Molecular Biology of the Cell
Vol. 3, 895–911, August 1992

Signal Recognition Particle Receptor is Important for Cell Growth and Protein Secretion in *Saccharomyces cerevisiae*

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Submitted March 24, 1992; Accepted June 17, 1992

In mammalian cells, the signal recognition particle (SRP) receptor is required for the targeting of nascent secretory proteins to the endoplasmic reticulum (ER) membrane. We have identified the *Saccharomyces cerevisiae* homologue of the α-subunit of the SRP receptor (SRα) and characterized its function in vivo. *S. cerevisiae* SRα is a 69-kDa peripheral membrane protein that is 32% identical (54% chemically similar) to its mammalian homologue and, like mammalian SRα, is predicted to contain a GTP binding domain. Yeast cells that contain the SRα gene (*SRP101*) under control of the *GAL1* promoter show impaired translocation of soluble and membrane proteins across the ER membrane after depletion of SRα. The degree of the translocation defect varies for different proteins. The defects are similar to those observed in SRP deficient cells. Disruption of the *SRP101* gene results in an approximately sixfold reduction in the growth rate of the cells. Disruption of the gene encoding SRP RNA (*SCR1*) or both *SCR1* and *SRP101* resulted in an indistinguishable growth phenotype, indicating that SRP receptor and SRP function in the same pathway. Taken together, these results suggest that the components and the mechanism of the SRP-dependent protein targeting pathway are evolutionarily conserved yet not essential for cell growth. Surprisingly, cells that are grown for a prolonged time in the absence of SRP or SRP receptor no longer show pronounced protein translocation defects. This adaptation is a physiological process and is not due to the accumulation of a suppressor mutation. The degree of this adaptation is strain dependent.

INTRODUCTION

Mammalian signal recognition particle (SRP)[1] acts to target ribosomes that synthesize secretory and membrane proteins to the endoplasmic reticulum (ER) membrane where they are translocated across the membrane into the ER lumen. In vitro assays have allowed the development of a detailed model describing the function of SRP in mammalian cells (Walter and Lingappa, 1986, for review). In brief, signal sequences that are expressed as part of a nascent protein chain emerging from the ribosome bind to SRP in a binding site contained on the 54-kDa SRP subunit (SRP54) (Krieg *et al.*, 1986; Kurzchalia *et al.*, 1986). This interaction causes SRP to bind tightly to the ribosome, effecting an elongation arrest or pause in the translation of the nascent secretory protein. Interaction of the engaged SRP with the SRP receptor, a heterodimeric membrane protein, that is anchored in the ER membrane (Gilmore *et al.*, 1982a; Meyer *et al.*, 1982; Tajima *et al.*, 1986) releases the translational arrest and allows the ribosome that is synthesizing the protein chain to become bound to other components in the ER membrane. These components, collectively termed a "translocon," allow the transfer of the growing protein chain across the membrane, presumably through a transient aqueous pore in the membrane (Gilmore and Blobel, 1985; Simon and Blobel, 1991). SRP and SRP receptor act catalytically in this

[1] *Abbreviations used:* bp, base pair; CPY, carboxypeptidase Y; DPAP-B, dipeptidyl aminopeptidase-B; DTT, dithiothreitol; ER, endoplasmic reticulum; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid; kb, kilobase; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SRα, SRP receptor α-subunit; SRβ, SRP receptor β-subunit; SRP, signal recognition particle; SRP54, 54-kDa subunit of SRP; Tris, tris-(hydroxymethyl)aminomethane.

process; they are not part of the ribosome-membrane junction that mediates the transfer of the protein chain across the lipid bilayer. Rather, they function to bring the ribosome to the correct target membrane and are then released from the ribosome (Gilmore and Blobel, 1983).

Interestingly, SRP54 and the α-subunit of the SRP receptor (SRα) both contain a GTPase domain (G-domain) (Bernstein *et al.*, 1989; Connolly and Gilmore, 1989; Römisch *et al.*, 1989). The G-domains of SRP54 and SRα are homologous to one another and define a new family of GTPases. The β-subunit of the SRP receptor (SRβ) also contains a GTPase domain, which is structurally distinct from those of SRP54 and SRα (Miller and Walter, unpublished data). The precise mechanistic role of each of the three GTPase domains that interact during the SRP-dependent protein targeting reaction remains to be elucidated. Cycles of GTP hydrolysis by SRP54 and SRP receptor may control the assembly of the SRP-signal sequence-ribosome complex and the assembly of components of the translocon. Thus, GTP hydrolysis may enhance the fidelity of and provide unidirectionality to the targeting reaction. Point mutations in the GTP binding sites of SRP54 and SRα block the translocation of proteins across the ER membrane (Rapiejko and Gilmore, 1992; Bernstein and Walter, unpublished data), indicating that both GTP binding sites are functionally important in the pathway.

Recently, components of a SRP have been identified and characterized in *Saccharomyces cerevisiae* (Hann and Walter, 1991; Hann *et al.*, 1992; Stirling and Hewitt, 1992). The yeast SRP is a 16S cytoplasmic ribonucleoprotein that is important for the targeting of membrane and secretory proteins to the ER membrane. *S. cerevisiae* SRP is not essential for cell growth, although cells in which SRP is genetically disrupted grow poorly. The translocation of a number of soluble and membrane proteins into the lumen of the ER is impaired in SRP deficient cells. Surprisingly, different proteins show translocation defects of varying severity. After SRP depletion, the membrane integration of dipeptidyl aminopeptidase B (DPAP-B) is inhibited by over 90%, the translocation of preKAR2p is inhibited by ~50%, and the translocation of preprocarboxypeptidase Y (preproCPY) is not affected. Thus, in the absence of SRP, proteins are targeted via an SRP-independent pathway to the ER membrane and are translocated in sufficient amounts to sustain cell growth. Some proteins, however, cannot efficiently use the SRP-independent pathway.

The approach that lead to the first identification of the yeast SRP took advantage of the phylogenetic conservation of the primary structure of the mammalian SRP54 and its prokaryotic homologue, the product of the *ffh* gene of *Escherichia coli*. Comparison of these evolutionary distant sequences allowed the identification of conserved blocks of amino acids that were used in a polymerase chain reaction (PCR)-based approach to isolate the gene (*SRP54*) encoding the corresponding protein from *S. cerevisiae* (Hann *et al.*, 1989). Here we have used a similar method to identify an SRα homologue in *S. cerevisiae*.

MATERIALS AND METHODS

Strains, Antibodies, and General Methods

Yeast strains used in this study are listed in Table 1. Genetic techniques were performed as described by Sherman *et al.* (1974), except where noted. Anti-DPAP-B serum was kindly provided by Tom Stevens, Institute of Molecular Biology, University of Oregon, Eugene; anti-KAR2p by Mark Rose, Biology Department, Princeton University, Princeton, NJ; anti-invertase, anti-CPY, anti-Sec61p, and anti-prepro-α-factor by Randy Schekman, Division of Biochemistry and Molecular Biology, University of California at Berkeley; and anti-$F_1\beta$ATPase by M. Yaffe, Department of Biology, University of California, San Diego. Recombinant DNA techniques were performed as described in Maniatis *et al.* (1982). Oligonucleotides were synthesized on a Milligen/Biosearch Cyclone Plus DNA synthesizer (Milligen/Biosearch, Division of Millipore, Novato, CA) and purified according to the manufacturer's instructions. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10–15% gradient gels and all Western blots were visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL) according to the instructions of the manufacturer.

PCR and Cloning of SRP101

PCR was performed on *S. cerevisiae* genomic DNA as described (Saiki *et al.*, 1988; Hann *et al.*, 1989; Kamb *et al.*, 1989). The oligonucleotide "A" had the sequence 5'-GGTGTNAA(T/C)GGNGTNGGNAA-3' (20mer, 512-fold degenerate) and encoded the amino acid sequence GVNGVGK. The reverse complement of oligonucleotide "B" with the sequence 5'-CN(G/C)CNGCNC(G/T)(A/G)AANGT(A/G)TC-3' (20mer, 4096-fold degenerate) encoded the amino acids DTFRAGA (Hann *et al.*, 1989). PCR products were separated on a 6% polyacrylamide gel. A 110-base pair (bp) band was excised from the gel and sequenced as described by Hann *et al.* (1989).

A genomic clone of the *SRP101* gene was isolated by screening a plasmid library of *S. cerevisiae* strain AB320 (Nasmyth and Tatchell, 1980) in the vector YEp13 using either the gel purified *S. cerevisiae* PCR product or, in later rounds of screening, an oligonucleotide derived from the PCR sequence. The complete 4.6-kilobase (kb) genomic insert together with flanking (pBR322 derived) vector sequences was excised by digestion with *Eco*RI and *Sal* I and cloned into Bluescript II SK (+) and SK (−) (Stratagene, La Jolla, CA) to generate plasmids pSRP101(+) and pSRP101(−), respectively. A series of deletions from the 5' end of the insert was generated using the method of Henikoff (1984) (using the Stratagene Exo-Mung kit) for the pSRP101(+) clone (Henikoff, 1984); a series of deletions from the 3' end was generated using the Kilo-sequencing method of Barnes (1987) for the pSRP101(−) clone (Barnes, 1987). Dideoxysequencing using Sequenase (United States Biochemicals, Cleveland, OH), ^{35}S-dATP, and electrolyte gradient gels (Sheen and Seed, 1988) was carried out on single stranded phagemid DNA isolated according to Stratagene protocols. Gaps in the sequence were filled by sequencing from synthetic oligonucleotide primers. Base number 1 of the DNA sequence deposited in Genbank (accession no. M77274) is the A of the presumptive initiating AUG codon.

Gene Disruption and Rescue

We constructed a DNA fragment suitable for gene disruption of the *SRP101* locus by PCR. A 2320-bp DNA fragment of the *ADE2* locus (bases 273–2593 [Stotz and Linder, 1990]) was amplified by PCR using two primers that each contained 27 bases of *ADE2* sequence at their

3′ ends (bases 273–300 and 2566–2593 of the *ADE2* sequence [Stotz and Linder, 1990]) and 72 bases of *SRP101* sequence at their 5′ ends (bases −1 to −72 and 1780–1846 of the *SRP101* sequence [Figure 5A]), respectively. The resulting 2474-bp fragment was introduced into an *ade2* diploid strain (strain W303) by one step gene replacement (Orr-Weaver *et al.*, 1981) using the LiOAc transformation procedure (Ito *et al.*, 1983). Yeast genomic DNA was prepared (Davis *et al.*, 1980) from Ade$^+$ transformants and was screened by PCR using oligonucleotides corresponding to bases −404 to −385 and 2136–2153 (Figure 5A, regions Y and Z) of the *SRP101* locus. The *ADE2* gene was found to be correctly integrated at the *SRP101* locus in 1 of 16 isolated Ade$^+$ transformants.

