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The Signal Recognition Particle in S. cerevisiae

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Summary

We have identified the Saccharomyces cerevisiae homolog of the signal recognition particle (SRP) and characterized its function in vivo. S. cerevisiae SRP is a 16S particle that includes a homolog of the signal sequence–binding protein subunit of SRP (SRP54p) and a small cytoplasmic RNA (scR1). Surprisingly, the genes encoding scR1 and SRP54p are not essential for growth, though SRP-deficient cells grow poorly, suggesting that SRP function can be partially bypassed in vivo. Protein translocation across the ER membrane is impaired in SRP-deficient cells, indicating that yeast SRP, like its mammalian counterpart, functions in this process. Unexpectedly, the degree of the translocation defect varies for different proteins. The ability of some proteins to be efficiently targeted in SRP-deficient cells may explain why previous genetic and biochemical analyses in yeast and bacteria did not reveal components of the SRP-dependent protein targeting pathway.

Introduction

The mammalian signal recognition particle (SRP) is a cytoplasmic ribonucleoprotein particle (RNP) that functions during the targeting of membrane-bound or soluble proteins into or across the membrane of the endoplasmic reticulum (ER) (see Walter and Lingappa, 1986, for review). SRP was purified from canine pancreas on the basis of its translocation-promoting activity in a heterologous in vitro system. Using this assay, it was demonstrated that SRP acts to couple protein translation with translocation across the ER membrane. As purified, mammalian SRP consists of six polypeptides and one RNA molecule (7SL RNA, here referred to as SRP RNA) (Walter and Blobel, 1982). An essential feature of SRP's function is its ability to bind to hydrophobic signal sequences on nascent preproteins as they emerge from the ribosome. Concomitant with signal sequence binding, SRP arrests or delays elongation of the nascent polypeptide and, by interaction with the ER-localized SRP receptor, targets the nascent chain–ribosome complex to the ER membrane (Gilmore et al., 1982; Meyer et al., 1982). There, the ribosome becomes engaged with membrane proteins, collectively termed the translocon, that catalyze the translocation of the nascent protein across the membrane into the lumen of the ER. While the cumulative data from in vitro studies have yielded a detailed model for SRP's function, there is as yet little information concerning how SRP activity contributes to protein translocation in living cells.

Recently, several lines of evidence have pointed to the presence of SRP homologs in cells from all three kingdoms. SRP RNA homologs have been identified in several yeast, prokaryotic, and archaebacterial species based on their phylogenetically conserved secondary structures containing a characteristic stem–loop structure (Poritz et al., 1988; Struck et al., 1988). Putative SRP RNAs in the yeasts Schizosaccharomyces pombe and Yarrowia lipolytica closely resemble mammalian SRP RNA; however, in the more widely studied yeast S. cerevisiae, no structurally or functionally analogous RNA has been identified. A second, independent line of evidence for SRP homologs in evolutionarily distant species comes from the isolation of genes from S. pombe, S. cerevisiae, and Escherichia coli that encode proteins homologous to the 54 kd SRP signal sequence–binding subunit, SRP54p (Bernstein et al., 1989; Römisch et al., 1989; Hann et al., 1989; Amaya et al., 1990). Immunoprecipitation studies have confirmed that in S. pombe and E. coli the SRP RNA and SRP54p homologs are physically associated (Poritz et al., 1990; Ribes et al., 1990). Both the S. pombe and E. coli genes encoding SRP RNA and SRP54p homologs are essential for growth (Brown and Fournier, 1984; Ribes et al., 1988; Brennwald et al., 1988; G. Philips, T. Silhavy, and J.-A. Wise, personal communications); to date, however, their role in protein secretion has not been demonstrated. Recently, in S. cerevisiae, Amaya and Nakano (1991) showed that depletion of SRP54p led to a defect in the translocation of two secretory proteins.

In contrast to these intriguing data hinting at the presence of SRP-like particles in all living cells, genetic selections in bacteria and yeast aimed at identifying the cellular secretion machinery have failed to yield SRP. Genetic analyses have identified genes encoding membrane components required for efficient translocation of several proteins in E. coli (*secA*, *secD–F*, *secY*) (Bieker et al., 1990; Schatz and Beckwith, 1990; Gardel et al., 1990) and in S. cerevisiae (*SEC61*, *SEC62*, and *SEC63*) (Deshaies and Schekman, 1987; Rothblatt et al., 1989). The products of several of these essential genes have been shown to interact in the respective organisms (Deshaies et al., 1991; Brundage et al., 1990; Bieker and Silhavy, 1990; Hartl et al., 1990).

Similarly, biochemical experiments done with cell-free translation and translocation systems prepared from E. coli and S. cerevisiae cell extracts have failed to demonstrate SRP-like activities. In in vitro translocation assays from these organisms, most proteins remained translocation competent after the completion of synthesis and release from the ribosome (Müller and Blobel, 1984; Rhoads et al., 1984; Hansen et al., 1986; Rothblatt and Meyer, 1986; Waters and Blobel, 1986). This indicated that translation and translocation could be uncoupled, which, together with in vivo data (Koshland and Botstein, 1982), led to the notion that in lower eukaryotic and bacterial cells targeting may occur by a fundamentally different, posttranslational mechanism. Indeed, other factors were

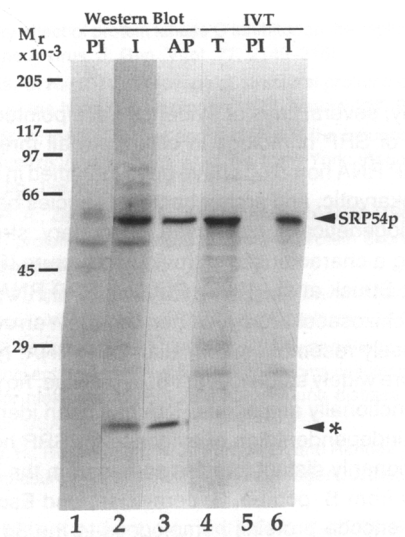

Figure 1. Characterization of α-SRP54p

Cell extracts were probed by Western blot analysis (see Experimental Procedures) using α-SRP54p as follows. Lane 1, preimmune serum ("PI," 1:250 dilution); lane 2, immune serum ("I," 1:500 dilution); lane 3, affinity-purified IgG ("AP," 1 mg/ml). Lanes 4–6 show the in vitro translation product ("IVT") of the *SRP54* gene translated in a yeast cell-free system. The total translation reaction analyzed by SDS–PAGE and visualized by autoradiography is shown in lane 4 ("T"). Lanes 5 and 6 show the products of immunoprecipitation reactions using either the preimmune (lane 5) or immune serum (lane 6). The asterisk indicates an SRP54p degradation product. Molecular weight markers are indicated.

identified that can associate with cytoplasmic preproteins and facilitate their posttranslational translocation: SecB, GroEL, and DnaK in E. coli (Collier et al., 1988; Phillips and Silhavy, 1990), and hsp70 in S. cerevisiae (Chirico et al., 1988; Deshaies et al., 1988).

In this study we have used antibodies to S. cerevisiae SRP54p as a starting point to characterize the function of yeast SRP. Our results suggest that yeast SRP, like its mammalian counterpart, functions in protein translocation across the ER membrane, but that an alternative protein targeting pathway(s) can substitute with surprising efficiency for most preproteins. In light of these results it is plausible that, because of the existence of redundant targeting pathways, previous genetic and biochemical analyses in yeast and bacteria did not reveal SRP or other components that function in the SRP-dependent protein targeting pathway.

