicating that they pleiotropically affect protein transport across and insertion into the ER membrane. Surprisingly, the mutants inhibited the translocation and insertion of different proteins to drastically different degrees. We have also shown that mutations in SEC61 and SEC63, which were previously isolated as mutants inhibiting the translocation of soluble proteins, also affect the insertion of membrane proteins into the ER. Taken together our data indicate that the process of protein translocation across the ER membrane involves a much larger number of gene products than previously appreciated. Moreover, different translocation substrates appear to have different requirements for components of the cellular targeting and translocation apparatus.

RECENT genetic studies in *Saccharomyces cerevisiae* have shown that the SEC61, SEC62, and SEC63 genes are required for secretory protein translocation into the lumen of the ER (Deshaies and Schekman, 1987; Rothblatt et al., 1989). These genes were identified because mutants retained in the cytoplasm a fusion protein that consisted of the prepro region of prepro-α-factor fused to histidinol dehydrogenase (HD),[1] a yeast cytoplasmic enzyme required for cells to grow on histidinol. Fused after a signal sequence, HD was sequestered to the lumen of the ER, where it was enzymatically inactive in vivo. Selection for mutant cells containing enzymatically active HD led to the identification of SEC61, SEC62, and SEC63.

SEC61, SEC62, and SEC63 are essential for cell growth. The three genes encode distinct ER membrane proteins that are associated with one another and with other yet uncharacterized proteins in a complex (Deshaies et al., 1991). This complex may form all or part of a polypeptide translocation apparatus. Mutants in each component of the complex inhibit the translocation of a variety of soluble proteins across the ER membrane (e.g., the precursors of α-factor (a secretory protein), immunoglobulin heavy chain binding protein (BiP) (an ER resident protein), and carboxypeptidase Y (CPY) (a vacuolar protein) (Deshaies and Schekman, 1987; Rose et al., 1989; Rothblatt et al., 1989; Sadler et al., 1989).

Interestingly, not all proteins passing through the secretory pathway were equally affected by mutations in SEC61, SEC-62, and SEC63; the translocation of pre-invertase showed little inhibition in mutant cells (Rothblatt et al., 1989) and the membrane integration of dipeptidylaminopeptidase B was not affected (Stirling et al., 1992).

Mutants in BiP, an ER lumenal protein (Rose et al., 1989), and mutants in the cytoplasmic 70K stress proteins also inhibit protein translocation (Deshaies et al., 1988). Both proteins may interact with the preproteins or with the translocation apparatus on either side of the membrane to facilitate membrane translocation.

In contrast to yeast, studies in mammalian systems have identified different molecular components as important for protein targeting to and translocation across the ER membrane. Specifically, cytoplasmic signal recognition particle (SRP), a ribonucleoprotein complex of six protein subunits and a 7S RNA, and the SRP receptor, a heterodimeric ER membrane protein, are required for protein translocation across the mammalian ER membrane in vitro (Walter and Blobel, 1980; Gilmore et al., 1982; Meyer et al., 1982; Walter and Blobel, 1982). Other ER membrane proteins have also been implied to play a role in protein translocation (Wiedmann et al., 1987; Krieg et al., 1989); however, to date, no mammalian protein homologous to Sec61p, Sec-62p, or Sec63p has been described. This lack of common components identified in the different organisms led to the idea that the mechanism of protein translocation may be fundamentally different. Indeed, biochemical studies showed

1. *Abbreviations used in this paper*: BiP, immunoglobulin heavy-chain binding protein; CPY, carboxypeptidase Y; HD, histidinol dehydrogenase; SRP, signal recognition particle.

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that protein translocation in mammalian cells occurs mostly co-translationally mediated by SRP and SRP receptor, whereas yeast ER membranes can translocate certain fully synthesized preproteins posttranslationally without an apparent need for these components (Hansen et al., 1986; Rothblatt and Meyer, 1986; Waters and Blobel, 1986).

Recently, however, the yeast and mammalian studies converged with the discovery of a yeast gene (SRP54) that is homologous to the 54-kD protein subunit of mammalian SRP (Bernstein et al., 1989; Hann et al., 1989; Römisch et al., 1989). Further studies showed that in yeast an SRP complex and an SRP receptor exist and, in analogy to their mammalian counterparts, function in protein translocation (Hann and Walter, 1991; S. Ogg, M. Poritz, and P. Walter, manuscript in preparation). Thus with respect to yeast a model is evolving in which different translocation pathways operate in parallel. Different translocation substrates may be able to utilize the different pathways to different degrees; some proteins like prepro-α-factor can be efficiently translocated posttranslationally and independent of SRP, whereas others may be more difficult to maintain translocation competent after release from the ribosome and may therefore strictly require co-translational targeting by SRP and SRP receptor.

From these results it is apparent that components other than Sec61p, Sec62p, and Sec63p participate in protein translocation across the ER membrane, and it is likely that many other components have not yet been identified. Under the premise that integral membrane proteins may have requirements for membrane integration distinct from those of soluble preproteins (such as prepro-α-factor whose prepro sequence was used in the selection for sec61, sec62, and sec63) we engaged in a genetic search for mutants that would block membrane protein integration. Our genetic selection mimicked the selection used to identify mutants in SEC61, SEC62, and SEC63 in that we used fusion proteins that employed HD as the reporter domain. Our approach differed, however, in that we used an integral membrane protein as the targeting domain. As shown in this report, we identified three novel complementation groups that show pleiotropic translocation defects. Mutants that fall into these groups show a surprising degree of substrate specificity. Furthermore, we show that the selection was not exhaustive. It is therefore likely that other genes involved in the process remain to be identified.