Disruption of the *SCR1* gene was performed exactly as in Hann and Walter (1991), except that the W303 strain was used. The disruption was confirmed by Southern hybridization as described (Hann and Walter, 1991). Because loss of either *SCR1* or *SRP101* yields only rho$^-$ cells, a diploid heterozygote resulting from a cross of these two deletions is also rho$^-$ and, consequently, unable to sporulate. Thus, we could not construct a strain bearing the double deletion of *SCR1* and *SRP101* by crossing strains bearing the single deletions. The double deletion was therefore constructed by the following procedure. A rho$^-$ haploid strain (SOY60, but MAT**a**) containing *scr1-Δ2* (*SCR1::HIS3*) was crossed with a rho$^+$ strain (SOY48) containing *srp101-Δ1* (*SRP101::ADE2*) complemented by plasmid pSO210 (see below). After selection on His$^-$, Ade$^-$, Ura$^-$ plates, diploids were scored for loss of pSO210 by growth on plates containing 5-fluoroorotic acid. The resulting diploid, heterozygous for both gene deletions, was sporulated and dissected by standard procedures.

Rescue of the haploid strain containing the *SRP101* deletion (*srp101-Δ1*) was performed by moving the complete genomic SRα insert of plasmid pSRP101(+) on a 7.0-kb *Pvu* I fragment into the *CEN6 ARS4*-containing *Pvu* I fragment of plasmids pRS316 and pRS314 (Sikorski and Heiter, 1989) to yield plasmids pSO210 and pSO220, respectively. Plasmid pSO210 contains *URA3* as a selectable marker, and pSO220 contains *TRP1* as a marker. These plasmids were transformed into the *SRP101/srp101-Δ1* diploid strain SOY34 by selecting for Ura$^+$ or Trp$^+$ transformants. Transformants were sporulated and tetrads dissected and tested by standard procedures (Sherman *et al.*, 1974).

Preparation of Antibodies to SRα

Antiserum was raised against a fusion protein expressed in *E. coli*, containing glutathione-S-transferase from *S. Japonicum* as the N-terminal domain linked to a fragment of SRα. This fusion protein was generated by a three step subcloning procedure. First, pUC5′Δ242 was generated by subcloning a 2.0-kb *Xba* I-*Hin*dIII fragment from pSRP101(+) encoding the C-terminal 379 amino acids of *SRP101* into the *Xba* I-*Hin*dIII sites of pUC18. Second, pUCSRP101 was constructed by subcloning a 740-bp PCR fragment encoding the first 242 amino acids of *SRP101* into the *Bam*HI-*Xba* I site of pUC5′Δ242. This regenerated the entire coding sequence of *SRP101* and removed all the DNA sequence information upstream of the start codon. Finally, subcloning a 950-bp *Bam*HI-*Hpa*I fragment from pUCSRP101 into the *Bam*HI-*Eco*RI site of pGEX-3X created the fusion protein plasmid, pGEXSRP101 (Smith and Johnson, 1988).

A 60-kDa fusion protein, containing amino acids 1–317 from SRα, was overproduced in *E. coli*. The protein was insoluble and was therefore solubilized in urea before further purification by differential centrifugation and preparative SDS-PAGE (Schloss *et al.*, 1988). Polyclonal rabbit antiserum against the SDS denatured fusion protein was prepared by Caltag (South San Francisco, CA).

The antibodies were purified by affinity chromatography. The fusion protein was coupled to CNBr activated Sepharose Cl-4B (Pharmacia, Piscataway, NJ) at a concentration of 6 mg/ml according to the manufacturers instructions. Bound antibodies were eluted as described (Walter and Blobel, 1983). Affinity purified antibodies were concentrated by precipitation with ammonium sulfate, resuspended in phosphate-buffered saline containing 50% glycerol and 0.02% NaN_3 at a concentration of 4 mg/ml, and stored at −80°C.

Cell Fractionation

Subcellular localization of SRα was performed essentially as in Deshaies and Schekman (1990). Briefly, strain W303 was grown to 2.0 OD_{600}/ml, and cells were harvested by centrifugation in a clinical centrifuge. After one wash in buffer A (100 mM tris(hydroxymethyl)aminomethane [Tris]-HCl, pH 9.4, 10 mM dithiothreitol (DTT), 10 mM NaN_3), cells were resuspended to a concentration of 20 OD_{600} cell equivalents per ml in buffer A and incubated for 10 min at 24°C. Cells were then pelleted and resuspended in spheroplast buffer (25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES]/KOH, pH 7.5, 20 mM DTT, 5 mM $MgCl_2$, 10 mM NaN_3) containing 1.3 M sorbitol to a concentration of 50 OD_{600} cell equivalents/ml. After adding 25 U/OD_{600} of lyticase (Sigma Chemicals, St. Louis, MO), cells were incubated with shaking at 30°C for 30 min. Spheroplasts were centrifuged through a cushion consisting of spheroplast buffer without DTT containing 1.9 M sorbitol at 8000 × *g* (7250 rpm) in a Beckman (Fullerton, CA) JS-13 rotor for 5 min at 4°C. Spheroplasts were resuspended in lysis buffer (200 mM sorbitol, 10 mM HEPES-KOH, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml each of the protease inhibitors pepstatin A, leupeptin, chymostatin, and antipain) to a concentration of 100 OD_{600} cell equivalents/ml. Lysis of the cells was achieved by gently vortexing (setting 3 on a scale of 1 to 10) the cell suspension three times in the presence of three-fourths volume zirconium oxide beads (Biospec Products, Bartlesville, OK). Each round of lysis consisted of 15 s of vortexing followed by 30 s incubation at 4°C.

The supernatant fraction was transferred to a new tube, and the beads were washed one time with one volume of lysis buffer. The supernatant and wash fractions were combined and adjusted to a final concentration of the cell lysate of 50 OD_{600} cell equivalents/ml. The cell lysate was subjected to a low-speed centrifugation of 200 × *g* (1200 rpm) in a Beckman JS-13 rotor for 4 min at 4°C.

The resulting supernatant fraction was further fractionated by centrifugation in a Beckman TL100 ultracentrifuge at 96 600 × *g* (50 000 rpm) in a TLA100 rotor for 25 min at 4°C. Mock extraction of the low-speed supernatant fraction was performed by addition of one fifth of the final volume of lysis buffer, incubation at 4°C for 20 min, and separation into pellet and supernatant fractions by centrifugation at 96 600 × *g* as described above. Extraction of the low-speed supernatant was performed by the addition of one fifth of the final volume of either 8 M urea, 3 M KOAc, or 2.5% Triton X-100. Samples were incubated for 20 min at 4°C and then separated into pellet and supernatant fractions by centrifugation at 96 600 × *g*. Sodium carbonate extraction was performed by the addition of the low-speed supernatant to 100 volumes of ice-cold 100 mM Na_2CO_3 (pH 11.5), incubation at 4°C for 30 min, and separation into pellet and supernatant fractions by centrifugation at 103 040 × *g* (50 000 rpm) in a Beckman TLA100.2 rotor.

Conditional Expression of SRP101

The plasmid pSO400 that contains *SRP101* under the control of the *GAL1* promoter was produced by a two-step subcloning procedure. A 2.6-kb *Bam*HI-*Hin*dIII fragment from pUCSRP101 (containing the entire coding sequence of *SRP101* but lacking all of the 5′ control region; see above for construction details) was subcloned into the *Bam*HI-*Hin*dIII site of pRS316 (Sikorski and Heiter, 1989), generating plasmid pSO213. In a second step, a 685-bp *Bam*HI fragment containing the *GAL1* promoter was inserted into the *Bam*HI site of pSO213, generating the plasmid pSO400. The *GAL1* shut-off, ^{35}S-methionine labeling, and denaturing immunoprecipitations were performed as described in Hann and Walter (1991).

Protease Protection Assay and Pulse-Chase Analysis

Protease protections were performed essentially as described by Deshaies and Schekman (1990). *S. cerevisiae* strain SOY46 and control cells were metabolically labeled for 7 min with ^{35}S-methionine after

Table 1. Yeast strains used in this study

Strain	Genotype	Reference
W303	*ade2-1/ade2-1 trp1-1/trp1-1 leu2-3, 112/leu2-3, 112 his3-11/his3-11 ura3-1/ura3-1 can1-100/can1-100* MAT**a**/MATα	Deshaies and Schekman (1990)
SOY34	*ade2-1/ade2-1 trp1-1/trp1-1 leu2-3, 112/leu2-3, 112 his3-11/his3-11 ura3-1/ura3-1 can1-100/can1-100 SRP101/srp101::ADE2 (srp101-Δ1)* MAT**a**/MATα	This study (see MATERIALS AND METHODS)
SOY35	*ade2-1 trp1-1 leu2-3, 112 his3-11 ura3-1 can1-100* MATα *SRP101* (Ade$^-$)	This study
SOY36	*ade2-1 trp1-1 leu2-3, 112 his3-11 ura3-1 can1-100* MAT**a** *srp101-Δ1* (Ade$^+$)	This study
SOY37	*ade2-1 trp1-1 leu2-3, 112 his3-11 ura3-1 can1-100* MAT**a** *srp101-Δ1* (Ade$^+$)	This study
SOY38	*ade2-1 trp1-1 leu2-3, 112 his3-11 ura3-1 can1-100* MATα *SRP101* (Ade$^-$)	This study
SOY46	*ade2-1 trp1-1 leu2-3, 112 his3-11 ura3-1 can1-100* MATα *srp101-Δ1* [pSO400]	This study
SOY48	*ade2-1 trp1-1 leu2-3, 112 his3-11 ura3-1 can1-100* MATα *srp101-Δ1* [pSO210]	This study
SOY53	*ade2-1 trp1-1 leu2-3, 112 his3-11 ura3-1 can1-100* MAT**a** *srp101-Δ1* [pSO220]	This study
SOY60	*ade2-1 trp1-1 leu2-3, 112 his3-11 ura3-1 can1-100 scr1-Δ2* MATα	This study
SOY61	*ade2-1 trp1-1 leu2-3, 112 his3-11 ura3-1 can1-100 srp101-Δ1* MATα	This study
SOY62	*ade2-1 trp1-1 leu2-3, 112 his3-11 ura3-1 can1-100 scr1-Δ2 srp101-Δ1* MATα	This study
SOY64	*ade2-1/ade2-1 trp1-1/trp1-1 leu2-3, 112/leu2-3, 112 his3-11/his3-11 ura3-1/ura3-1 can1-100/can1-100 srp101-Δ1/srp101-Δ1* MAT**a**/MATα [pSO400]	This study
SOY70	SOY46 [rho$^-$]	This study
RSY457	*sec65-1 ura3-52 ade2-1 trp1-Δ1 leu2-3, 112 his3* MATα	Stirling *et al.* (1992)
MYY147	*mas1-1 leu2-3,112 his3 ura3-52* MAT**a**	Witte *et al.* (1988)