Results

Antibodies to Yeast SRP54p Precipitate scR1 RNA from S. cerevisiae

We previously identified a gene in S. cerevisiae that encodes a protein with high homology to the 54 kd subunit of the mammalian SRP (Hann et al., 1989). To determine whether this protein was a component of a putative yeast SRP, we prepared antibodies against the gene product, SRP54p. A glutathione-S-transferase–SRP54p fusion protein was expressed in E. coli, purified by affinity chromatography, and injected into rabbits, yielding a polyclonal antiserum (α-SRP54p). By Western blot analysis, α-SRP54p (Figure 1, lane 2) but not the preimmune serum (lane 1) recognized proteins of the predicted molecular weight (60 kd) in crude cell lysate. Since multiple bands were recognized by α-SRP54p, the antibodies were further purified by affinity chromatography on a Sepharose column containing immobilized fusion protein. After affinity purification, α-SRP54p IgG recognized a single protein species of the predicted size (Figure 1, lane 3). The lower molecular weight band (marked by an asterisk) was frequently observed in cell extracts and probably represented a proteolytic breakdown product of SRP54p.

To further examine the specificity of α-SRP54p, we synthesized S. cerevisiae SRP54p in a yeast in vitro translation extract programmed with a synthetic mRNA derived from the cloned gene. Note that the in vitro translation product (Figure 1, lane 4) comigrated with the band recognized by Western blot analysis (lanes 2 and 3). Furthermore, the in vitro translation product was immunoprecipitated by α-SRP54p (Figure 1, lane 6) but not by the preimmune serum (lane 5). With additional support from the α-SRP54p Western blot in Figure 5B, which shows the absence of the 60 kd band in cell extracts prepared from SRP54p-deficient cells (*srp54-Δ1*), we conclude that α-SRP54p specifically recognized SRP54p.

To test whether SRP54p was assembled into an RNP complex, we performed native immunoprecipitations from S. cerevisiae cell extracts with α-SRP54p. Coimmunoprecipitated RNA species were extracted and fractionated on polyacrylamide gels. The RNA was visualized either by ethidium bromide staining (Figure 2A) or by labeling at the 3′ end with [^{32}P]pCp and RNA ligase and then detected by autoradiography (Figure 2B). Note that a single RNA species (labeled "scR1") was specifically immunoprecipitated with α-SRP54p (Figures 2A and 2B, lanes 3) but not with preimmune serum (Figures 2A and 2B, lanes 2). The band was competed with excess SRP54p fusion protein (Figure 2A, lane 4). The immunoprecipitated RNA species comigrated with scR1, a previously identified RNA of unknown function (Felici et al., 1989) that is also visible in the total cytoplasmic RNA fraction (Figures 2A and 2B, lanes 1).

Northern blot analysis confirmed that the RNA coimmunoprecipitated with SRP54p was indeed scR1. RNA extracted from the unfractionated cell extract (Figure 2C, lane 1) or from the pellet of an immunoprecipitation with α-SRP54p (Figure 2C, lane 3), but not with preimmune serum (Figure 2C, lane 2), contained a band of the expected size that hybridized at high stringency to an oligonucleotide probe complementary to scR1. As expected, the presence of excess SRP54p fusion protein inhibited the coimmunoprecipitation of scR1 (Figure 2C, lane 4).

These immunoprecipitation experiments suggested that S. cerevisiae SRP54p and scR1 are associated in a ribonucleoprotein complex. While scR1 is notably larger (519 nucleotides) than any of the other known eukaryotic SRP RNA homologs (250–300 nucleotides), it is, like SRP RNA in other species, the most abundant nonribosomal cytoplasmic RNA (Felici et al., 1989). To examine further the

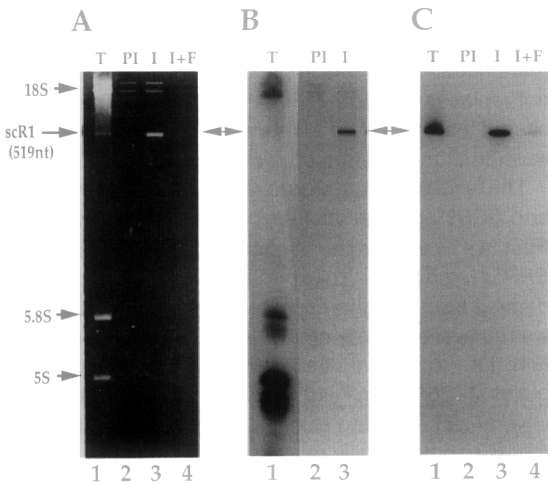

Figure 2. scR1 Coimmunoprecipitates with SRP54p

RNA was extracted from total S. cerevisiae cytoplasmic extract (lanes T) or from the pellets of native immunoprecipitation performed with either α-SRP54p preimmune serum (lanes PI), α-SRP54p immune serum (lanes I), or α-SRP54p immune serum plus excess SRP54p fusion protein (lanes I+F) (see Experimental Procedures). The RNA was examined by ethidium bromide staining (A), 3′ end labeling with [^{32}P]pCp (B), or Northern blot hybridization with an oligonucleotide complementary to scR1 (C). The predominant cellular RNA species are indicated.

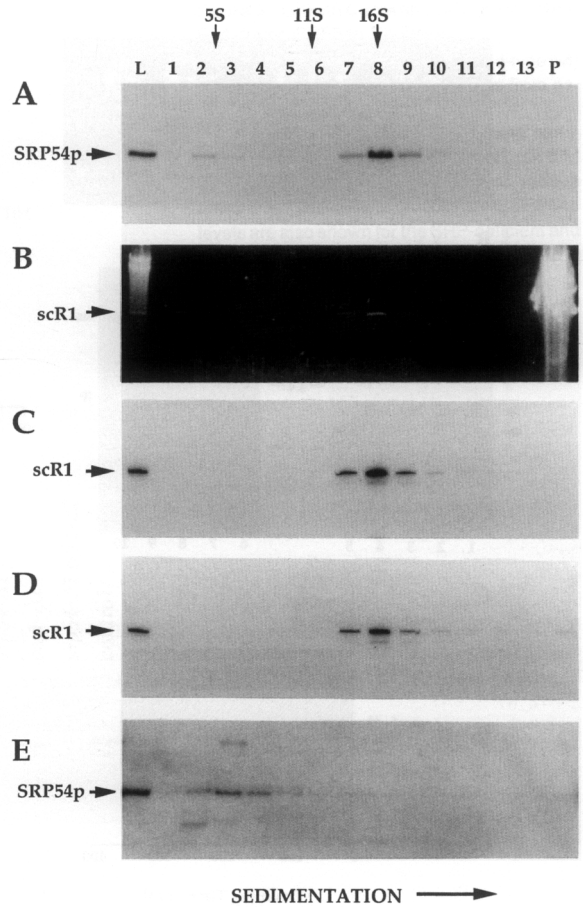

Figure 3. scR1 and SRP54p Cosediment in Sucrose Density Gradients

Crude S. cerevisiae cell extracts were layered over a 13 ml 5%–20% sucrose gradient and centrifuged for 15 hr at 40,000 rpm. Equivalent amounts of the load fraction (lanes L) and fractions from the gradient (lanes 1–13), including the pellet fraction (lanes P), were analyzed by Western blot with α-SRP54p (40 μl of each fraction) (A), ethidium bromide staining of extracted RNA (50 μl of each fraction) (B), Northern blot hybridization of total RNA with an scR1-specific probe (50 μl of each fraction) (C), and Northern blot analysis as in (C) but of RNA coimmunoprecipitated with α-SRP54p (300 μl of each fraction) (D). (E) shows a Western blot analysis with α-SRP54p of sucrose gradient fractions from a *scr1-Δ1* strain cell extract. Sedimentation values of protein standards are indicated.