Materials and Methods

Antibodies, Reagents, and Media

anti-BiP was obtained from Mark Rose (Princeton University, Princeton, NJ), anti-invertase from Johnny Ngsee (Stanford University, Stanford, CA), anti-α-factor from William Hansen (this lab), and anti-carboxypeptidase Y from Randy Schekman (University of California, Berkeley, CA). The preparation of anti-HD is described elsewhere (Green and Walter, 1991). Histidinol was obtained from Sigma Chemical Co. (St. Louis, MO) and used at a concentration of 10 mM on agar plates. For testing the growth of cells in the presence of 10 mM histidinol, agar plates contained SD medium (0.67% yeast nitrogen base without amino acids, 20 g/liter agar, 20 g/liter glucose). Supplements included 0.1 mg/ml leucine, 0.1 mg/ml adenine, 0.1 mg/ml tryptophan, and 0.1 mg/ml uracil. Trasylol was obtained from Mobay Corp. (New York). [^{35}S]Methionine was obtained from ICN Biomedicals Inc. (Irvine, CA).

SD-CAS medium was used for the growth of cells containing plasmids marked with URA3. SD-CAS contains the same ingredients as SD medium plus casamino acids (0.7%), tryptophan, and adenine (0.1 mg/ml each). The casamino acid supplement contains leucine and histidine, but the uracil concentration is too low for normal cell growth of ura3 mutants. We took advantage of the low uracil concentration in SD-CAS to detect cells lacking plasmids marked with URA3 which grew to very small colonies. These small colonies were then tested for normal growth on SD-CAS agar plates plus uracil (0.1 mg/ml).

Complementation Analysis

For some of the complementation analyses two procedures were used which were based on two different phenotypes exhibited by the SEC70, SEC71, and SEC72 mutants. In the first, a plasmid containing LEU2 and either SEC61, SEC62, SEC63, or KAR2 was transformed into strains NG30 (sec70-3)/pA^{255}invHD, NG31 (sec71-1)/pA^{255}invHD, and NG32 (sec72-1)/pA^{255}invHD. Transformed cells were detected on agar plates containing adenine, histidine, and tryptophan (0.1 mg/ml each) as described (Green and Walter, 1992). Cells were then streaked on similar agar plates except that 10 mM histidinol was substituted for histidine. Confluent growth on agar plates containing histidinol was scored as a failure to complement a sec70, sec71, and sec72 mutation. The plasmids used for this analysis are described in Table I.

Reduced cell growth rates which are characteristic of SRP54 mutants (Hann and Walter, 1991) and SEC70, SEC71, and SEC72 mutants were also used as a phenotype to test for complementation. This was done by crossing strain BHY147 with strains NG22, NG27, NG28, and the parent strain JC1-3C. Cells were then cured of pSRP54 (see above). The cells obtained from all four crosses grew at similar rates, indicating that the new mutants were not alleles of SRP54. The strains and plasmids used for this analysis are described in Table I.

Pulse-labeling and Immunoprecipitation and Western Blot Analysis

Cells were pulse-labeled with [^{35}S]methionine for 5 min at 30°C as described elsewhere (Green and Walter, 1992). CSa42 (sec61^{ts}), RDMY-20 (sec62^{ts}), and D8 (sec63^{ts}) cells were pulse labeled at the permissive temperature (22°C). Immunoprecipitations and analysis by SDS-PAGE were as described (Green and Walter, 1992). Prepro-α-factor was detected by Western blot analysis. Cell extracts from NG30 (sec70-3), NG31 (sec71), NG32 (sec72), or IG27-1 (Sec$^+$) cells were prepared from cells grown to OD_{600} = 1 ml cells were resuspended in 0.2 ml of 10% TCA and broken by vortexing with glass beads. The precipitated protein was fractionated by SDS-PAGE, blotted onto nitrocellulose, and detected with anti-α-factor antibodies followed by alkaline phosphatase-labeled secondary antibodies (purchased from Bio-Rad, Richmond, CA).

Fusion Protein Constructs

The construction of the plasmids containing gene fusions A^{189}invHD and A^{255}invHD were described elsewhere (Green and Walter, 1992). Sec62p-Invertase contains an N-terminal fragment (187 residues) of Sec62p fused to an invertase fragment that contains all of the mature invertase sequence except for two residues from its N-terminus (Deshaies and Schekman, 1990). Sec63p-Invertase contains an N-terminal fragment (133 residues) of Sec63p fused to an invertase fragment that contains all of the mature invertase except for two residues from its N-terminus (D. Feldheim, personal communication; Sadler et al., 1989).

Results

Isolation of Mutants

To isolate mutants defective in membrane protein integration, we used a gene fusion, A^{255}invHD, which encodes the NH_2-terminal 255 amino acids of arginine permease fused to a spacer comprised of a fragment of cytoplasmic invertase, which in turn was fused to histidinol dehydrogenase (HD) (Green and Walter, 1992). As depicted in Fig. 1, the NH_2-terminal 255 amino acids of arginine permease contain at least three putative transmembrane segments (also see Green and Walter, 1992). HD catalyses the last step in the

Table I. Strains and Plasmids Used in This Study

Strain	Genotype	Source
JC1-3C	MATa, ade5-1, trp-1-1, his4-401, ura3-52, leu2-1, HOL1-1	Christine Guthrie (University of California, San Francisco, CA)
FC2-12B	MATα, trp1-1, leu2-1, ura3-52, his4-401, HOL1-1, can1-1	Christine Guthrie
IH27-1	MATα, his4-401, trp1, ura3, can1, HMRα, HMLα	John Chant (University of California, San Francisco, CA)
CSa42	MATa, sec61-3, his4-401, ura3-52, HOL1-1, trp1-1, leu2-3,-112	Colin Stirling (University of California, Berkeley, CA)
RDMY-20	MATα, sec62-1, his4-401, ura3-52, HOL1-1, trp1-1, leu2-3,-112	Colin Stirling
D8	MATα, sec63-1, his4-401, ura3-52, HOL1-1, trp1-1, leu2-3,-112	Colin Stirling
BHY147	MATα, trp1, lys2, his3, ura3, ade2, srp54::LYS2 + [pSRP54]	Byron Hann (University of California, San Francisco, CA)
NG21	MATa, ade5-1, trp-1, his4-401, ura3-52, leu2-1, HOL1-1, sec70-2	This study
NG22	MATa, ade5-1, trp-1, his4-401, ura3-52, leu2-1, HOL1-1, sec70-3	This study
NG23	MATa, ade5-1, trp-1, his4-401, ura3-52, leu2-1, HOL1-1, sec70-4	This study
NG27	MATa, ade5-1, trp-1, his4-401, ura3-52, leu2-1, HOL1-1, sec71-1	This study
NG28	MATa, ade5-1, trp-1, his4-401, ura3-52, leu2-1, HOL1-1, sec72-1	This study
NG30	MATα, trp1, his4, ura3, ade5-1,sec70-3, HOL1-1	This study
NG31	MATα, trp1, his4-401, ura3,leu2, sec71-1, HOL1-1	This study
NG32	MATα, trp1, his4, ura3, leu2, sec72-1, HOL1-1	This study