repression of SRα synthesis for 20 h. Cell extracts were prepared as described above. Extracts were digested with proteinase K (0.5 mg/ml) for 20 min on ice and terminated by the addition of phenylmethylsulfonyl fluoride to a final concentration of 10 mM. In the mock digestion, proteinase K was preincubated with phenylmethylsulfonyl fluoride for 20 min at 4°C and then added to the extracts. Samples were precipitated by addition of an equal volume of 20% trichloroacetic acid. Pellets derived from 1 OD_{600} equivalents of cells were resuspended in 40 μl of a solution of 3% SDS, 100 mM Tris base, 3 mM DTT, incubated for 5 min at 100°C, frozen in liquid nitrogen, and stored at −80°C. Subsequent denaturing immunoprecipitations and pulse-chase analysis were performed as in Hann and Walter (1991).

Strain Constructions of SOY64 and SOY70

SOY64 was constructed by crossing SOY53 with SOY46 (see Table 1). Before the cross, SOY46 was repeatedly streaked over a 4-wk period onto plates containing glucose to allow the cells to undergo adaptation. Ura$^+$, Trp$^+$, Ade$^+$ diploids were selected on Ura$^-$, Ade$^-$, Trp$^-$ plates containing galactose as the carbon source, allowing SRα expression from pSO400. Approximately 95% of these cells were rho$^-$, whereas the remaining 5% were rho$^+$. One rho$^+$ colony was chosen for further strain construction. This colony was streaked onto rich plates containing 2% galactose to allow loss of pSO220. Single colonies from this plate were tested for growth in the absence of Trp. Several colonies were found that were Trp auxotrophs but still prototrophs for Ura, suggesting that these cells had lost pSO220 but still retained pSO400. These cells were designated strain SOY64. After sporulatuion, tetrads were dissected onto YEP plates containing 2% galactose and 2% sucrose as the carbon sources. One tetrad was chosen in which all four spores formed colonies with normal growth rates. Cells derived from each spore were Trp$^-$ and Ade$^-$, but Ura$^+$. These cells were used for the analysis shown in Figure 12A.

SOY70 was made by streaking out SOY46 onto a rich plate containing 2% glucose as well as 2 μg/ml of ethidium bromide. Individual colonies were tested for growth on YEP glycerol/ethanol plates; SOY70 was unable to grow on carbon sources requiring respiration.

RESULTS

Yeast SRP101 Gene Encodes an SRα Homologue

Although the G-domains of mammalian SRα and SRP54 are similar, sequence alignments reveal regions in which the G-domains of both proteins are more closely related to their corresponding prokaryotic homologues, FtsY and Ffh, than to one another. We therefore used the alignment of all four G-domains to identify short contiguous blocks of amino acid sequence that are either highly conserved across all four sequences or that are unique to the SRα/FtsY and SRP54/Ffh subfamilies (Bernstein *et al.*, 1989; Römisch *et al.*, 1989). These sequences allowed us to design oligonucleotide probes that could be used in PCR to identify genes encoding putative SRα homologues in yeast.

For this purpose, we synthesized two pools of degenerate oligonucleotides. One pool encoded a seven amino acid stretch (designated A in Figure 1) that is conserved between mammalian SRα and FtsY but distinct from eukaryotic SRP54 (both mammalian and yeast) and Ffh. A second pool contained the reverse complements of sequences encoding a seven amino acid stretch (designated B in Figure 1) that is conserved between SRα and FtsY, as well as SRP54 from mammals and yeast. We used PCR to amplify DNA products from genomic DNA of both *Schizosaccharomyces pombe* and *S. cerevisiae* using the two oligonucleotide pools as primers. Because the spacing between regions A and B is conserved between mammalian SRα and FtsY, we predicted that amplification of the desired yeast genes would yield products of 110 bp. Indeed, both PCRs yielded abundant products of the expected size as part of a complex mixture. The 110-bp PCR products were purified by gel electrophoresis and sequenced directly as described (Hann *et al.*, 1989) (see also MATERIALS AND METHODS and Figure 1). The DNA amplified from both yeast species predicted protein sequences in

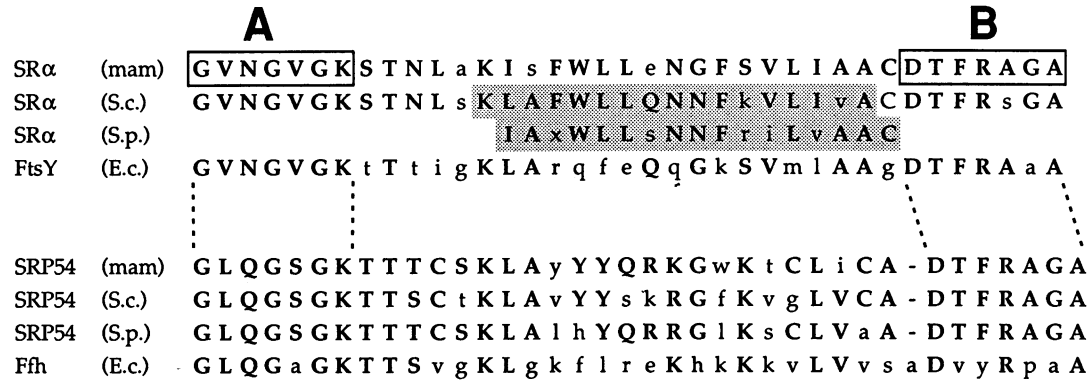

Figure 1. PCR strategy for SRα cloning. The sequences (single letter amino acid code) of the relevant portions of the SRα and SRP54 protein families are aligned. The amino acid sequences used to design the degenerate PCR oligonucleotides A and B are boxed. The amino acid sequences that could be unambiguously determined from the PCR products are shaded; the *S. cerevisiae* sequence includes amino acids determined from the genomic clone (outside the shaded area, see Figure 2). The x in the *S. pombe* sequence could not be determined. Capital letters indicate two or more identical amino acid residues in a given position (SRα and SRP54 related sequences are treated independently in this analysis). The nucleotide sequences that could be determined from the PCR fragments are: 5′ AAAGCTAGCG TTTTGGTTAC TGCAAAATAA TTTCAAGGTC TTAATTGTTG CTTG 3′ (*S. cerevisiae*) and 5′ AAATCGCGTN TTGGCTTTTA TCTAACAACT TTCGAATCTT AGTTGCTGCC TGCGACA 3′ (*S. pombe;* N could not be determined unambiguously). The sequences of mammalian SRα (mam) and mammalian SRP54 (mam) are identical in this region between different species (canine and human for SRα and canine and mouse for SRP54). The quest for a complete SRα gene from *S. pombe* was not further pursued as part of this study.

the expected orientation and reading frame (shaded in Figure 1) that were similar to the corresponding region of mammalian SRα and FtsY. Moreover, the amino acid sequences were distinct from known sequences of SRP54 in *S. cerevisiae* and *S. pombe* (Figure 1). This suggested that the amplified DNA segments were derived from genes encoding protein homologues of SRα in both yeast species.

We proceeded to isolate a genomic clone of the *S. cerevisiae* gene using the 110-bp PCR product as a hybridization probe to screen a plasmid library. Sequence analysis of the cloned *S. cerevisiae* gene revealed a 621 amino acid open reading frame (predicted molecular mass of 69.25 kDa) with 44% sequence identity (67% chemical similarity) to mammalian SRα in the G-domain and 19% identity (41% chemical similarity) in the amino terminal domain (Figure 2). We refer to the gene as *SRP101* and to its protein product as SRα.

Yeast SRα is a Peripheral Membrane Protein

To identify SRα in yeast cells, we raised rabbit antibodies to a fusion protein containing a portion of SRα linked to glutathione transferase (see MATERIALS AND METHODS). The antibodies specifically recognized SRα as a single protein of the predicted molecular mass in lysates from wild-type cells but not in lysates from cells in which the *SRP101* gene had been genetically disrupted (*srp101-Δ1* cells, see below) (Figure 3).

To characterize the intracellular localization of SRα, we fractionated yeast cell homogenates by differential centrifugation. Samples from each fraction were subjected to SDS-PAGE followed by Western blot analysis with anti-SRα and a control antibody to a known ER membrane protein, Sec61p (Stirling *et al.*, 1992) (Figure 4A). After low-speed centrifugation, SRα and Sec61p were each equally distributed between the pellet fraction (Figure 4A, lane 3) and the supernatant fraction (Figure 4A, lane 2). The presence of the two proteins in the low-speed pellet is presumably due to incomplete cell lysis. On further centrifugation at 100 000 × *g*, SRα and Sec61p were quantitatively recovered in the pellet fraction (Figure 4A, lane 5), suggesting that both proteins were associated with intracellular membranes.