association of SRP54p and scR1, we fractionated crude S. cerevisiae cell extracts by velocity sedimentation on sucrose density gradients. Fractions were collected from the gradients and analyzed in each of four ways. First, gradient fractions were analyzed by Western blot analysis with α-SRP54p (Figure 3A). Second, RNA was extracted from each gradient fraction and visualized with ethidium bromide after gel electrophoresis (Figure 3B). Third, the gel shown in Figure 3B was subjected to Northern blot analysis, probing with an scR1-specific oligonucleotide (Figure 3C). Finally, each gradient fraction was immunoprecipitated with α-SRP54p, and coimmunoprecipitated RNA was detected by Northern blot analysis with the scR1-specific oligonucleotide (Figure 3D). These data demonstrate that all of the SRP54p and scR1 present in the cell extracts were stably associated in a complex that sedimented in a well-defined peak centered at 16S.

SRP54 and *SCR1* in S. cerevisiae Are Not Essential for Cell Growth

Taken together, these results strongly suggested that scR1 is the SRP RNA homolog in S. cerevisiae. This notion raised an interesting paradox. Hughes and coworkers previously made an internal deletion in the chromosomal gene of scR1 that removed roughly half of the scR1 sequence. Surprisingly, cells containing the disrupted gene were viable (Felici et al., 1989). Assuming that the deletion destroyed the RNA's function, this result is in contrast to the SRP RNA homologs in other yeasts. In both S. pombe and Y. lipolytica, the SRP7 RNA genes were shown to be essential for growth (Brennwald et al., 1988; Ribes et al., 1988; He et al., 1989). Furthermore, we and others have previously reported that *SRP54* is an essential gene in S. cerevisiae (Hann et al., 1989; Amaya et al., 1990). If SRP54p and scR1 function as indispensable components of the same complex, then gene disruption of either component should show a similar growth phenotype. It remained possible, however, that scR1 is essential for growth but that large regions of the molecule can be deleted without loss of function, as is the case for the U2 RNA in S. cerevisiae (Shuster and Guthrie, 1988).

To test this possibility, we asked whether SRP54p was still associated with the truncated form of scR1, scR1-Δ1, expressed in cells bearing the chromosomal gene disruption. After immunoprecipitation with α-SRP54p, no scR1-Δ1 was detected in the pellet fraction (data not shown), suggesting that SRP54p was not stably associated with

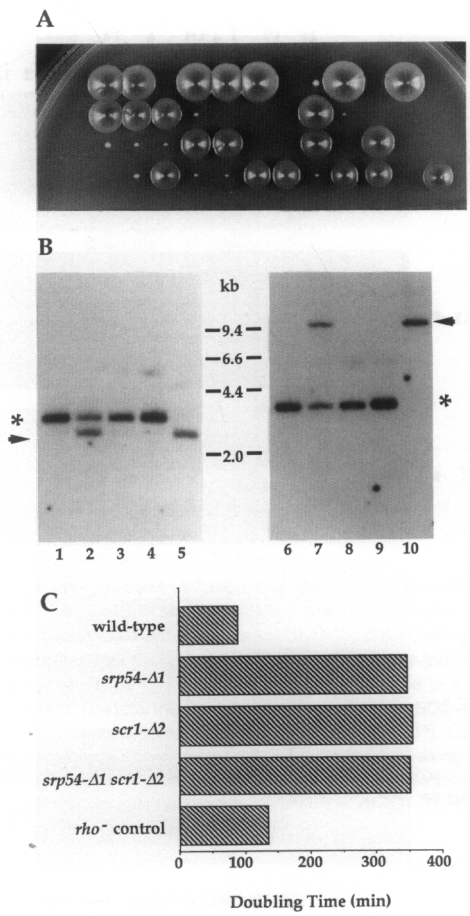

Figure 4. Neither *SCR1* nor *SRP54* Is Essential for Cell Growth

(A) Yeast colonies are shown that were derived from tetrad dissections of a diploid strain in which one of the chromosomal copies of *SRP54* has been disrupted.

(B) Southern blot analyses were performed on genomic DNA isolated from the wild-type parent strain (lanes 1 and 6), a diploid transformant containing the *scr1::HIS3* gene disruption (lanes 2 and 7), or cells derived from surviving spores dissected from a single tetrad: either His$^-$ (lanes 3, 4, 8, and 9) or His$^+$ (lanes 5 and 10). The DNA was digested with EcoRI (lanes 1–5) or ClaI (lanes 6–10). Fragments corresponding to the intact gene are marked by asterisks; fragments corresponding to the disrupted gene are marked by arrows. As predicted, the hybridizing EcoRI DNA fragment from the intact gene (3.5 kb) is 0.5 kb larger than that of the disrupted gene (3.0 kb).

(C) Growth rates in YEPD medium of strains in which the chromosomal *SRP54* gene, *SCR1* gene, or both were disrupted. For comparison the growth rates are shown of isogenic wild-type and *rho* control strains.

scR1-Δ1. This conclusion was supported by a second approach in which cell extracts from *scr1-Δ1* cells were fractionated by sedimentation on sucrose density gradients (Figure 3E). Western blot analysis of the gradient fractions showed that SRP54p in extracts prepared from *scr1-Δ1* cells sedimented near the top of the gradient, as expected for free SRP54p.

Two important conclusions can be drawn from these results. First, the results provide further evidence that scR1 and SRP54p are associated in a complex, since a mutation in scR1 changes the sedimentation properties of SRP54p. Second, in extracts of the *scr1-Δ1* strain, SRP54p sedimented in a position expected for monomeric SRP54p and no other RNA was coimmunoprecipitated (data not shown). This implied that scR1 was apparently not replaced by another RNA in this strain. Thus we concluded that in *scr1-Δ1* cells the putative SRP-like particle was structurally disrupted, yet the cells were viable.

The results prompted us to reinvestigate whether *SRP54* and *SCR1* are essential for cell growth in S. cerevisiae. First, we examined the phenotype of a gene disruption of *SRP54* (*srp54-Δ1*). While the initial appearance of colonies derived from daughter spores following tetrad dissection was consistent with a lethal phenotype for the gene disruption (i.e., 2:2, alive:dead), incubation of the dissection plates at 30°C for 7–10 days revealed the presence of several slow-growing colonies (Figure 4A). While we and others had previously reported that *SRP54* was essential for growth (Hann et al., 1989; Amaya et al., 1990), these results were most likely obtained because the dissection plates were not incubated at 30°C long enough for the slow-growing colonies to appear.