Plasmid	Markers	Source
pA^{189}invHD	CAN1::HIS4, URA3, 2μ	Green and Walter (1992)
pA^{255}invHD	CAN1::HIS4, URA3, 2μ	Green and Walter (1992)
pCS15	SEC61, LEU2, CEN6, ARSH4	Dave Feldheim and Randy Schekman (University of California, Berkeley, CA)
pRD15	SEC62, LEU2, CEN6, ARSH4	Dave Feldheim and Randy Schekman
pDF26	SEC63, LEU2, CEN6, ARSH4	Dave Feldheim and Randy Schekman
pMR713	KAR2, LEU2, CEN4, ARS1	Mark Rose (Princeton University, Princeton, NJ)
pDF13	SEC63::SUC2, URA3, 2μ	Dave Feldheim and Randy Schekman
pRD33	SEC62::SUC2, URA3, 2μ	Dave Feldheim and Randy Schekman
pDF14	SEC63, LEU2, 2μ	Dave Feldheim and Randy Schekman
pSRP54	SRP54, URA3, CEN6, ARSH4	Byron Hann (University of California, San Francisco, CA)

biosynthesis of histidine. When HD is expressed in the cytoplasm of his4 mutant cells it allows for growth on histidinol, a metabolic precursor to histidine. A^{255}invHD, however, did not allow his4 mutant cells to grow on histidinol (Green and Walter, 1992) because upon membrane integration of the fusion protein HD became localized to the lumen of the ER (see Fig. 1) where it was enzymatically inactive in vivo.

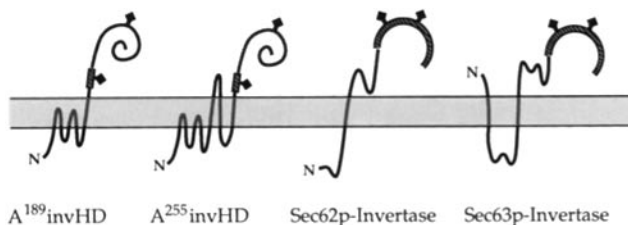

Figure 1. Schematic presentation of the proposed topologies of the membrane fusion proteins. The proposed topologies of A^{189}invHD, A^{255}invHD, Sec62p-Invertase, and Sec63p-Invertase in the ER membrane are schematically indicated (see Materials and Methods). Arginine permease, Sec62p, and Sec63p derived sequences are drawn as solid lines. The invertase spacer fragment is indicated as a striped box, the invertase fragment contained in Sec62p-Invertase and Sec63p-Invertase is substantially larger. The HD domain is indicated as a shaded spiral. Core glycosylation is indicated by black diamonds. The cartoon attempts to illustrate the relative transmembrane configurations. An arbitrary number of core-glycosylation units is shown.

We used the A^{255}invHD fusion construct in a selection for mutants that would be defective in the proper integration of this membrane protein. Such mutants would be expected to retain a sufficient amount of the HD moiety of the fusion protein in the cytoplasm to allow for growth of his4 mutant cells on histidinol. For this selection we used the strain JC1-3C (his4) (Table I) bearing A^{255}invHD on a plasmid. Five independent JC1-3C (his4)/pA^{255}invHD cell cultures were mutagenized with ethanemethyl sulfonate (Deshaies and Schekman, 1987). One half of the cells were killed by the mutagenesis. Approximately 10^6 cells from each culture were plated on agar plates containing histidinol (see Materials and Methods; Parker and Guthrie, 1985). Twenty mutant colonies appeared after five to seven days (4×10^{-6} mutational frequency).

Since we were interested in mutations in the cellular membrane protein integration apparatus, we wished to eliminate plasmid-linked mutations. Mutations linked to pA^{255}invHD would be expected to show a dominant phenotype in diploid cells. To identify dominant mutations, cells from each of the twenty colonies were crossed with FC2-12B (his4) cells (Table I). We found that eight of the mutants were recessive,

since the diploid cells derived from the cross did not grow in the presence of histidinol, but grew when histidine was added. The other 12 mutants were not examined further. The haploid cells of the eight recessive mutants were cured of pA^{255}invHD (see Materials and Methods) and retransformed with pA^{255}invHD. All eight mutants grew on medium containing histidinol, confirming that the recessive mutations were indeed unlinked to pA^{255}invHD. Two of the mutants showed a slight temperature-sensitive growth phenotype at 37°C; however, none of the mutations was lethal at elevated or reduced temperature.