The physical association of SRα with intracellular membranes was examined by extracting the low-speed supernatant fraction with various reagents known to perturb protein-membrane interactions. After centrifugation of the extracts at 100 000 × *g*, supernatant and pellet fractions were subjected to SDS-PAGE and immunoblotted with anti-SRα and anti-Sec61p (Figure 4B). SRα remained associated with the membrane fraction after treatment with 1.6 M urea (Figure 4B, lanes 3 and 4) or high salt (Figure 4B, lanes 5 and 6). In contrast, when membranes were disrupted with non-ionic detergent, most of SRα was released into the 100 000 × *g* supernatant fraction (compare lanes 7 and 8). The presence of a portion of Sec61p in the 100 000 × *g* detergent pellet may be due to its association with other proteins in the ER that remain insoluble after detergent extraction.

To test whether SRα is an integral membrane protein, we diluted the low-speed supernatant with a large excess of a solution of sodium carbonate at pH 11.5 and centrifuged as above. Under alkaline conditions peripheral, but not integral, membrane proteins are ex-

Mammal
S. cer.
Mammal
S. cer.
Mammal
S. cer.
E. coli
Mammal
S. cer.
E. coli
Mammal
S. cer.
E. coli
G
Mammal
S. cer.
E. coli
Mammal
S. cer.
E. coli
Mammal
S. cer.
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Mammal
S. cer.
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Mammal
S. cer.
E. coli
I
II
III

tracted from membranes (Fujiki *et al.*, 1982). SRα was quantitatively recovered in the supernatant fraction, whereas Sec61p remained in the pellet fraction (Figure 4B, lanes 9 and 10). These extraction properties of SRα are consistent with the view that yeast SRα, like its mammalian homologue, is a peripheral ER membrane protein. Mammalian SRα is thought to be anchored in the ER membrane through an interaction with SRβ, which contains a bona fide transmembrane domain (Miller, Tajima, Lauffer, and Walter, unpublished data). Yeast SRα does not contain any obvious hydrophobic amino acid stretches characteristic of transmembrane regions. Moreover, in yeast cells in which the yeast SRα is overexpressed, a large portion of the protein is found soluble in a high-speed supernatant, indicating that attachment sites to the membrane are limiting. From the data shown, we can conclude that SRα is membrane associated; however, due to the intrinsic limitations of the fractionation scheme, we presently cannot conclude with certainty that SRα is associated with the ER. Attempts to show an ER localization of SRα in yeast cells directly by immunofluorescence have been unsuccessful, presumably due to the low abundance of the protein.

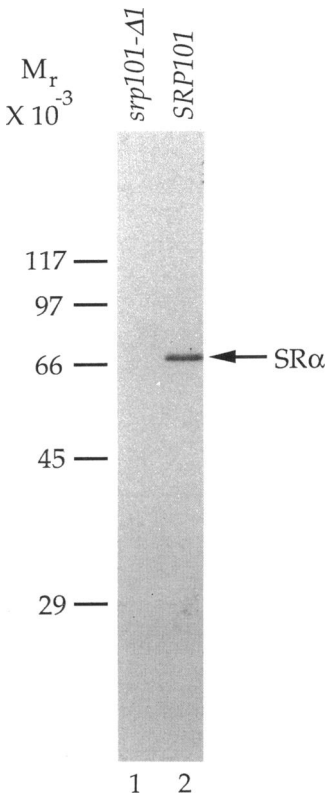


Figure 3. Characterization of anti-SRα. Cell lysates (0.2 OD_{600} cell equivalents per lane) were probed by Western blot analysis using affinity purified anti-SRα. Lane 1 contains lysate from cells deleted for the *SRP101* gene (SOY36), and lane 2 contains lysate from wild-type cells (W303). The positions of molecular weight standards and SRα are indicated.

Yeast Cells Require SRP101 for Efficient Growth

To analyze the physiological role of SRα, we constructed a strain in which the *SRP101* gene was deleted using a one-step gene replacement (Rothstein, 1983). The entire coding region of *SRP101* was replaced with a selectable marker, the *ADE2* gene (schematically shown in Figure 5A). In brief, we used a novel approach based on PCR to construct a linear DNA fragment containing the *ADE2* gene flanked by DNA sequences derived from the 5′ and 3′ flanking regions of the *SRP101* locus (Figure 5A, PCR step). This DNA was introduced into diploid *ade2*$^-$cells (strain W303) by transformation and selection of Ade$^+$ colonies (Figure 5A, gene replacement step). Integration of the *ADE2* gene at the *SRP101* locus was confirmed by PCR analysis (Figure 5B, compare lanes 1 and 2). For this analysis, oligonucleotides corresponding to regions Y and Z in Figure 5A, which lie outside the regions of homology used for integration (W and X in Figure 5A), were used in an amplification reaction of genomic DNA from wild-type cells (W303) or from an Ade$^+$ transformant (strain SOY34). As expected, amplification of DNA containing the wild-type *SRP101* locus (Figure 5B, lanes 1 and 2) yielded a predicted 2.4-kb PCR product. Integration of the *ADE2* gene into the *SRP101* locus (*srp101-Δ1*) was revealed by the presence of a larger 3.0-kb PCR product (Figure 5B, lane 2). The identity of these PCR products was confirmed by restriction analysis. Sporulation of diploid SOY34 (*SRP101/srp101-Δ1*) cells produced tetrads containing two to four viable spores. PCR analysis of the four colonies produced from spores of a single tetrad confirmed that the deletion allele *srp101-Δ1* cosegregated with the *ADE2* gene (Figure 5B).

All *SRP101* spores were Ade$^-$, formed normal colonies on plates, and grew in culture with a doubling time similar to that of the parent W303 strain (~80 min). In

Figure 2. Alignment of the amino acid sequences of SRα homologues. The deduced amino acid sequence of mammalian SRα (canine; Lauffer *et al.*, 1985), *S. cerevisiae* SRα (this work), and FtsY (*E. coli*; Gill *et al.*, 1986) are aligned. Two or more identical amino acids at a single position are shown in capital letters. Amino acids of similar chemical properties are boxed (Dayhoff *et al.*, 1972) as described by Bernstein *et al.* (1989) and Hann *et al.* (1989). Gaps are indicated with dashes. The positions of three regions matching GTP-binding consensus sequences are indicated above the alignment by roman numerals (Dever *et al.*, 1987). The amino acid sequence of the third conserved motif *S*KxD (box III) predicted to contact the guanine ring of GTP differs by a conservative substitution in *S. cerevisiae* SRα from the consensus *T*KxD defined for all other known members of the SRP54/SRα subfamily of GTPases. The beginning of the G-domain, as defined by Bernstein *et al.* (1989), is marked. The primary structures of SRα homologues from two additional species are known but for clarity omitted from this alignment. These proteins are the products of the *PilA* gene from *Neisseria gonorrhoeae* (Taha *et al.*, 1991) and the *SSO* gene from *Sulfolobus solfataricus* (Ramirez and Matheson, 1991). As for *E. coli* FtsY and eukaryotic SRα, the primary structures of these proteins are very similar in the G-domain but diverge in N-terminal regions.

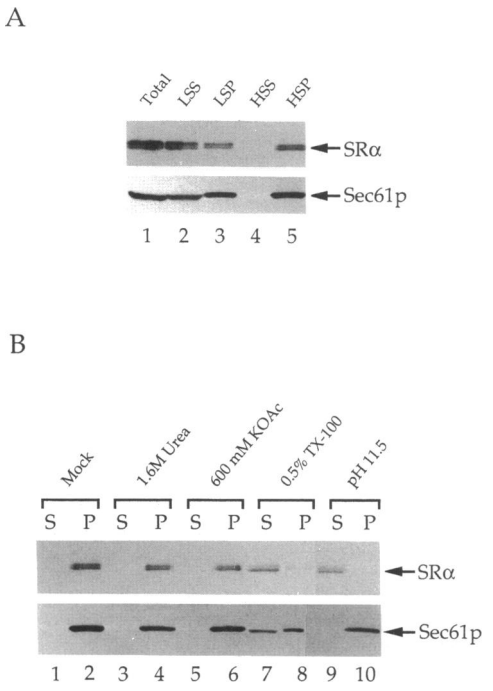


Figure 4. Subcellular localization of SRα. (A) Wild-type cell extracts were fractionated as detailed in MATERIALS AND METHODS. Cell fractions were analyzed by Western blot using affinity purified anti-SRα and anti-Sec61p. Each lane contains 0.2 OD_{600} cell equivalents of the fraction: lane 1, cell lysate; lane 2, low-speed supernatant; lane 3, low-speed pellet; lane 4, high-speed supernatant; lane 5, high-speed pellet. (B) A portion of the low-speed supernatant (as shown in lane 3 of A) was diluted with lysis buffer (lanes 1 and 2) or adjusted to final concentrations of 1.6 M urea (lanes 3 and 4), 0.6 M KOAc (lanes 5 and 6), 0.5% Triton X-100 (lanes 7 and 8), or 0.1 M Na_2CO_3, pH 11.5 (lanes 9 and 10). The samples were centrifuged at 100 000 × *g* and the supernatant fractions (lanes 1, 3, 5, 7, and 9) and pellet fractions (lanes 2, 4, 6, 8, and 10) were analyzed by Western blot using affinity purified anti-SRα and anti-Sec61p. Only the relevant portions of each immunoblot are shown. SRα was frequently observed as a doublet, presumably due to some proteolysis that occurred during cell fractionation. Note that no doublet was observed when cell extracts were prepared rapidly and under denaturing conditions (see Figure 3).