To examine the phenotype of cells in which *SCR1* had been entirely eliminated, we constructed a strain in which the structural gene encoding scR1 was completely deleted (*scr1-Δ2*) (see Experimental Procedures). Tetrad dissection resulted in a growth phenotype identical to that shown above for *srp54-Δ1* cells (data not shown). Marker selection and Southern blot analysis (Figure 4B) confirmed that these slow-growing colonies represented cells containing the gene disruption. To examine whether cells containing either gene disruption had accumulated suppressor mutations, the strains were backcrossed against the wild-type parent strain and, after sporulation, redissected. The resulting daughter cells showed the same pattern of growth as in the initial gene disruption dissection (data not shown), suggesting that the growth of these strains was not the result of a mutation in an unlinked gene. When *srp54-Δ1* or *scr1-Δ2* cells were grown in liquid culture, their growth rates were approximately 4 times slower than that of the parent strain (Figure 4C).

If SRP54p and scR1 functionally collaborate as part of a yeast SRP and if the SRP is completely disrupted in *srp54-Δ1* and *scr1-Δ2* cells, then we would expect that cells disrupted in both genes should have the same growth phenotype as cells disrupted in either gene alone. This notion was confirmed by our results. We constructed a strain containing the double gene disruption *srp54-Δ1 scr1-Δ2* (by a cross and a dissection). *srp54-Δ1 scr1-Δ2* cells were viable and, as shown in Figure 4C, grew at the same rate as *srp54-Δ1* or *scr1-Δ2* cells. All cells containing gene disruptions of *srp54-Δ1* and/or *scr1-Δ2* were *rho*$^-$, as previously reported for *scr1-Δ1* cells (Felici et al., 1989). We concluded that the cells were *rho*$^-$ because a diploid strain resulting from a cross of the cells with a known *rho*° strain (FCY207) were also respiratory deficient. The SRP-deficient strains apparently lost their ability to respire during or soon after germination, as we were not able to obtain respiratory-competent cells even if cells were transferred to selective (e.g., glycerol) plates shortly after germination. As a control for the *rho*$^-$ phenotype, we crossed *srp54-Δ1* (MAT**a**) cells with *scr1-Δ2* (MATα) cells.

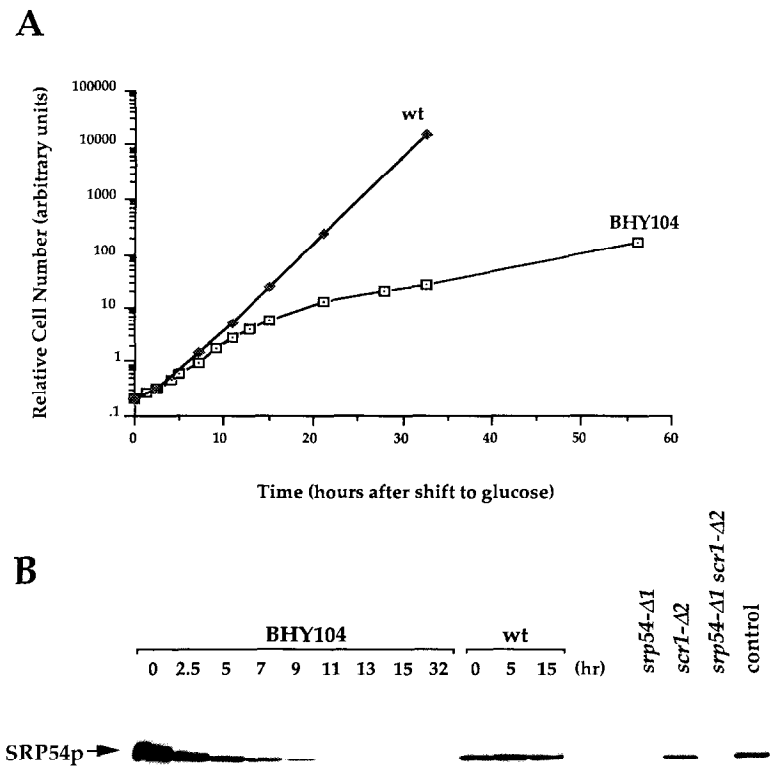

Figure 5. Growth Curve and SRP54p Western Blot during SRP54p Depletion

(A) Growth curves of BHY104 and wild-type (wt) yeast after changing carbon sources from galactose to glucose.

(B) The cellular levels of SRP54p were monitored by Western blot analysis in BHY104 and wild-type (right-hand lanes) yeast at various times after galactose to glucose shift. SRP54p levels are also shown for the SRP-deficient and *rho*⁻ control strains. The position of SRP54p is indicated.

These cells were *rho*⁻ and grew only slightly slower then the wild-type strain (Figure 4C).

SRP54p and scR1 Are Required for Efficient Protein Translocation across the ER Membrane

The data presented so far demonstrate that both SRP54p and scR1 are components of a putative yeast SRP that, while not essential, is important for cell growth. To ask questions regarding the cellular function of the putative yeast SRP, we engineered a plasmid (pGalSRP54) that would allow the conditional expression of *SRP54* in vivo from the *GAL1* promoter. This construct was transformed into a diploid cell in which one of the chromosomal *SRP54* copies was disrupted. After sporulation and tetrad dissection on galactose-containing agar plates, a haploid strain, BHY104, was obtained that contained both the gene disruption and pGalSRP54. The resulting cells were able to grow as well as wild-type cells when galactose was used as a carbon source (Figure 5A, early time points). In these cells SRP54p was about 20-fold overexpressed from the *GAL1* promoter compared with wild-type cells as judged by Western blot analysis with α-SRP54p (Figure 5B, compare 0 hr lanes). After BHY104 cells were switched to glucose-containing medium, the amount of SRP54p decreased rapidly. By 5–7 hr after the carbon source switch, the amount of SRP54p was approximately equal to levels in wild-type cells (Figure 5B), and by 15 hr SRP54p was undetectable. Shortly after this time BHY104 cells began to grow at a slower rate that was approximately equal to that of *srp54-Δ1* cells (Figure 4C).

If the putative yeast SRP functions like its mammalian counterpart in targeting proteins for translocation across the ER membrane, then one would expect this process to be disrupted in the absence of SRP54p. To test this notion directly, we monitored the fate of several newly synthesized soluble and membrane proteins known to be translocated across the ER membrane (Figure 6). For this purpose, BHY104 cells were pulse labeled with [^{35}S]methionine at various time points after switching off SRP54p synthesis. We chose time points between 0 and 15 hr after the switch to glucose medium (i.e., before the cells started to show a growth defect) (Figure 5A). Individual proteins were immunoprecipitated from cell extracts and analyzed by SDS–PAGE. This analysis allowed us to monitor translocation because each of the proteins examined undergoes some type of covalent modification following entry into the ER lumen: either signal sequence cleavage, core glycosylation, or both.