Complementation Analysis

To group the mutations by complementation analysis, each of the eight mutants was crossed with strain IH27-1 (his4). Strain IH27-1 was used because diploid cells were found to sporulate with high frequency. After sporulation, tetrads were dissected and then germinated. Each mutation segregated 2:2 (wild-type:mutant) from at least three of four tetrads suggesting that a single mutant gene was responsible for cell growth on histidinol. After the cross, each of the eight mutations was obtained in both *MATα* and *MATa* strains. Thus, we were able to cross each mutant with the other seven mutants so that all possible combinations were obtained in diploid cells, which we tested for growth on agar plates containing histidinol. Six mutants were allelic since the diploid cells grew on agar plates containing histidinol. The remaining two mutants were not allelic to each other or to the other six mutants. Thus, the eight isolated mutations fall into three complementation groups which we named SEC70, SEC71, and SEC72. During the mutant selection some of the plates contained more than one SEC70 mutant. Three of the six isolated mutants of SEC70, sec70-2 (strain NG21; Table I), sec70-3 (strain NG22), and sec70-4 (strain NG23), were isolated from independently mutagenized cell pools. The SEC71-1 mutant and sec72-1 mutant were named strains NG27 and NG28 (Table I). The haploid strains of the opposite mating type, NG30 (sec70-3), NG31 (sec71-1), and NG32 (sec72-1) (Table I), were obtained by tetrad dissection from crosses between strains IH27-1 and NG22, NG27, and NG28, respectively. To further establish that a single mutation was responsible for growth on histidinol, strains NG30, NG31, and NG32 were backcrossed with strain JC1-3C/pA^{255}invHD. 10 tetrads were examined from each cross and tested for growth on histidinol. Each of the 30 tetrads yielded two germinated spores which grew on agar plates containing histidinol and two which failed to grow on agar plates containing histidinol. Interestingly, we found that the sec70-3, sec71-1, and sec72-1 mutations cosegregated with a cell growth defect. For sec70-3 this defect resulted in colonies on agar plates that were about one-third the size of the Sec^{+} strains. This growth defect was about one-half for strains containing either sec71-1 or sec72-1.

To determine whether the newly defined complementation groups are allelic to known translocation mutants, we next tested whether mutations in SEC61, SEC62, and SEC63, which block the translocation of soluble proteins across the ER membrane, would also block the integration of A^{255}invHD and thus allow his4 mutant cells expressing the fusion protein to grow on histidinol. It was previously shown that sec61^{ts}, sec62^{ts}, and sec63^{ts} mutant cells show protein translocation defects even when grown at permissive temperature (Deshaies and Schekman, 1987; Rothblatt et al., 1989). We therefore transformed sec61, sec62, and sec63 mutant cells with plasmid pA^{255}invHD and tested for growth on agar plates containing histidinol. Strains CSa42 (sec61, his4)/pA^{255}invHD, and D8 (sec63, his4)/pA^{255}invHD, but not the control strain JC1-3C (Sec^{+}, his4)/pA^{255}invHD grew at 22°C on agar plates containing histidinol. Interestingly, strain RDMY-20 (sec62, his4)/pA^{255}invHD did not grow under these conditions. This indicated that the tested mutant alleles of SEC61 and SEC63, but not that of SEC62 were defective in the membrane integration of A^{255}invHD.

These results were confirmed by a biochemical analysis. A^{255}invHD has been shown to become core glycosylated upon localization of the HD domain to the ER lumen (Green and Walter, 1992). We used the molecular weight shift associated with the glycosylation of A^{255}invHD as an indicator of membrane integration. Sec^{+} and sec61, sec62, and sec63 mutant cells bearing pA^{255}invHD were pulse labeled with [^{35}S]methionine, and A^{255}invHD was immunoprecipitated with anti-HD antibodies (Green and Walter, 1992). In agreement with the genetic data described above, a faster migrating band corresponding to unglycosylated A^{255}invHD was obtained in the sec61 and sec63 mutant strains (Fig. 2, lanes *2* and *4*). This band comigrated exactly with in vitro translated A^{255}invHD (not shown; Green and Walter, 1992). Note that a small amount of unglycosylated A^{255}invHD was also detected in the Sec^{+} control strain and the sec62 mutant strain (Fig. 2, lanes *1* and *3*). This indicated that some HD was present in the cytoplasm in the Sec^{+} control cells,

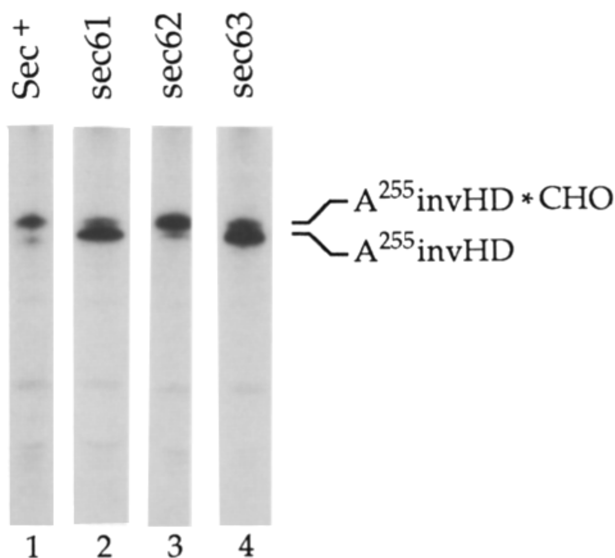

Figure 2. Analysis of A^{255}invHD in sec61, sec62, and sec63 cells. JC1-3C (*Sec^{+}*)/pA^{255}invHD cells (lane *1*), CSa42 (*sec61-3*)/pA^{255}invHD cells (lane *2*), RDMY-20 (*sec62-1*)/pA^{255}invHD cells (lane *3*), and D8 (*sec63-1*)/pA^{255}invHD cells (lane *4*) were pulse labeled with [^{35}S]methionine at the permissive temperature as described (see Materials and Methods and Green and Walter, 1992). Proteins were precipitated with anti-HD antibodies and displayed by SDS-PAGE. The position of A^{255}invHD and glycosylated A^{255}invHD (*A^{255}invHD*CHO*) are indicated.