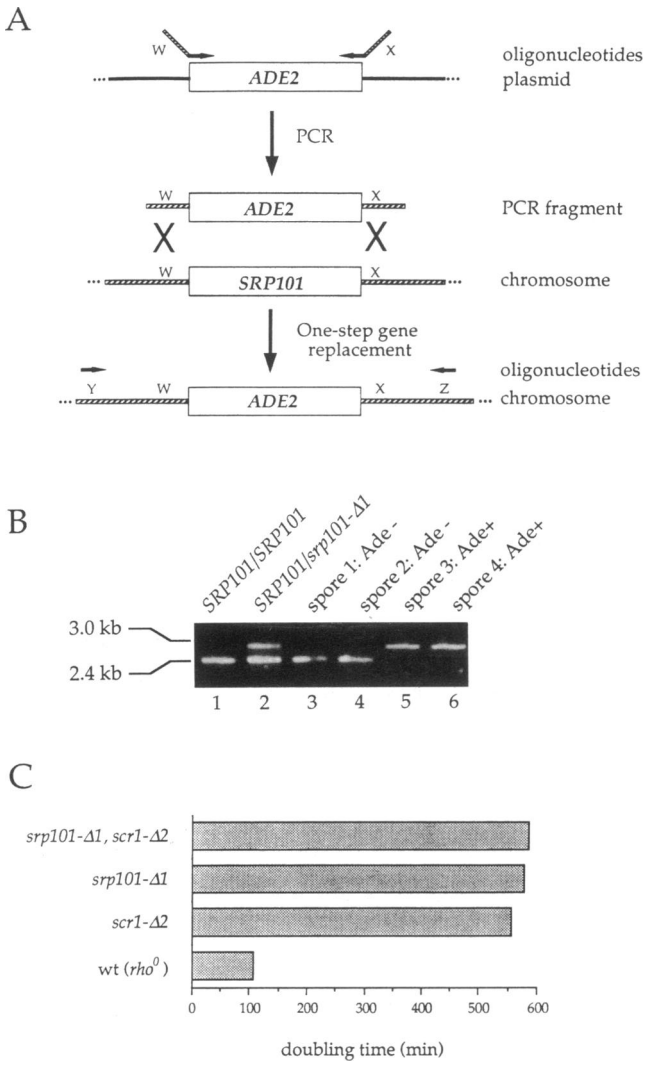


Figure 5. Gene disruption of *SRP101*. (A) The gene disruption procedure is depicted (see MATERIALS AND METHODS). Oligonucleotides composed of sequences from both the *ADE2* gene and sequences from regions W and X of the *SRP101* gene were used in a PCR reaction to generate a DNA fragment (PCR fragment) containing the *ADE2* gene of *S. cerevisiae* flanked at either end by sequences from the 5′ and 3′ untranslated regions of the *SRP101* locus. Upon transformation, the end sequences directed the integration of the linear DNA fragment into the *SRP101* gene (one-step gene replacement). PCR confirming the gene disruption was performed on yeast genomic DNA using the oligonucleotides corresponding to regions Y and Z. (B) PCR reactions confirming the gene disruption were performed on genomic DNA from the wild-type parent strain (lane 1), SOY34, the diploid transformant heterozygous for *SRP101*, and the *srp101::ADE2* gene disruption (*srp101-Δ1*; lane 2) or cells derived from the surviving spores of a single tetrad that are either Ade^+ (lanes 5 and 6) or Ade^- (lanes 3 and 4). The PCR products were separated on a 1% agarose gel and visualized by ethidium bromide staining. (C) Growth rates in YEPD medium of strains in which the chromosomal copies of *SRP101*, *SCR1* (see MATERIALS AND METHODS), or both *SCR1* and *SRP101* have been disrupted.

contrast, all *srp101-Δ1* spores were Ade^+, formed unusually small colonies on plates, were unable to grow on carbon sources requiring respiration, and grew about sixfold slower in liquid culture when compared with an isogenic *SRP101* rho^- strain (Figure 5C).

To show that the genomic clone of *SRP101* is functional in vivo, we transformed the SOY34 (*SRP101/srp101-Δ1*) cells with a centromeric yeast vector carrying both the *URA3* and *SRP101* genes (pSO210; see MATERIALS AND METHODS). Ura^+ transformants were sporulated and dissected. We recovered Ade^+ Ura^+ colonies with wild-type growth rates indicating that the *srp101-Δ1* allele was complemented by the plasmid carrying the cloned *SRP101* gene. As expected, every Ade^+ spore that grew at the wild-type rate was also Ura^+. Southern analysis revealed that *SRP101* is present in

single copy in the haploid yeast genome. Taken together, these results indicate that SRα is encoded by a single gene that is important but not essential for cell growth. Low stringency Southern blots, however, performed with a probe derived from the *SRP54* gene encoding a structurally related protein failed to detect any *SRP101* sequences. Likewise, no *SRP54* sequences were detected using *SRP101* sequences as a probe. Thus, it remains possible that other distantly related homologues of the SRα/SRP54 subfamily of GTPases exist in yeast.

If SRP and SRα function in sequential steps of a common protein targeting pathway, then the absence of both SRP and SRα should be no more detrimental to cell growth than the absence of either component alone. To test this directly, we constructed strain SOY60 in which the SRP RNA gene was deleted (*scr1-Δ2*) and strain SOY62 in which the genes encoding SRP RNA and SRα were both deleted (*srp101-Δ1/scr1-Δ2*). SOY60 and SOY62 were both constructed in the same strain background (W303) used elsewhere in this study. The results depicted in Figure 5C show that cells bearing the double gene deletion grow with a doubling time that is comparable with that of cells bearing either gene deletion alone.

Conditional Expression of SRα

To characterize the molecular nature of the defects in SRα deficient cells, we constructed a strain (SOY46) that allows the conditional expression of SRα. SOY46 is a haploid strain bearing the *srp101-Δ1* deletion allele that is complemented by a plasmid (pSO400) containing *SRP101* under control of the *GAL1* promoter. SOY46 cells grew as well as wild-type cells when galactose was used as a carbon source (Figure 6A, early time points). Under these conditions, the level of SRα was ~50-fold higher than found in wild-type cells, as estimated from an immunoblot analysis using anti-SRα antibodies (Figure 6B, compare lanes 1 and 7). The level of SRα decreased rapidly, when SOY46 cells were switched to growth medium containing glucose. After 16 h of growth on glucose containing medium, the amount of SRα was approximately equal to the amount found in wild-type cells (Figure 6B, compare lanes 4 and 7). By 19 h, SRα had fallen below wild-type levels and by 32 h was barely detectable by immunoblot analysis (Figure 6B, lanes 5 and 6; also see Figure 11B, lane 1). Concomitant with the drop of SRα levels, SOY46 cells began to grow at a slower rate (Figure 6A), rapidly approaching the growth rate of *srp101-Δ1* cells with a doubling time of ~10 h.

Yeast SRα is Required for Efficient Protein Translocation Across the ER Membrane

To test whether yeast SRα performs a function in facilitating protein translocation similar to that of its mammalian homologue, we depleted cells of SRα and

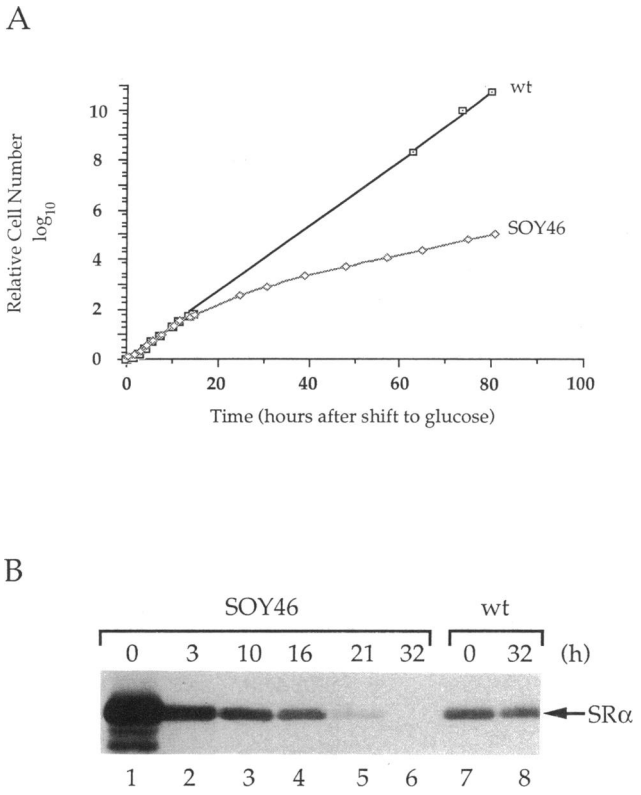
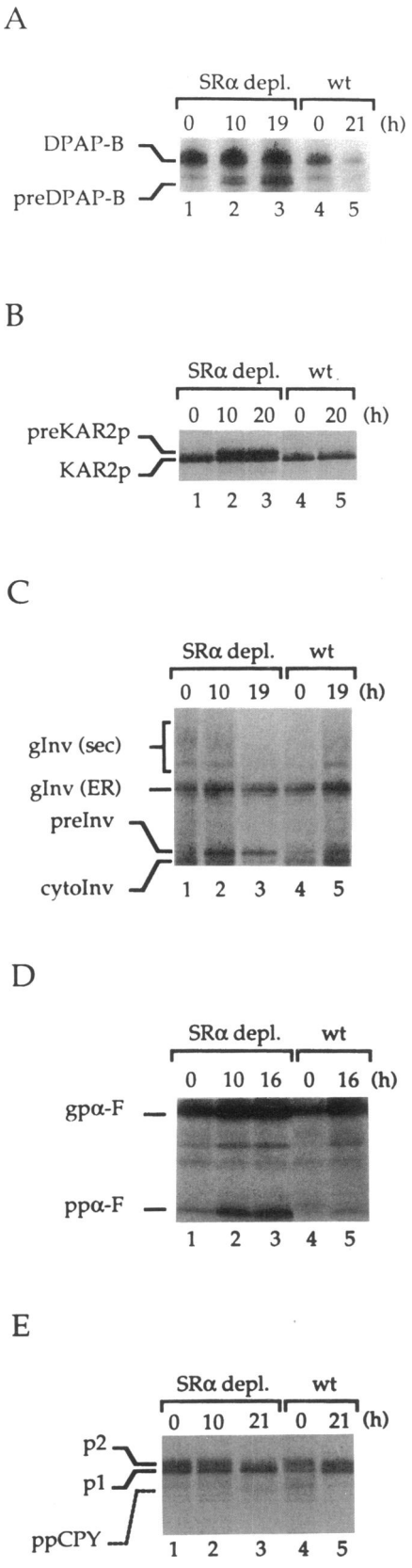


Figure 6. Growth curves and immunoblot analysis of SRα depletion. (A) The growth curves of SOY48 (*SRP101* expressed from its own promoter, labeled wt) and SOY46 (*SRP101* expressed from the *GAL1* promoter) are shown. At time 0, the carbon source was changed from galactose to glucose as detailed in Hann and Walter (1991). (B) Cellular SRα was monitored by Western blot analysis. Lanes 1–6 contain cell lysates prepared from SOY46 cells and lanes 7 and 8 contain lysate prepared from SOY48 cells at the indicated times after switch to glucose medium. The position of SRα is indicated.

monitored the maturation of several newly synthesized soluble and membrane proteins known to be translocated across the ER membrane. SOY46 cells were pulse labeled for 7 min with ^{35}S-methionine at various time points after the repression of SRα synthesis. Cell extracts were prepared from the labeled cells, and individual proteins were immunoprecipitated with specific antibodies and analyzed by SDS-PAGE. This allowed us to monitor the efficiency with which different proteins were translocated into the ER. For all substrates analyzed, translocation across the ER is accompanied by well-characterized modifications (signal sequence cleavage and/or glycosylation) that alter their gel mobility.