In four of the five proteins examined, a translocation defect was observed upon SRP54p depletion, as inferred from the accumulation of unmodified precursor protein. Surprisingly, however, the degree of the defect varied greatly between the translocation substrates that were examined. Dipeptidyl aminopeptidase B (DPAP-B), a type II integral membrane protein, was the most severely affected (Figure 6A). Pulse-labeled wild-type cells or

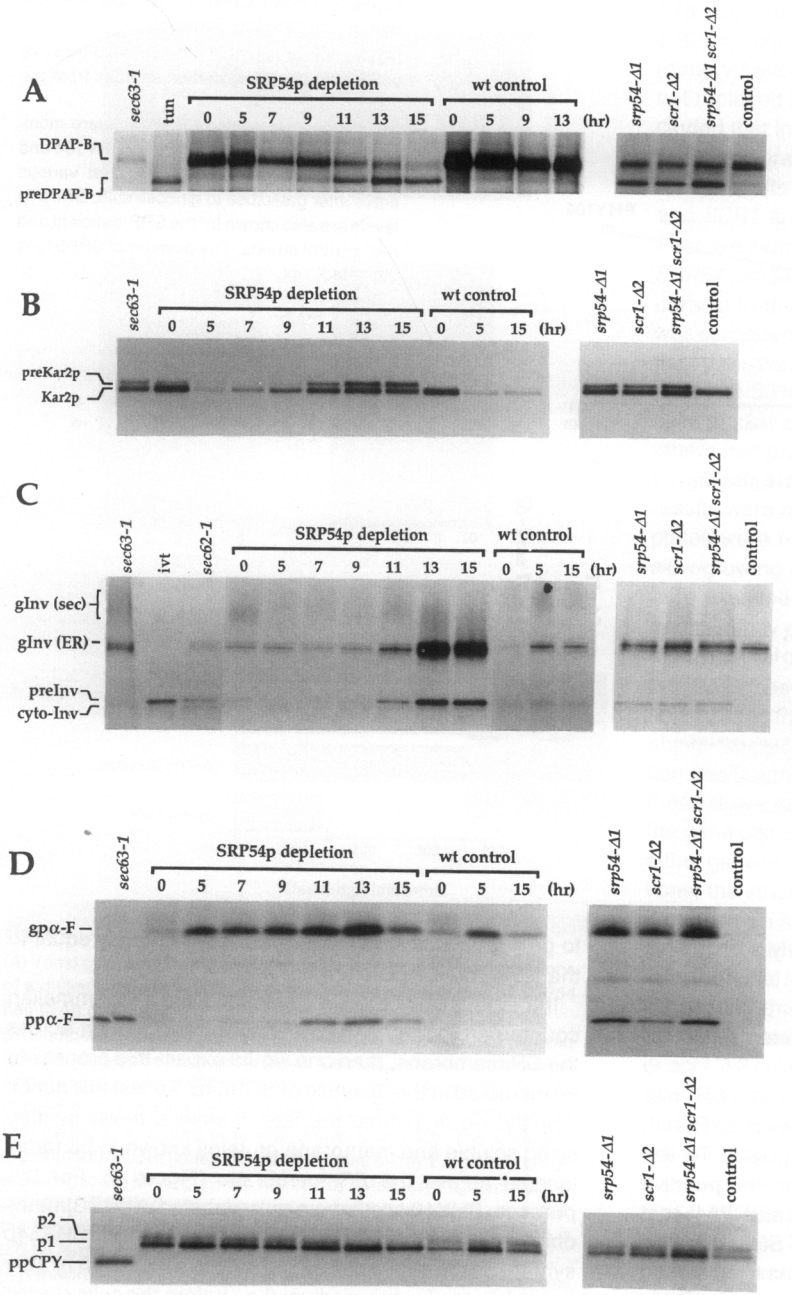

Figure 6. Accumulation of Precursor Proteins during Depletion of SRP54p

BHY104 (cells containing *SRP54* under control of the *GAL1* promoter) or wild-type cells were pulse labeled for 7 min before (time = 0 hr) and at various times after galactose to glucose medium shift (time = 5–15 hr). Cell extracts were prepared as described in Experimental Procedures, and immunoprecipitations were done from SDS-denatured cell extracts using antibodies against the proteins indicated below. Immunoprecipitations are also shown from *sec63-1* cells and, for invertase, *sec62-1* cells (each labeled for 7 min after a 1 hr incubation at the nonpermissive temperature) and from *srp54-Δ1* cells, *scr1-Δ2* cells, *srp54-Δ1 scr1-Δ2* cells, and the *rho*$^-$ control strain.

(A) Dipeptidyl aminopeptidase B; mature ("DPAP-B") and precursor ("preDPAP-B") forms are indicated. Tunicamycin-treated cells ("tun") serve as a marker for precursor.

(B) Kar2p; mature ("Kar2p") and precursor ("preKar2p") forms are indicated.

(C) Invertase; cytoplasmic ("cyto-Inv"), precursor ("preInv"), core-glycosylated ER form ("gInv (ER)"), and secreted, outer chain–glycosylated ("gInv (sec)") forms are indicated. For size comparison, the in vitro translation product of preinvertase ("ivt") is shown.

(D) Prepro-α-factor; precursor ("ppα-F") and glycosylated ("gpα-F") forms are indicated.

(E) Carboxypeptidase Y; precursor ("ppCPY"), ER-glycosylated glycosylated ("p1"), and Golgi-glycosylated ("p2") forms are indicated.

BHY104 cells before switch to glucose medium (Figure 6A, 0 hr lanes) contained the mature, glycosylated form of DPAP-B. Over time after the switch to glucose, increasing proportions of unglycosylated DPAP-B precursor accumulated (Figure 6A, preDPAP-B forms). This band comigrated with unglycosylated DPAP-B synthesized in wild-type cells incubated in tunicamycin (Figure 6A, lane tun). At the latest time point, 15 hr after shutoff of SRP54p synthesis, more then 90% of DPAP-B synthesized during the 7 min pulse was untranslocated.

In comparison, Kar2p showed a maximal translocation defect of only about 50% (Figure 6B). Kar2p is not glycosylated, and the translocation defect is therefore inferred from the accumulation of a slower-migrating band representing preKar2p, which contains the unprocessed signal sequence. Note that this band comigrated with preKar2p accumulated in *sec63* mutant cells (Figure 6B, lane *sec63-1*), which are known to be defective in ER translocation (Rothblatt et al., 1989). Invertase (Figure 6C) and prepro-α-factor (Figure 6D) were affected to a lesser extent. In each case, unmodified precursor forms of the proteins accumulated at the later time points after shutoff of SRP54p synthesis. These bands comigrated with the precursor forms that accumulated in *sec62* or *sec63* mutant cells and the in vitro translation product (Figure 6C, lane ivt) of preinvertase. In contrast to the four transloca-

tion substrates shown in Figure 6A–6D, carboxypeptidase Y (CPY) showed no detectable translocation defect, even at the late time points (Figure 6E). No untranslocated pre-proCPY (ppCPY) was detected even after overexposure of the autoradiograms.

For DPAP-B, Kar2p, invertase, and α-factor, with slight variations between individual experiments, translocation defects were first evident between 7 and 9 hr after switch to glucose medium and clearly manifested by 11 hr. The onset of the defect occurred at about the time that the amount of cellular SRP54p dropped below the level present in wild-type cells (see Figure 5B). No effects on the translocation efficiency of any of the tested proteins were detected when the isogenic wild-type control strain was switched to the different carbon source (Figures 6A–6E, "wt control" lanes).