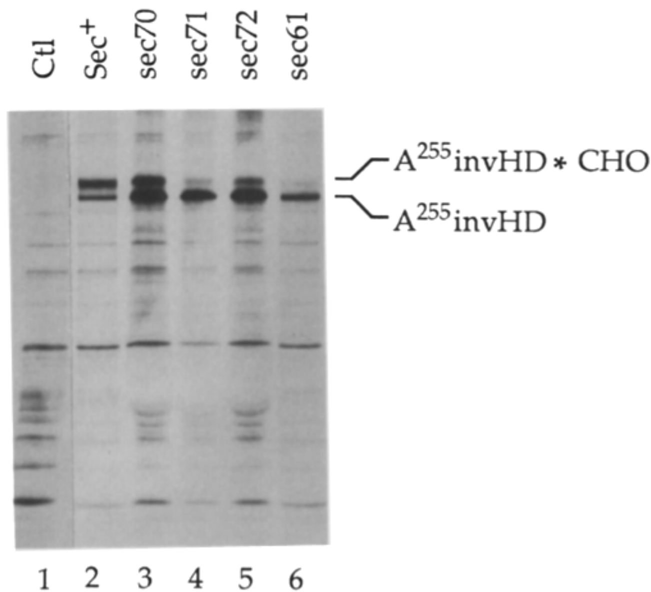

Figure 3. Analysis of A^{255}invHD in sec70, sec71, and sec72 cells. As described in Fig. 2, A^{255}invHD was immunoprecipitated from pulse-labeled NG22 (*sec70-3*)/pA^{255}invHD cells (lane *3*), NG27 (*sec71-1*)/pA^{255}invHD cells (lane *4*), and NG28 (*sec72-1*)/pA^{255}invHD cells (lane *5*), and as controls, from CSa42 (*sec61-3*)/ pA^{255}invHD cells (lane *6*), JC1-3C (*Sec*$^+$)/pA^{255}invHD cells (lane *2*), and JC1-3C (*Sec*$^+$) cells that did not contain a plasmid (lane *1*). Proteins were precipitated with anti-HD antibodies and displayed by SDS-PAGE. The position of A^{255}invHD and glycosylated A^{255}invHD (*A^{255}invHD*CHO*) are indicated.

although the genetic data indicated that this level was insufficient to support growth on histidinol.

Since the membrane integration of A^{255}invHD was impaired in sec61 and sec63 mutant strains, it was important to test whether any of the newly identified mutants were allelic to the known translocation mutants. CSa42 (sec61, his4) was crossed with strains NG30 (sec70, his4)/pA^{255}invHD, NG31 (sec71, his4)/pA^{255}invHD, and NG32 (sec72, his4)/pA^{255}invHD and, analogously, strain D8 (sec63, his4) was crossed with strains NG21 (sec70, his4)/pA^{255}invHD, NG27 (sec71, his4)/pA^{255}invHD, and NG28 (sec72, his4)/ pA^{255}invHD. None of the diploid cells derived from these crosses grew on agar plates containing histidinol, thus indicating that the mutants complemented each other. We can therefore conclude that sec70-3, sec71-1, and sec72-1 are not allelic to sec61-3 and sec63-1. The sec62 mutant strain could not be similarly tested, since the integration of A^{255}invHD was not impaired in sec62 cells (see above and Fig. 2, lane *3*).

In the second test, sec70, sec71, and sec72 mutant cells were transformed with low copy number plasmids (CEN) bearing the wild-type genes of SEC61, SEC62, and SEC63 and were tested for growth on histidinol (see Materials and Methods). Each of the transformed mutants grew on agar plates containing histidinol, indicating that SEC61, SEC62, and SEC63 did not complement the sec70, sec71, and sec72 defects. Furthermore, the transformed mutants exhibited a reduced growth rate, described above, that is characteristic of the sec70, sec71, and sec72 defects. A multi-copy plasmid containing SEC63, pDF14, was transformed into strain NG31 (sec71-1)/pA^{255}invHD, and cells were tested for growth on agar plates containing histidinol. These cells grew to small colonies compared to cells that were placed on agar plates containing histidine, indicating that the overproduction of SEC63 may have partially compensated for the defective insertion of A^{255}invHD into the ER membrane. In similar experiments we determined that none of the three new complementation groups was allelic to SRP54 or to KAR2 encoding BiP, which was recently shown (Rose et al., 1989) to play a role in ER protein translocation (see Materials and Methods).

Analysis of Membrane Protein Integration in sec70, sec71, and sec72 Cells

To corroborate the genetically inferred membrane protein integration defect of sec70, sec71, and sec72 cells, we assayed the integration of A^{255}invHD directly by immunoprecipitation. As shown in Fig. 3, JC1-3C (Sec$^+$)/pA^{255}invHD cells contained predominantly the glycosylated form of A^{255}invHD (Fig. 3, lane *2*) and, as expected, no immunoreactive band was detected in JC1-3C control cells lacking the plasmid (Fig. 3, lane *1*). As described above, a small amount of unglycosylated A^{255}invHD was also present in JC1-3C (Sec$^+$)/ pA^{255}invHD cells (Fig. 3, lane *2*). In contrast, however, sec70, sec71, and sec72 mutant cells contained markedly increased amounts of the unglycosylated form of A^{255}invHD (Fig. 3, lanes *3*, *4*, and *5*, respectively). As shown above, sec61 cells accumulated almost exclusively the non-glycosylated form of A^{255}invHD (Fig. 3, lane *6*). These results lend strong support to the notion that sec70, sec71, and sec72 mutant cells are indeed defective in the membrane integration of A^{255}invHD. We repeated the immunoprecipitation of A^{255}invHD from mutant strains obtained from two backcrosses against Sec$^+$ strains (see above), and similar results were obtained (data not shown).