Figure 7 shows the results of such an analysis at time points during the course of SRα depletion. Under these conditions, four of the five proteins analyzed showed an accumulation of cytoplasmic precursor forms, indicating that protein translocation was perturbed as a consequence of the reduced SRα level. The degree to



which translocation was affected, however, varied among the different proteins. KAR2p, a soluble ER resident protein, and DPAP-B, a vacuolar integral membrane protein, showed the greatest defect. In the case of KAR2p, the translocation defect is inferred from the accumulation of a more slowly migrating band representing preKAR2p, which contains the uncleaved signal sequence (Figure 7B, lanes 2 and 3, preKAR2p); in the case of DPAP-B, the defect is inferred from the accumulation of a faster migrating band representing unglycosylated DPAP-B (Figure 7A, lanes 2 and 3, preDPAP-B). At the later time point, 20 or 21 h after repressing the synthesis of SRα, about one half of the KAR2p or DPAP-B synthesized remained untranslocated. The translocation of invertase and prepro-α-factor were affected to a lesser extent, but for both proteins significant amounts of precursor forms (Figure 7, C, lanes 2 and 3, preInv, and D, lanes 2 and 3, ppαF) accumulated after repression of SRα synthesis. In contrast to the four translocation substrates discussed above, CPY showed no detectable translocation defect on SRα depletion as indicated by the absence of a band migrating in the position of its cytoplasmic unglycosylated prepro-form (Figure 7E, ppCPY). No effect was observed on the translocation of any of the four substrates when an isogenic strain carrying *SRP101* controlled by its own promoter was switched from galactose to glucose (Figure 7, A, B, D, and E, wt lanes).

Note that translocation defects were already apparent at the first timepoint in the analysis shown in Figure 7, which was taken 10 h after switch of the SOY46 cells from galactose to glucose, yet at this time the intracellular SRα level as judged by Western analysis was still above that found in wild-type cells (Figure 6B). This apparent paradox can be reconciled if we assume that in SOY46 cells (which overproduce SRα from the *GAL1* promoter) only a portion of the SRα is properly assembled at the membrane and functional, whereas another portion remains unassembled and nonfunctional. As discussed above, fractionation of SOY46 cells grown on galactose indicated that a large portion of SRα remained

Figure 7. Precursor protein accumulation on depletion of SRα. SOY46 cells (*GAL1* promoter regulated *SRP101*) and SOY48 cells (wild-type *SRP101*) were grown as described in Figure 6A. Before (0 h) and at the indicated times after changing the carbon source from galactose to glucose cells were pulse labeled for 7 min with ^{35}S-methionine. Cell extracts were prepared and immunoprecipitations were performed using antibodies against the following proteins. (A) Dipeptidyl aminopeptidase-B; mature DPAP-B and the nonglycosylated precursor preDPAP-B are indicated. (B) KAR2p; mature KAR2p and precursor preKAR2p (containing the uncleaved signal sequence) are indicated. (C) Invertase; cytoplasmic cytoInv, precursor preInv (containing the uncleaved signal sequence and lacking carbohydrate attachment), core glycosylated ER form gInv (ER), and secreted outer chain glycosylated gInv (sec) forms are indicated. (D) α-Factor; precursor pp-αF and core-glycosylated gp-αF forms are indicated. (E) Carboxypeptidase Y; ER glycosylated p1 and Golgi modified p2 forms are indicated, as well as the predicted location of preproCPY ppCPY.

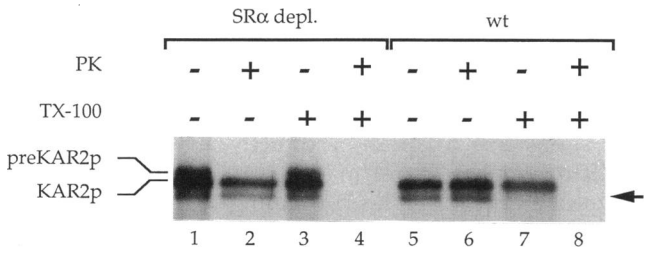


Figure 8. Accessibility of accumulated preKAR2p to protease. SOY46 cells (*GAL1* promoter regulated *SRP101*) and SOY48 cells (wild-type *SRP101*) were grown as described in Figure 6 and pulse labeled at 20 h after the shift from galactose to glucose. Extracts were prepared under nondenaturing conditions (see MATERIALS AND METHODS) from SRα depleted (lanes 1–4) and wild-type cells (lanes 5–8) and either digested with 0.5 mg/ml proteinase K (PK) or mock digested in the presence (lanes 3, 4, 7, and 8) or absence (lanes 1, 2, 5, and 6) of 0.5% Triton X-100. After inactivation of the protease, immunoprecipitations were performed with anti-KAR2p antibodies, followed by SDS-PAGE. The position of mature KAR2p and preKAR2p are indicated. A protected fragment of KAR2p (indicated with an arrow) migrates slightly faster than the mature form of KAR2p.

in a cytoplasmic supernatant. Accordingly, upon SRα depletion, only a portion of the SRα remaining in the cells as detected in Figure 6B may be functional. Alternatively, SRα expression from SOY46 cells could be nonuniform during the depletion experiment, giving rise to cell populations that are heterogeneous with respect to their levels of SRα. At the 10-h timepoint then, there could exist a population of cells that contain higher than wild-type levels of SRα and show no translocation defect, in addition to a population of cells that contain lower than wild-type levels of SRα and show a defect in protein translocation. The Western blot shown in Figure 6B would not distinguish these two populations.

The data presented suggest that the SRα facilitates protein translocation across the membrane of the ER. Because translocation was assayed indirectly in the experiments shown in Figure 7, we used a protease protection assay to ask whether the precursor proteins detected were retained in the cytoplasm or had, in fact, entered the ER. To this end, control cells or SOY46 cells (taken 20 h after SRα synthesis had been repressed), were pulse labeled, and crude cell extracts were prepared. The extracts were treated with proteinase K. PreKAR2p that was present in SOY46 cell extracts (Figure 8, lane 1, preKAR2p) but not in the control cell extracts (Figure 8, lane 5) was completely digested by proteinase K (Figure 8, lane 2). In contrast, mature KAR2p was resistant to proteolysis (Figure 8, lanes 2 and 6, KAR2p), presumably because it was sequestered within ER membrane vesicles. When membrane vesicles in the extract were disrupted with detergent, KAR2p became accessible to protease and was digested under these conditions (Figure 8, lanes 4 and 8). Thus, we conclude that preKAR2p accumulated in the cytoplasm in SRα depleted cells.

The accumulation of preKAR2p in the cytoplasm could be explained in two ways. First, cellular depletion of SRα could cause a kinetic delay in the targeting of preKAR2p to the ER membrane. The precursor observed after the pulse labeling would then represent an intermediate in the translocation process and would be expected eventually to reach the lumen of the ER. Alternatively, the depletion of SRα could impair the efficiency of targeting rather than the rate of the process, allowing only a portion of the nascent preproteins to become productively engaged with the translocation machinery. To distinguish between these possibilities, we performed a pulse-chase analysis. Both control and SOY46 cells were pulse labeled for 2 min with ^{35}S-methionine 20 h after repressing the synthesis of SRα, followed by addition of a large excess of nonradioactive methionine. The forms of KAR2p were examined by immunoprecipitation at timepoints during a 16-min chase period. The results are presented in Figure 9. From the quan-

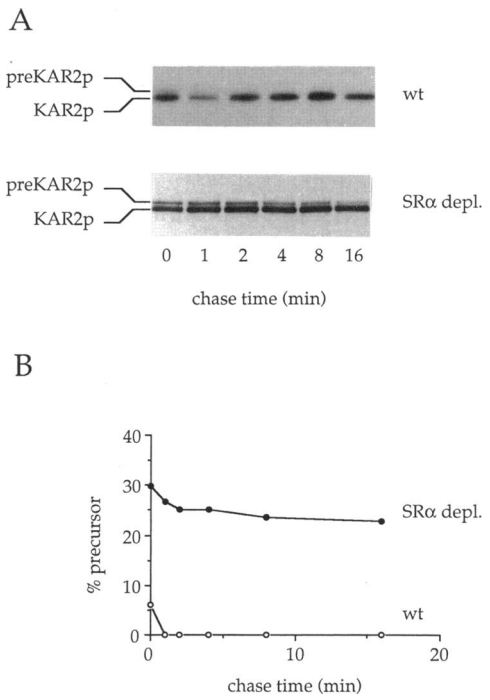


Figure 9. Pulse-chase of preKAR2p after SRα depletion. Twenty hours after switch from galactose to glucose medium, SOY48 cells (wild-type *SRP101*) and SOY46 cells (*GAL1* promoter regulated *SRP101*) were pulse labeled for 2 min with ^{35}S-methionine and chased for 0, 1, 2, 4, 8, or 16 min after the addition of excess unlabeled methionine. (A) Extracts prepared from each time point were subjected to immunoprecipitation with anti-KAR2p antibodies, followed by SDS-PAGE and autoradiography. The position of mature KAR2p and preKAR2p are indicated. (B) The data in A were quantitated using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). Note that the percent translocation, as calculated from the ratio of preKAR2 and KAR2, was less in this experiment than during the corresponding SRα depletion experiments shown in Figure 7. This is due to the longer pulse time used in Figure 7, which allowed more preKAR2p to accumulate.