The translocation phenotype of each protein was also examined in the strains containing the SRP gene disruptions—*srp54-Δ1* cells, *scr1-Δ2* cells, *srp54-Δ1 scr1-Δ2* cells—and the *rho*⁻ control strain described above (Figures 6A–6E, correspondingly labeled lanes on the righthand side). Note that for DPAP-B the translocation defect of strains containing either or both gene disruptions was less severe than the "terminal" phenotype seen in the later time points after the SRP54p shutoff in BHY104 cells. This suggests that after prolonged growth in the absence of SRP, cells are able to adapt in a way that lessens the translocation defects. This could, for example, be due to the induction of the heat shock response. Indeed, we observed by Western blot analysis that hsp70 levels were increased by 2- to 3-fold both in the SRP gene disruption strains and in BHY104 cells 18–20 hr after shutoff of SRP54p synthesis (not shown). As expected, translocation was not impaired in the control strain for any of the tested proteins (Figures 6A–6E, "control" lanes). Note that this control is invalid for prepro-α-factor (Figure 6D), since diploid cells do not produce this protein.

These data provide evidence that disruption of SRP leads to ER translocation defects in vivo. Since translocation was inferred from the covalent modifications of the translocated proteins, we wished to confirm directly that the detected precursor proteins were retained in the cytoplasm. We used protease protection in crude cell extracts to determine whether Kar2p had entered the lumen of the ER. Thirteen hours after SRP54p synthesis was stopped, BHY104 cells were pulse labeled and cell extracts were prepared and treated with proteinase K. Note that preKar2p (Figure 7, labeled "p"), but not Kar2p (labeled "m"), was completely digested by the protease (Figure 7, compare lanes 5 and 6). If the protease treatment was performed in the presence of detergent to disrupt the protecting lipid bilayer, Kar2p was also digested (Figure 7, lanes 4 and 8). A protease-resistant digestion product of Kar2p (labeled with an asterisk) migrated slightly faster than Kar2p. Thus, we have demonstrated that the accumulation of preKar2p after switching off SRP54p synthesis was due to impaired translocation across the ER membrane, and not simply a defect in signal peptide processing. The argument that protein translocation was directly impaired was further strengthened by the observation that CPY was efficiently translocated and efficiently glycosylated after depletion of SRP54p. This implied that the glycosylation defect observed for the other protein substrates could not have resulted from a defective glycosylation apparatus, but rather must have been due to a defect of the substrate in reaching the lumen of the ER.

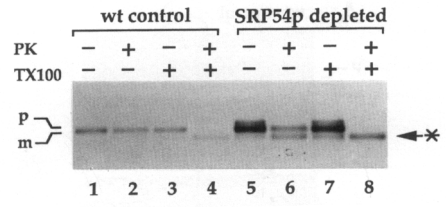

Figure 7. Protease Protection of PreKar2p following SRP54p Depletion

At a late time point (13 hr) after switching from galactose to glucose medium, wild-type yeast ("wt control") or BHY104 cells ("SRP54p depleted") were pulse labeled for 7 min. Extracts were prepared and either digested with 0.5 mg/ml proteinase K (lanes 2, 4, 6, 8) or mock treated (lanes 1, 3, 5, 7) in the presence (lanes 3, 4, 7, 8) or absence (lanes 1, 2, 4, 5) of 0.4% Triton X-100. After inactivation of the protease, the cell extracts were immunoprecipitated with anti-Kar2p. The mature ("m") and precursor ("p") forms are indicated. The asterisk indicates a protease-resistant digestion product.

All analyses presented so far were done by pulse labeling cells for 7 min with [^{35}S]methionine. The presence of untranslocated preKar2p could thus be explained in two ways. One possibility is that translocation was slowed down in cells lacking SRP54p. The observed preKar2p would therefore represent an accumulated kinetic intermediate that eventually would become translocated. Alternatively, translocation could be inefficient, allowing only a certain fraction of the newly synthesized protein to engage productively with the translocation apparatus. To address this question, we pulse labeled wild-type cells or BHY104 cells 13 hr after SRP54p shutoff. After a 2 min incubation with [^{35}S]methionine, an excess of nonradioactive methionine was added and the biosynthetic forms of Kar2p were examined at various time points over a 16 min chase period. The results, shown in Figure 8, indicate that during the chase period the majority of the preKar2p was not processed to the mature form. The data therefore suggest that the pulse-labeling analysis identified two distinct pools of Kar2p: a portion of newly synthesized protein that was rapidly translocated and converted to mature Kar2p, and another portion, the material accumulated as preKar2p, that was translocation incompetent or was translocated at a significantly reduced rate.

Two Unexpected Phenotypes of SRP54p-Depleted Cells

Surprisingly, in addition to the defect in protein translocation into the ER, cells depleted of SRP54p showed a significantly decreased rate of ER to Golgi transport. This effect is best seen in Figure 6E. Two glycosylated forms of CPY are resolved: p1 corresponds to the core-glycosylated ER form and p2 to a Golgi form that has acquired further carbohydrate residues. Note that as SRP54p was depleted from the cells, levels of the p2 form progressively diminished.

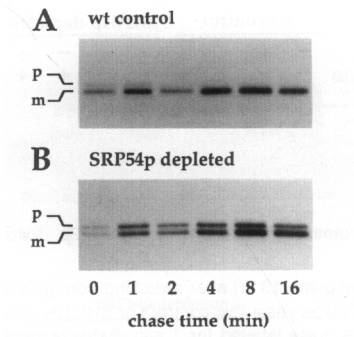

Figure 8. Pulse-Chase Analysis of PreKar2p following SRP54p Depletion

At a late time point (13 hr) after a switch from galactose to glucose medium, wild-type yeast (A) or BHY104 cells (B) were pulse labeled for 2 min with [^{35}S]methionine and then chased after addition of an excess of unlabeled methionine for 0, 1, 2, 4, 8, or 16 min. Cell extracts were prepared from each time point and were immunoprecipitated with anti-Kar2p antibodies. The mature ("m") and precursor ("p") forms are indicated.

These data are consistent with the observation that ER to Golgi transport has slowed down and therefore less p1 has reached the Golgi compartment within the time interval determined by the 7 min pulse labeling procedure. α-Factor (Figure 6D) and invertase (Figure 6C) show similar effects, consistent with this interpretation. Both show an increased amount of the corresponding ER form (labeled "gpα-F" and "gInv," respectively) accumulating at the later time points. Since the entry of DPAP-B into the ER was almost completely blocked at the later time points, its ER to Golgi transport rate could not be assessed from these data (Figure 6A).

A second surprising result was obtained from a control experiment in which we investigated whether the translocation defects in SRP54p-depleted cells were specific for the ER translocation system. We tested the import of a mitochondrial protein in SRP54p-depleted cells. As shown in Figure 9, we observed a defect in the import of the β subunit of F_1 ATPase into the mitochondrial matrix space. As for ER proteins, the import defect was inferred from the accumulation of the unprocessed precursor form (Figure 9, "preF$_1$β"). The kinetics of the defect during the depletion of SRP54p in BHY104 cells parallel those of the ER-targeted proteins. A lesser defect was seen in *srp54-Δ1*, *scr1-Δ2*, or *srp54-Δ1 scr1-Δ2* cells.

Discussion

We have identified and have begun to characterize the SRP homolog in S. cerevisiae. Multiple lines of evidence suggest structural and functional similarities of the yeast SRP with its mammalian homolog. First, using antibodies against SRP54p we showed that S. cerevisiae SRP is an RNP containing scR1 as its RNA component. Second, genetic evidence indicates that both of the SRP subunits are equally important, and the loss of both SRP54p and scR1 is no more detrimental than the loss of either component alone. These in vivo data support the notion that SRP54p and scR1 are associated with one another in a complex whose function requires each component. Third and most important, cells whose SRP has been disrupted show defects in the translocation of proteins across the ER membrane. This phenotype suggests a functional role of S. cerevisiae SRP in protein translocation. These data are in agreement with results from in vitro studies of mammalian SRP and, together with recent studies from Amaya and Nakano (1991), provide evidence regarding SRP's role in vivo.