We next tested whether a similar defect could also be observed for other membrane protein constructs. First we tested A^{189}invHD (Fig. 1) which is similar to A^{255}invHD, but only contains the NH_2-terminal 189 amino acids of arginine permease. As indicated in Fig. 1, A^{189}invHD lacks two putative transmembrane segments which are present in A^{255}invHD. Both A^{189}invHD and A^{255}invHD place the HD domain on the lumenal side of the ER membrane (Green and Walter, 1992). A^{189}invHD products synthesized in vivo were analyzed as above by immunoprecipitation after pulse labeling of cells. As expected, in Sec$^+$ control cells all of the A^{189}invHD was immunoprecipitated as the glycosylated form (Fig. 4 *A*, lane *1*), whereas in CSa42 (sec61-1)/ pA^{189}invHD cells all of the A^{189}invHD was found in the non-glycosylated form (Fig. 4 *A*, lane *5*) which comigrated with the in vitro translation product (not shown). The corresponding analysis of NG27 (sec71-1)/pA^{189}invHD and NG28 (sec72-1)/pA^{189}invHD cells (Fig. 4 *A*, lanes *3* and *4*) showed that both mutants were severely impaired in the membrane integration of A^{189}invHD and accumulated considerable amounts of the unglycosylated form of the protein. Surprisingly, however, NG22 (sec70-3)/pA^{189}invHD cells showed no detectable defect in the integration of the fusion protein (Fig. 4 *A*, lane *2*). Thus the sec70-3 mutation appears to inhibit selectively the integration of A^{255}invHD, but not of A^{189}invHD (compare Fig. 3, lanes *3* with Fig. 4 *A*, lane *2*). Simi-

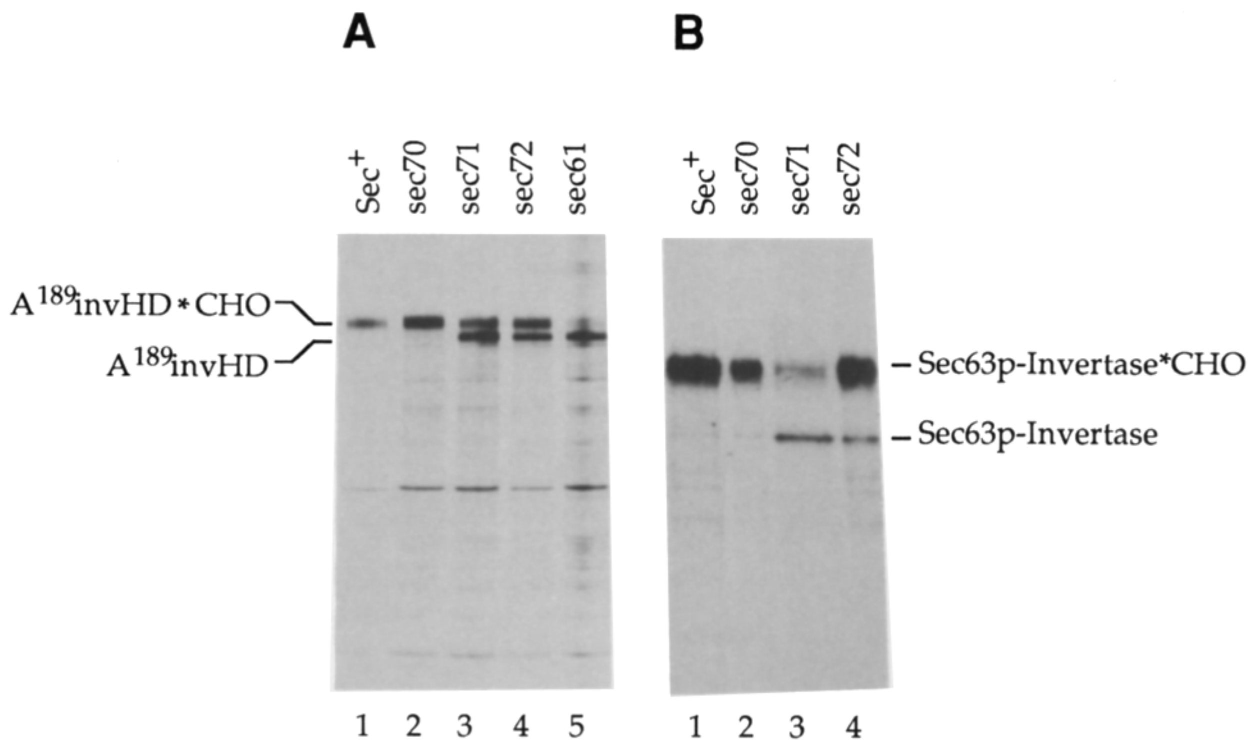

Figure 4. Analysis of other membrane fusion proteins in sec70, sec71, and sec72 cells. (*A*) As described in Fig. 2, A189invHD was immunoprecipitated from pulse-labeled NG22 (*sec70-3*)/pA189invHD cells (lane 2), NG27 (*sec71-1*)/pA189invHD cells (lane 3), and NG28 (*sec72-1*)/pA189invHD cells (lane *4*), and as controls, from CSa42 (*sec61-3*)/pA189invHD cells (lane *5*), and JC1-3C (*Sec+*)/pA189invHD cells (lane *1*). Proteins were precipitated with anti-HD antibodies and displayed by SDS-PAGE. The positions of A189invHD and glycosylated A189invHD (*A189invHD*CHO*) are indicated. (*B*) Sec63p-Invertase was immunoprecipitated from pulse-labeled NG22 (*sec70-3*)/pDF13 cells (see Table I) (lane *2*), NG27 (*sec71-1*)/pDF13 cells (lane *3*), and NG28 (*sec72-1*)/pDF13 cells (lane *4*), and as control, from JC1-3C (*Sec+*)/pDF13 cells (lane *1*). Proteins were precipitated with anti-invertase antibodies and displayed by SDS-PAGE. The positions of Sec63p-Invertase and glycosylated Sec63p-Invertase (*Sec63p-Invertase*CHO*) are indicated.

larly, the analysis of the other two alleles of sec70 showed no accumulation of unglycosylated A189invHD for sec70-2 and only a very minor effect for sec70-4 (data not shown).