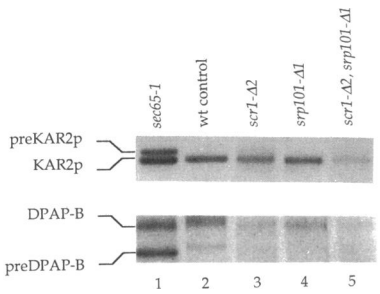


Figure 10. Precursor proteins do not accumulate in SRα (*srp101-Δ1*) or SRP RNA (*scr1-Δ2*) deletion strains. Strains (SOY60, SOY61, SOY62) containing the indicated chromosomal gene deletions and a wild-type strain were pulse labeled for 7 min with ^{35}S-methionine. Cell extracts were prepared and immunoprecipitations were performed using antibodies against KAR2p and DPAP-B. Precursor and mature forms of each protein are indicated. *Sec65-1* cells were similarly labeled and immunoprecipitated to provide markers for the gel mobility of preKAR2p and preDPAP-B.

titative analysis of the data (Figure 9B), it is apparent that most of the accumulated preKAR2p did not chase into the mature form during the chase period. These data suggest that the translocation defect observed after removal of SRα is not due to a kinetic delay but represents a reduction of the efficiency with which preproteins can be translocated. This analysis identifies two distinct pools of KAR2p: one portion that was translocated and processed to mature KAR2p during the time of the pulse and one portion, the accumulated preKAR2p, that was rendered translocation incompetent.

Cells can Adapt to the Absence of SRα and SRP

The defects in protein translocation demonstrated in Figures 7–9 were observed in cells in which SRα was progressively depleted after repression of its synthesis. To determine the effects of long-term SRα depletion, we performed similar experiments to monitor translocation defects in *srp101-Δ1* mutant cells. Surprisingly, we detected no translocation defects in *srp101-Δ1* cells when ^{35}S-methionine pulse-labeled extracts were immunoprecipitated with anti-KAR2 and anti-DPAP-B antibodies (Figure 10, lane 4). This result was unexpected because the corresponding experiment performed with yeast SRP, a component of the same pathway, showed only minor amelioration of translocation defects in deletion strains (Hann and Walter, 1991). Because a different strain (TR1) was used in the previous study, we repeated the experiment with cells containing a deletion of the SRP RNA gene, *SCR1*, in the W303 strain background used in this study (SOY60). In these cells and in cells containing the double deletion *scr1-Δ2* and *srp101-Δ1* (SOY62), translocation defects of both KAR2p and DPAP-B are barely detectable (Figure 10, lanes 3 and 5).

Thus, it appears that W303 cells are capable of adapting efficiently to the absence of SRα. Translocation defects were observed when SRα was depleted from cells rapidly after shutoff of its synthesis yet were not apparent when cells bearing the genetic disruption of SRα were grown for a prolonged time in the absence of SRα. To show this adaptation directly, we grew SOY46 (*SRP101* under control of the *GAL1* promoter) for an extended time on plates containing glucose, i.e., under conditions that repress SRα synthesis from the *GAL1* promoter. Analogous to the phenotype of *srp101-Δ1* cells, neither preDPAP-B nor preKAR2p were detected above the level found in wild-type control cells (Figure 11A). Thus, the translocation defects that are maximal between 10 and 20 h after shutoff of SRα synthesis (Figure 7, A and B, lanes 2 and 3) are no longer apparent after growth in the absence of SRα for a prolonged time. This reduction in the translocation defects was not due to increased SRα levels. Western blot analysis confirmed that the intracellular SRα level remained depressed even after prolonged growth on glucose plates (Figure 11B, lane 3).

Curiously, although cells grown for prolonged time in the absence of SRα are able to diminish their apparent protein translocation defects, they do not increase their growth rate as a result of this adaptation (compare Figure 6A and 5C).

To distinguish whether the adaptation was due to the accumulation of a simple suppressor mutation or was due to a reversible physiological change on SRα depletion, we performed a back cross experiment. Haploid SOY46 cells were grown on glucose plates to allow adaptation to occur. These cells were then mated with the strain SOY53 that, like SOY46, contains the chromosomal *srp101-Δ1* deletion but a different plasmid (pSO220) bearing *SRP101* under control of its own pro-

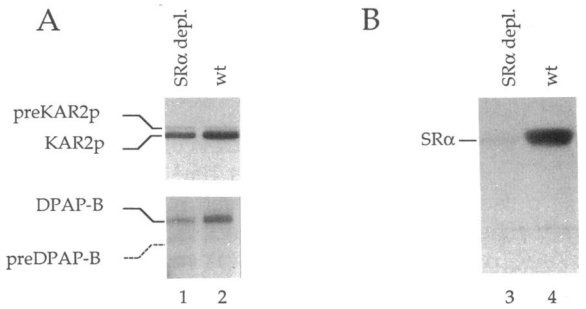


Figure 11. Precursor proteins do not accumulate after extended depletion of SRα. After prolonged growth in glucose medium, SOY48 cells (wild-type *SRP101*) and SOY46 cells (*GAL1* promoter regulated *SRP101*) were assayed for both levels of SRα protein and precursor protein accumulation. Before the assay, cells were maintained for ~10 wk on glucose Ura$^-$ plates. (A) Cells were pulse labeled with ^{35}S-methionine for 7 min. Cell extracts were prepared and immunoprecipitations performed using antibodies against KAR2p and DPAP-B. The positions of both precursor and mature forms of each protein are indicated. (B) Cellular SRα levels in SOY46 and SOY48 cells were monitored by Western blot as described in Figure 3. The position of SRα is indicated.

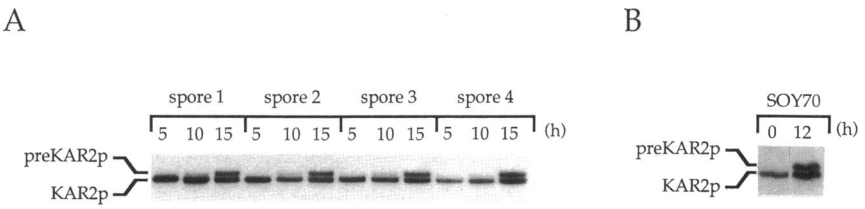


Figure 12. Genetic suppression or loss of respiratory function are not the cause of adaptation. Cells were pulse labeled with ^{35}S-methionine for 7 min. Cell extracts were prepared and immunoprecipitations performed using antibodies against KAR2p. The positions of both precursor and mature forms of KAR2p are indicated. (A) Cells from the four spores of a single tetrad (labeled spore 1 through spore 4) after sporulation of SOY64 were pulse labeled at the indicated times after repression of expression of SRα. (B) SOY70 cells were pulse labeled at the indicated times after repression of SRα synthesis.

moter. In contrast to the SOY46 cells used in the mating, SOY53 cells had never been depleted of SRα, were not adapted, and were still rho$^+$. Once mating had occurred, cells (SOY64; see MATERIALS AND METHODS) were isolated from the resulting diploid strain (homozygous for the *srp101-Δ1* deletion and containing the plasmids pSO220 and pSO400) that had lost pSO220 and thus only contained *SRP101* under control of the *GAL1* promoter. After sporulation and tetrad dissection, cells were maintained on galactose-containing media allowing expression of SRα. If adaptation results from a suppressor mutation, then we would predict that the adapted phenotype would segregate two-to-two when four spores from a single tetrad are analyzed. If, on the other hand, adaptation is a reversible event, we would expect that none of the four spores from a single tetrad would show the adapted phenotype. We assessed the adaptation of cells derived from the four spores obtained from a single tetrad by repressing the expression of SRα after shift to glucose-containing media. As shown in Figure 12A, the amount of preKAR2p that accumulated in cells derived from each spore at different timepoints after repression of SRα synthesis was identical. Thus, we conclude that adaptation is not due to a single suppressor mutation but is caused by a reversible alteration of cellular physiology.

Accumulation of Mitochondrial Precursors

We previously observed that cells in which SRP was genetically disrupted rapidly lost the ability to grow on fermentable carbon sources (Felici *et al.*, 1989; Hann and Walter, 1991). Furthermore, we showed that mitochondrial precursor proteins accumulate in SRP-depleted cells (Hann and Walter, 1991). We thus asked whether similar phenotypes would be observed in SRα deficient cells. Similar to the results obtained for SRP mutant cells, we found that all strains containing deletions of SRP101 were rho$^-$, indicating that mitochondrial function in these cells was impaired. As shown in Figure 13, at late timepoints during the SRα depletion, we observed a defect in the import of the β-subunit of the F_1-ATPase ($F_1\beta$) into the mitochondrial matrix space. As with KAR2p and DPAP-B, no import defect was observed in *srp101-Δ1* cells.

To test whether the loss of respiratory function causes adaptation, we constructed strain SOY70, which is rho$^-$ (see MATERIALS AND METHODS) and allows regulated expression of SRα from the *GAL1* promoter. As shown in Figure 12B, preKAR2p accumulated after repression of SRα synthesis in SOY70 cells, indicative of the nonadapted phenotype. Thus, we conclude that the acquisition of the rho$^-$ phenotype, which occurs after growth of cells in the absence of SRα, is not the cause for the observed adaptation.

DISCUSSION

We have isolated a gene from *S. cerevisiae* that encodes a homologue of the mammalian SRα protein. Several lines of evidence suggest that the yeast SRα not only shares structural similarities with its mammalian counterpart but also serves an analogous function. First and most important, yeast cells in which SRα has been depleted show defects in the translocation of proteins across the ER membrane. Second, the growth defects of *srp101-Δ1* cells and the severity to which different proteins are compromised in their translocation across the ER in SRα deficient cells resemble that of cells in which SRP has been disrupted (Hann and Walter, 1991).

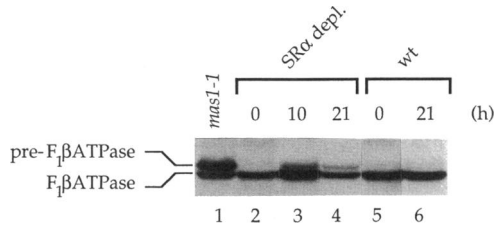


Figure 13. Mitochondrial precursor accumulation on SRα depletion. SOY46 (*GAL1* promoter regulated *SRP101*), SOY48 (wild-type *SRP101*), and MYY147 (*mas1-1*) cells were grown and labeled as described in Figure 7. Cell extracts were subjected to immunoprecipitation with antibodies to the β-subunit of the F_1 ATPase, followed by SDS-PAGE. Precursor (pre-$F_1\beta$ATPase) and mature ($F_1\beta$ATPase) forms are indicated.