Structural Features of S. cerevisiae SRP

In addition to SRP54p and scR1, the S. cerevisiae SRP undoubtedly contains other, yet unidentified, subunits. This is suggested by the 16S sedimentation coefficient and by analogy to other known eukaryotic SRPs. In separate studies carried out collaboratively with us, Selinger and coworkers have used antibodies raised against the S. pombe SRP54p to immunoprecipitate specific polypeptides from metabolically labeled S. pombe cell extracts (Brennwald, Altohoff, Stevens, Selinger, B. C. H., P. W., and Wise, unpublished data). The sizes of the coprecipitated proteins are remarkably similar to those of the well-characterized mammalian SRP proteins. While we expect to identify other protein subunits of S. cerevisiae SRP, the data presented here indicate that scR1 is the only RNA component.

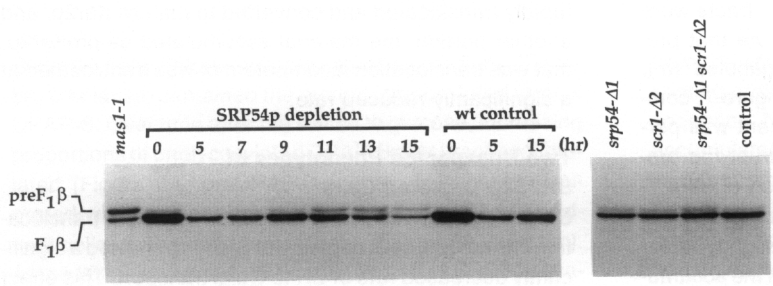

Figure 9. Accumulation of F$_1$β ATPase Precursor during the In Vivo Depletion of SRP54p

As in Figure 6, BHY104 or wild-type cells were pulse labeled for 7 min before (0 hr) and at various times after (5–15 hr) galactose to glucose shift. Immunoprecipitations were done on cell extracts using anti-F$_1$β ATPase antibodies. As a positive control, immunoprecipitations were also done from *mas1-1* cell extracts that were labeled after growth for 1 hr at the nonpermissive temperature (*mas1-1* cells are defective in mitochondrial import; Witte et al., 1988). The translocation phenotype is also shown for the single and double SRP gene disruption strains and in the *rho*$^-$ control strain. Mature ("F$_1$β") and precursor forms ("preF$_1$β") are indicated.

Based on its abundance and intracellular localization, the RNA subunit, scR1, was previously proposed to function as a possible SRP RNA homolog in S. cerevisiae (Felici et al., 1989). Subsequently, we have shown that scR1 contains a conserved sequence element near the 5′ end of the RNA that in the mammalian SRP RNA constitutes part of the binding site for the SRP 9/14 protein (Strub et al., 1991). The initially proposed secondary structure (Felici et al., 1989), however, did not resemble the phylogenetically conserved SRP RNA consensus structure. Since the RNA is about twice as large as mammalian or S. pombe SRP RNA, it is not clear which regions in scR1 correspond to the identifiable domains in the other SRP RNAs. In particular, a well-conserved stem–loop consensus structure, the so-called domain IV motif, cannot be readily identified in scR1. In contrast to scR1, the domain IV motif has been identified in every putative SRP RNA homolog from all three kingdoms. Presently, we do not know how to resolve this evolutionary paradox. A definitive secondary structure awaits further experimentation.

In Vivo Effects of SRP Disruption

Surprisingly, we discovered that neither *SCR1* nor *SRP54* was essential for cell growth. The possibility remained, however, that the products of other, redundant genes replaced SRP54p and scR1 directly. Several observations argue against this possibility. First, the double gene disruption of *SCR1* and *SRP54* was no more detrimental than either disruption alone. If the crippled SRP that lacked either one of the two components still played an essential role in cell growth, then disruption of both may have led to a synthetic enhancement of the defect. Second, attempts to identify redundant gene products were unsuccessful. In low stringency Southern blots no related genes were detected, and, similarly, no other RNA was coimmunoprecipitated with SRP54p from *scr1-Δ1* cell extracts. Finally, secretion defects and a reduced growth rate, closely resembling those of the SRP-deficient cells, were observed in S. cerevisiae cells containing a gene disruption in the putative α subunit of the SRP receptor, SRα (Ogg, Poritz, and P. W., unpublished data). By analogy with its mammalian homolog, SRα would be predicted to function at a later step in the same pathway. From these results we conclude that the entire SRP-dependent targeting pathway is nonessential in S. cerevisiae.

Since yeast cells are able to grow in the absence of SRP, all essential membrane and extracytoplasmic soluble proteins must still be correctly localized at efficiencies great enough to allow for cell survival. From the SRP54p depletion experiments shown in Figure 6, it was apparent that a lack of functional SRP affected the integration or translocation of different proteins to dramatically different extents. On one end of the spectrum, the membrane integration of DPAP-B was strongly dependent on SRP and was almost completely inhibited in SRP54p-depleted cells. At the other extreme, the translocation of preproCPY was not affected by the depletion of SRP54p. The other proteins that were examined showed slight (preinvertase and prepro-α-factor) or intermediate (preKAR2) translocation defects in SRP54p-depleted cells.

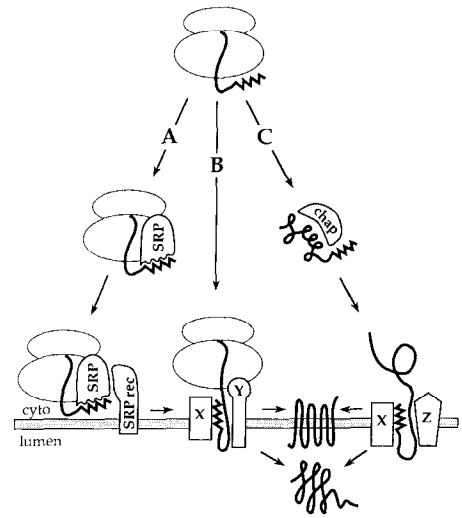

Figure 10. Model for Different Putative Targeting Pathways to the Yeast ER

Pathway A shows SRP-dependent cotranslational targeting. The signal sequence is recognized on the ribosome by SRP; after interaction with the SRP receptor, the ribosome becomes engaged with the translocon (components X and Y). The nascent protein becomes translocated into the ER lumen or integrated into the ER membrane. Pathway B shows SRP-independent cotranslational targeting. SRP and SRP receptor are bypassed and the ribosome attaches to the ER membrane via an interaction indistinguishable from that achieved in pathway A. Attempts to identify such a pathway across mammalian ER were negative (Garcia and Walter, 1988). Pathway C shows SRP-independent posttranslational targeting. For translocation to be posttranslational, protein synthesis has to be terminated and the precursor has to be released from the ribosome into a soluble pool. Translocation competence may be maintained by interactions with chaperonins. The preproteins interact with the translocon (components X and Z), which is not necessarily identical to that in used in pathways A and B to affect its translocation or integration.