Very similar results were obtained when the integration of a Sec63p-Invertase fusion protein (see Materials and Methods) (contained on the plasmid pDF13) (Table I) was tested in the mutant strains. The membrane topology of Sec63p-Invertase is shown in Fig. 1. Note that after pulse labeling the Sec+ control cells and NG22 (sec70-3)/pDF13 cells accumulate mostly the glycosylated, i.e., integrated form of Sec63p-Invertase (Fig. 4 *B*, lanes *1* and *2*). In contrast, NG27 (sec71-1)/pDF13 and NG28 (sec72-1)/pDF13 cells accumulate substantial amounts of the unglycosylated, i.e., non-integrated form of Sec63p-Invertase (Fig. 4 *B*, lanes *3* and *4*). A fourth membrane protein tested was a Sec62p-Invertase fusion (Fig. 1), which, interestingly, showed no integration defect in NG22 (sec70-3), NG27 (sec71-1), or NG28 (sec72-1) cells (data not shown).

Thus the integration of the different chimeric membrane proteins was impaired differentially by the sec70, sec71, and sec72 mutations. These data are summarized in Table II. There is no obvious correlation between the presumed membrane protein topology (Fig. 1) and the severity of the defect in the different mutant strains.

Analysis of Protein Translocation in sec70, sec71, and sec72 Cells

We have shown above that mutations that block the translocation of secretory proteins across the ER membrane also inhibited the integration of membrane proteins (Fig. 2). We were therefore interested to test whether, conversely, the mutants isolated as membrane protein integration defective

*Table II. Inhibition of Protein Insertion into the ER**

Protein	sec70-3	sec71-1	sec72-1
pA255invHD	+++‡	+++	+++
Sec63p-Invertase	−	++	+
pA189invHD	−	+++	++
Sec62p-Invertase	−	−	−
α-factor	+	+	++
BiP	++	−	−
CPY	+	+++	++
Invertase	−§	−	−

* Protein insertion was detected by immunoprecipitating each protein from either NG30, NG31, or NG32 cells, and then testing for glycosylation or, for BiP, for signal cleavage after a 5-min pulse of [35S]methionine. α-Factor was detected by Western blotting as detailed in Materials and Methods and in Fig. 5 *C*. Strains NG22, NG27, and NG28 were used for the immunoprecipitations of A189invHD, A255invHD, and invertase. The membrane protein insertion assay for glycosylation measured only the translocation of the extracytoplasmic domains; the insertion of other segments were not tested. A189invHD, A255invHD, Sec63p-Invertase, and Sec62p-Invertase were plasmid encoded. The other proteins were encoded in single copy by chromosomal genes.
‡ (+) indicates insertion was inhibited by 10% or less. (++) Indicates insertion was inhibited by 10 to 50%. (+++) Indicates insertion was inhibited by more than 50%.
§ In the case of invertase, a small amount of pre-invertase (<10%) was detected in sec70-3 cells in two out of three independent experiments.

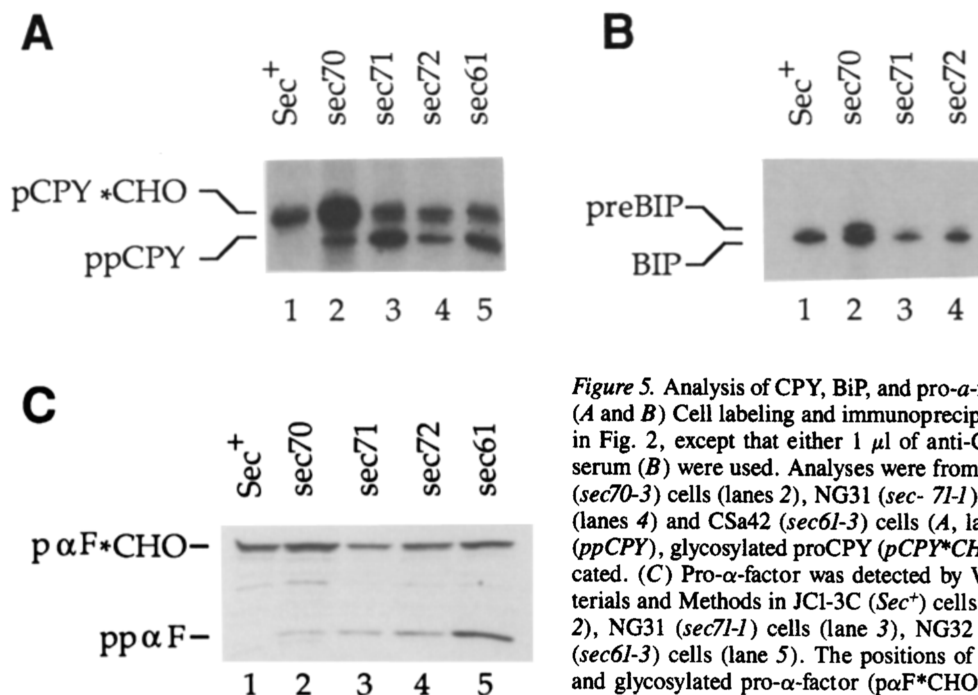

Figure 5. Analysis of CPY, BiP, and pro-*α*-factor in sec70, sec71, and sec72 cells. (*A* and *B*) Cell labeling and immunoprecipitations were performed as described in Fig. 2, except that either 1 μl of anti-CPY serum (*A*) or 0.5 μl of anti-BiP serum (*B*) were used. Analyses were from JC1-3C (*Sec*⁺) cells (lanes *1*), NG30 (*sec70-3*) cells (lanes *2*), NG31 (*sec- 71-1*) cells (lanes *3*), NG32 (*sec72-1*) cells (lanes *4*) and CSa42 (*sec61-3*) cells (*A*, lane *5*). The positions of prepro-CPY (*ppCPY*), glycosylated proCPY (*pCPY*CHO*), pre-BiP and mature BiP are indicated. (*C*) Pro-α-factor was detected by Western blotting as described in Materials and Methods in JC1-3C (*Sec*⁺) cells (lane *1*), NG30 (*sec70-3*) cells (lane *2*), NG31 (*sec71-1*) cells (lane *3*), NG32 (*sec72-1*) cells (lane *4*), and CSa42 (*sec61-3*) cells (lane *5*). The positions of cytoplasmic prepro-α-factor (*ppαF*) and glycosylated pro-α-factor (pαF*CHO) are indicated.