Furthermore, cells lacking both SRP and SRα show a growth defect indistinguishable from cells in which either component alone was deleted. This suggests that yeast SRα and SRP function in the same pathway. Third, its subcellular localization is consistent with the notion that yeast SRα, like its mammalian homologue, is a peripheral ER membrane protein. Taken together, these findings suggest that the mechanism and the components mediating SRP/SRP receptor dependent protein targeting are evolutionarily conserved.

Structural Features of Yeast SRα

Mammalian SRα is attached to the ER membrane presumably through an interaction with SRβ that, based on its primary structure, is a bona fide integral membrane protein (Miller, Tajima, Lauffer, and Walter, unpublished data). Based on proteolytic dissection studies, this interaction is thought to be mediated by regions of SRα that are located proximal to its N-terminus (Lauffer *et al.*, 1985). Sequence comparison of yeast and mammalian SRα reveals a striking similarity that extends across the entire coding sequence. This extensive homology and the similarity in the extraction conditions that allow the release of yeast and mammalian SRα from the membrane suggest that yeast SRα may also be complexed to a putative yeast SRβ chain in the ER membrane. Thus, in cells overproducing SRα, only a portion of the protein may assemble into a functional heterodimeric SRP receptor. Oligomeric assembly in the ER membrane may be limited by the number of available SRβ chains. The sequence similarity between yeast and mammalian SRα is strongest in the G-domain and includes the consensus sequences that, as predicted from the known x-ray structures of H-ras and EF-Tu, form loops on the surface of the domain that contact the bound GTP (Bourne *et al.*, 1991). The strong phylogentic sequence conservations in the G-domain reaffirm the notion that the GTPase function of SRα is important for its biological activity.

Role of SRα in Elongation Arrest Release

SRα was originally purified from mammalian cells (Gilmore *et al.*, 1982b) based on its activity to release the SRP mediated translation arrest of presecretory proteins (Walter and Blobel, 1981). These in vitro studies led to the notion that SRα is required for releasing SRP-induced translational arrest. In the absence of SRα, protein synthesis would not proceed beyond a short "arrested fragment" of the presecretory protein. To date, such a tight arrest has only been observed in in vitro assays composed of heterologous components, e.g., mammalian SRP and a wheat germ translation system. In assays composed of exclusively mammalian components, the SRP-induced elongation arrest is transient, thus causing a kinetic delay in protein elongation (Wolin and Walter, 1989). We previously have speculated that the effects of SRP on presecretory protein synthesis may help to maintain proteins in a translocation competent state and/or may provide a potential regulatory point to couple translation to the secretory needs of the cell.

In the experiments described here, presecretory protein synthesis is not detectably reduced as compared with overall protein synthesis when SRα is depleted from cells. We therefore conclude that the lack of SRP receptor in vivo does not lead to a tight elongation arrest that is not released in the absence of SRα. In future studies, it will be interesting to measure directly the elongation rates of presecretory protein synthesis in SRα-depleted cells to test whether yeast SRP causes elongation pausing in vivo as observed in the mammalian in vitro system. Interestingly, in a different yeast, *Yarrowia lipolytica*, it recently was shown that mutations in SRP RNA can cause an inhibition of the translation of a secretory protein (He *et al.*, 1992; Yaver *et al.*, 1992).

Functional Role of Yeast SRα in ER Targeting

From previous studies of SRP, we concluded that in yeast, protein targeting to the ER can occur by redundant pathways (Hann and Walter, 1991). The data presented here for the SRP receptor confirm this notion. In the absence of SRP and SRP receptor, proteins must be targeted to and translocated across the ER membrane by alternative SRP and SRP receptor independent targeting pathways. Although the translocation of some proteins is severely impaired on disruption of the SRP/SRP receptor dependent targeting pathway, the mutant cells are viable. Thus, every protein that has to cross the ER membrane during its biogenesis and that is essential for cell viability can use an alternate targeting pathway sufficiently well to sustain cell growth.

Most of the proteins analyzed were impaired in their translocation across the ER membrane in SRP and/or SRP receptor depleted cells. We consider it likely that these proteins are cotranslationally targeted by SRP and SRP receptor to the ER membrane in wild-type cells and that they become rerouted into alternative targeting pathways only in the mutant cells lacking SRP and/or SRP receptor function. Some preproteins, e.g., preproCPY, that can be targeted as efficiently in the absence of SRP as in its presence may have evolved signal sequences that do not interact efficiently with SRP. Thus, preproCPY may not use the SRP dependent targeting pathway even in wild-type cells (Bird *et al.*, 1987; Hann and Walter, 1991).

The molecular details of alternative targeting pathways in these mutant cells are presently unclear. There are two conceptually distinct but not mutually exclusive possibilities. First, SRP/SRP receptor independent targeting could occur posttranslationally; precursor proteins are released from ribosomes and maintained soluble and translocation competent by interactions with cytosolic chaperonins and other putative targeting fac-

tors. The folding characteristics of a particular preprotein may thus determine how efficiently it can be maintained in a translocation competent form. This could explain why translocation defects observed in SRP and SRP receptor deficient cells vary in magnitude for different proteins. Consistent with this hypothesis, in vitro studies have shown that some fully synthesized precursor proteins can be translocated efficiently across yeast microsomal membranes. Prepro-α-factor, for example, can be efficiently translocated posttranslationally in vitro and shows only minor translocation defects in SRP and SRP receptor depleted cells in vivo.

According to a second hypothesis, SRP/SRP receptor independent targeting could occur cotranslationally; ribosomes synthesizing precursor proteins engage with the ER membrane independent of SRP and SRP receptor but before termination of protein synthesis. If cotranslational targeting is obligate for a given precursor protein, then the kinetics of its elongation would affect the efficiency of its membrane translocation. If elongation is slow, for example, then a longer time window would be available for the nascent precursor protein to engage with the ER membrane before it is completed and released from the ribosome.

The pulse-chase data in Figure 9 show that in SRα-depleted cells, the accumulated pool of cytosolic preKAR2p molecules is not translocation competent. Although we cannot rule out that some of these preKAR2p molecules are translocated very slowly, such slow kinetics could not account for the rapid appearance of translocated KAR2p at the earliest time point. Similar kinetic data were previously obtained in SRP-depleted cells (Hann and Walter, 1991). These data are consistent with either model outlined above. The observed translocation of preKAR2p may occur by a rapid posttranslational mechanism, and a factor mediating this reaction may be limiting. Therefore, only a portion of the precursor proteins would be able to interact with this factor and be translocated. The other portion would be rapidly rendered translocation incompetent. Alternatively, it is possible that preKAR2p requires cotranslational targeting and cannot be maintained translocation competent if released from the ribosome. In this case, the translocated portion of the KAR2p molecules would be synthesized exclusively by ribosomes that are engaged with the ER membrane during KAR2p synthesis.

Growth and translocation defects of SRα-depleted cells resemble those observed in SRP-depleted cells (Hann and Walter, 1991). After shutoff of SRα or SRP54 synthesis, the translocation of KAR2p and DPAP-B was severely impaired, the translocation of invertase and pro-α-factor showed intermediate defects, and the translocation of preproCPY was not affected. There are some quantitative differences in the relative magnitudes of the translocation defects. The translocation of DPAP-B, for example, was impaired to >90% in SRP-depleted cells (Hann and Walter, 1991), yet only impaired to ~50% in SRα-depleted cells (Figure 7). Most likely these differences arose because different strains were used in these studies.

Cells that have been grown in the absence of SRP or SRP receptor for a prolonged time "adapt" to the lack of these components. In the W303 strain used in this study, the adaptation results in almost undetectable translocation defects among the preproteins examined. In the TR1 strain used previously (Hann and Walter, 1991), a similar adaptation was observed that was less pronounced. SRα-depleted cells in which translocation defects are no longer detectable grow with a sixfold increased doubling time, indicating that the adaptation process cannot restore cell physiology to a wild-type state. The molecular nature of the adaptation process is presently unknown. From the presented data, we can rule out, however, that the adaptation is a direct consequence of a suppressor mutation or of the rho$^-$ phenotype that cells acquire after growth in the absence of SRα. According to the two models discussed above, translocation in adapted cells could be enhanced either because limiting components mediating alternate targeting pathways are induced and/or because the rate of protein elongation is reduced. The latter possibility would provide a plausible mechanism by which translocation can be improved but cell growth remains impaired. More efficient protein translocation would be obtained at the expense of efficient protein synthesis. Further studies of the molecular nature of the adaptation process are required to distinguish between these possibilities and may provide valuable information as to the mechanism of SRP/SRP receptor independent protein targeting. From the available data, it is still conceivable that the efficiency of protein translocation only appears to be improved in adapted cells. Cells may have improved a "housekeeping" pathway that allows mislocalized precursor proteins to be degraded more rapidly. This would change the precursor to product ratio that presently is the only measure for translocation efficiency.

Surprisingly, the import of the β-subunit of the F_1-ATPase ($F_1\beta$) into mitochondria was impaired in SRα-depleted cells. A similar phenotype was observed in SRP-depleted cells. As discussed previously (Hann and Walter, 1991), the impaired mitochondrial import could be an indirect consequence of cumulative defects in cells after disruption of the SRP/SRP receptor dependent targeting pathway. Alternatively, it remains possible that SRP and SRα are directly involved in mitochondrial protein import. Models entertaining this latter notion would need to explain, however, how SRα, which is thought to be restricted in its intracellular localization to the ER membrane, could also participate in mitochondrial import. At present, the available data do not allow us to distinguish conclusively between different possibilities. On SRα or SRP depletion, cells rapidly acquired a rho$^-$ phenotype (see RESULTS; Felici *et al.*,

1989; Hann and Walter, 1991). This loss of mitochondrial function could be a direct consequence of the impaired protein import.

ACKNOWLEDGMENTS

We thank Mark Rose, Randy Schekman, Tom Stevens, and Mike Yaffe for strains and antibodies. We thank Jodi Nunnari for suggesting the novel gene replacement scheme and Ray Deshaies, Byron Hann, Tim Stearns, and Debbie Zimmerman for comments on the manuscript. This work was supported by a predoctoral fellowship from the HHMI to S.C.O. and by grants from the Alfred P. Sloan Foundation and the NIH to P.W. The Genbank accession number for the *SRP101* sequence is M77274.

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