would also block the complete translocation of soluble proteins. We tested for the accumulation of untranslocated proteins in the strains NG30 (sec70-3), NG31 (sec71-1), and NG32 (sec72-1). Fig. 5 shows the analysis for carboxypeptidase Y (CPY, a vacuolar protein), BiP (encoded by KAR2, a soluble ER resident protein), and pro-α-factor (a secretory protein). Note that the translocation of CPY and pro-α-factor was impaired in all three mutant strains, as apparent from the accumulation of unglycosylated preproCPY (Fig. 5 *A*, lanes *2–4*) and unglycosylated prepro-α-factor (Fig. 5 *C*, lanes *2–4*). Similar defects were observed in CSa42 (sec61-1) cells (Fig. 5, *A* and *C*, lanes *5*), but not in control Sec⁺ cells (Fig. 5, *A* and *C*, lanes *1*). A very different result was obtained when the translocation of BiP was assessed in these cells. BiP is not glycosylated and therefore the accumulation of a slower migrating form, preBiP, containing the uncleaved signal sequence is indicative of a translocation defect. Note that only NG30 (sec70-3) cells show an accumulation of preBiP (Fig. 5 *B*, lane *2*), whereas BiP appeared efficiently translocated in NG31 (sec71-1) and NG32 (sec72-1) cells (Fig. 5 *B*, lanes *3* and *4*) and in Sec⁺ control cells (Fig. 5 *B*, lane *1*). A fourth soluble protein, invertase, was not detectably impaired in sec70-3, sec71-1, and sec72-1 mutant cells (data not shown).

Discussion

Mutations in SEC70, SEC71, and SEC72 were selected using a strategy designed to find mutants that were impaired in their ability to integrate a membrane protein. During the analysis of the mutant cells we found that mutations in each of the three new complementation groups also blocked the translocation of soluble proteins across the ER membrane. This shows that the defects observed in these mutants were not restricted to membrane protein integration, but more pleiotropically impaired protein traffic across the ER membrane. Since the soluble proteins that were monitored in this analysis were encoded by their endogenous chromosomal genes, this demonstrated that the effects of the mutations were not limited to the membrane proteins tested which were artificial fusion proteins encoded by plasmids.

Our data did not address the questions of whether the observed mutant phenotypes were due to defective targeting of the proteins to the ER membrane or due to a defective membrane integration/translocation machine. In vitro assays using membrane and soluble fractions of mutant cell extracts and characterizations of the affected gene products after identification, cloning, and genetic manipulation will directly address this issue. Progress toward this goal has been made. We have recently cloned and sequenced a gene that complements the sec71 mutant. This gene encodes a 87-kD hydrophilic protein of unknown function.

The mutations in SEC70 (identified by three independently isolated alleles) conferred a remarkable phenotype: cells correctly localized the HD moiety of A^{189}invHD to the extracytoplasmic side of the ER membrane, but showed an increased mislocalization of the HD moiety of A^{255}invHD (Figs. 3 and 4). Since A^{255}invHD differs from A^{189}invHD only by the additional presence of two putative transmembrane segments, this suggests that SEC70 function may be specifically required to facilitate the proper integration of these latter hydrophobic protein segments. Thus we would predict that the SEC70 gene product exerts its function at the ER membrane during the translocation/integration process. This interpretation appears most plausible since A^{189}invHD was correctly targeted to the ER and A^{255}invHD contained all the same targeting signals contained in A^{189}invHD, including an internal signal sequence identified previously (Green et al., 1989). However, it still remains possible that, despite these similarities, A^{189}invHD may have been targeted to the ER membrane by a different pathway than that

used by A^{255}invHD, one that was not impaired in sec70 mutants.

Surprisingly, different membrane and soluble proteins were impaired to different degrees during their integration into or translocation across the ER membrane in the different mutant strains (see Table II). There is no obvious pattern emerging that would allow us to categorize the substrate proteins and thus to predict whether a given protein would be affected by a particular mutation. Rather it appears from these data that individual proteins are differentially sensitive to the mutations. This could imply that different cellular components may be used in different pathways that lead to the trafficking of proteins to and across the ER membrane. For example, our data suggest that BiP can be translocated efficiently across the ER membrane without SEC71 and SEC72 function, whereas Sec63p-Invertase can be inserted into the membrane without SEC70 function. Alternatively, all the gene products defined by the mutations collaborate in the targeting and translocation process of all protein substrates, but subtle defects in the isolated alleles produce more pronounced translocation defects for some proteins, yet are silent for others.

It is clear from these data, that the requirements on the translocation machine differ vastly for different translocation substrates. Whether these differences are due to different signal sequences or different passenger domains remains to be analyzed by altering these parameters systematically. Intriguingly, none of the mutations identified to date, i.e., the mutations described here and mutations in SEC61, SEC62, and SEC63 (Rothblatt et al., 1989) shows a marked inhibition of pre-invertase translocation. Thus for unknown reasons, invertase appears to be a particularly permissive translocation substrate.

Since cells mutant in SEC61 and SEC63 also showed defects in the membrane integration of A^{255}invHD, we expected our selection to yield mutations in these genes. Surprisingly however, none of our mutants was complemented by SEC61, SEC62, and SEC63. Similarly, the selection applied by Schekman and co-workers in which sec61, sec62 and sec63 mutants were isolated did not identify other genes which are now known to be involved in protein targeting and translocation, such as BiP (Rose et al., 1989) and components in the SRP-dependent targeting pathway (Hann and Walter, 1991; S. Ogg, M. Poritz, and P. Walter, manuscript in preparation). It is possible that using a different translocation substrate or using less stringent selection criteria (e.g., we did not require our mutants to be conditionally lethal) have allowed us to identify new complementation groups. Given the small number of mutants isolated and our failure to isolate mutations in SEC61, SEC62, and SEC63, it is clear that this selection has not been exhaustive and that many as yet unidentified genes may also participate in protein transport across or integration into the ER membrane.

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