Targeting to the ER via an Alternative, SRP-Independent Pathway(s)

Taken together, these observations imply that SRP-deficient cells utilize an alternative, SRP-independent targeting pathway(s) to the ER membrane (Figure 10). In particular, SRP-independent targeting may occur by a posttranslational mechanism for which there is evidence in S. cerevisiae from in vitro studies (Figure 10, pathway C). The question arises as to why some proteins are more dependent on SRP for translocation than others. If the SRP in S. cerevisiae performs a function analogous to that ascribed to mammalian SRP, then it would act as an adapter between the cytoplasmic translation and the ER membrane translocation apparatus. According to this analogy, S. cerevisiae SRP would assure the cotranslational recognition of signal sequences, catalyze the attachment of the nascent protein–ribosome complex to the ER membrane, and thereby allow protein translocation to occur concomitant with polypeptide elongation (Figure 10, pathway A). Thus, we would expect disruption of SRP in S. cerevisiae to affect most strongly those proteins whose membrane translocation must proceed coupled to their translation. The degree to which a given protein is dependent on SRP would be determined by the properties of that

particular preprotein, specifically by how effectively it can be maintained in the cytoplasm in a translocation-competent state. This could be a function of its folding characteristics, i.e., its tendency to aggregate or to fold too tightly for subsequent translocation, or of the affinity of the preprotein for chaperonins, such as hsp70, that help maintain its translocation competence. As we have shown for preKar2p in Figure 8, only a portion of the precursor molecules synthesized in SRP54p-depleted cells were translocated into the ER lumen. We interpret this as the consequence of a kinetic race to the ER membrane in which a portion of synthesized preKar2p engaged with the ER translocation machine in time to become translocated, and the other fraction was rendered translocation incompetent before productive engagement.

While this model is plausible, our results do not directly address the mechanism of SRP-independent protein targeting. In particular, the observation that SRP can be bypassed in vivo does not necessarily imply that residual translocation occurs posttranslationally. We cannot distinguish, for example, whether the translocation-competent fraction of preKar2p was fully synthesized and released from the ribosome prior to encountering the translocon in the ER membrane (Figure 10, pathway C), or whether translocation was observed only for the fraction of nascent preKar2p that engaged with the translocon prior to termination of protein synthesis (Figure 10, pathway B). In the latter example, the process may not require SRP but may still be dependent on a ribosome–membrane junction.

Similarly, the extent to which an alternative targeting pathway(s) is used in wild-type cells remains to be determined. Though prepro-α-factor was translocated rather efficiently in SRP-deficient cells, it is possible that in wild-type cells targeting is mediated exclusively by SRP. Indeed, if SRP interacts with signal sequences emerging from the ribosome, then it would have the "first pick" in recognizing and targeting nascent chains. In this regard, in vitro studies in heterologous systems indicate that the signal sequence of prepro-α-factor and that of preinvertase are efficiently recognized by mammalian SRP (Hansen et al., 1986; Garcia and Walter, 1988). It is possible, therefore, that in wild-type cells most proteins are not targeted via an alternative pathway(s).

A notable exception may be preproCPY, which contains a signal sequence that is not recognized by mammalian SRP in vitro (Bird et al., 1987). Consistent with this observation, preproCPY was the only protein tested that showed no translocation defect in SRP-deficient yeast cells in vivo (Figure 6E). The signal sequence of preproCPY contains a number of glycine residues, which are not normally found in signal sequences. Bird et al. (1987) analyzed signal sequence mutants in which these residues were systematically changed into hydrophobic amino acids and showed that the signal sequence could thus be changed into one that was readily recognized by mammalian SRP. Thus, CPY may have evolved so as to weaken the binding of its signal sequence to SRP and hence may be preferentially shunted into an SRP-independent pathway (Figure 10, pathway B or C). Bird et al. (1987) entertained very similar ideas in the insightful discussion in their paper.

Do the SRP-Dependent and SRP-Independent Pathways Converge?

An important question is whether the SRP-dependent and the SRP-independent pathways converge at a common translocon in the ER membrane (Figure 10, components labeled X, Y, and Z). Clearly, the molecular requirements for translocation in the two pathways could be different: the selected translocons may share components (Figure 10, component X) but need not be identical. The available data indicate that different translocation substrates may indeed have different requirements for the translocon. A genetic selection in S. cerevisiae has identified three genes to date, *SEC61*, *SEC62*, and *SEC63*, encoding interacting ER membrane proteins that are required for protein translocation. Interestingly, there was a striking inverse correlation between the severity of translocation defects in *sec63* mutant cells and any of the SRP disruption strains. For example, at their nonpermissive temperature *sec63* cells accumulated mostly unprocessed prepro-α-factor and preproCPY, but the integration of DPAP-B was unaffected (Figure 6E). A similar trend is observed in *sec61* and *sec62* cells (Deshaies and Schekman, 1987; Rothblatt et al., 1989; J. Rothblatt, personal communication). In contrast, in the SRP54p-depleted cells DPAP-B was most strongly affected and α-factor and CPY showed only minor or no precursor accumulation. While the complementary nature of these two classes of molecules is compelling, its significance is not known. Furthermore, the degree to which allele specificity determines the subset of proteins affected in *sec61*, *sec62*, and *sec63* cells remains to be tested. Nevertheless, the choice of translocation substrates used in the genetic identification of *SEC61*, *SEC62*, and *SEC63* may provide an explanation of why this genetic approach did not identify SRP or SRP receptor.

The data regarding preinvertase are somewhat enigmatic. In vitro studies showed that preinvertase could not be translocated posttranslationally (Hansen and Walter, 1988; Ngsee et al., 1989). Thus it was surprising to find that translocation of invertase was not severely affected by SRP54p depletion (Figure 6C). Furthermore, *sec63* mutant cells showed no impairment in preinvertase translocation when shifted to the nonpermissive temperature (Figure 6C), and only minor defects were noted in *sec61* and *sec62* mutant cells (Figure 6C; Rothblatt et al., 1989). Preinvertase therefore appears to be particularly permissive in vivo regarding its requirements on the cellular targeting and translocation machine.

Phenotypes Other Than ER Translocation Defects in SRP-Deficient Cells

We consider it likely that the decreased rate of ER to Golgi transport in SRP-deficient cells resulted from the gradual loss of a particular luminal or membrane-bound transport factor(s) during SRP depletion. According to this interpretation, it may be possible to restore the rate of ER to Golgi transport in SRP-deficient cells to wild-type levels by supplying increased levels of the limiting protein(s) by overproduction.

A more intriguing observation is the impaired import of the β subunit of the F_1 ATPase ($F_1\beta$) into mitochondria in

SRP-deficient cells. This effect could be an indirect consequence of cumulative defects in the cells after SRP depletion. In particular, mitochondrial import of $F_1\beta$ is known to be highly sensitive to the levels of cytoplasmic hsp70 in vivo and in vitro (Deshaies et al., 1988; K. Pfanner, personal communication). Since nontranslocated ER preproteins accumulate in SRP-deficient cells, it is conceivable that these proteins effectively deplete free pools of hsp70 and hence limit the amount available to facilitate mitochondrial import. Two observations, however, argue against this explanation. First, if hsp70 were limiting in cells, the heat shock response would be mounted (DiDomenico et al., 1982). By Western blot analysis hsp70 was induced after shutoff of SRP54p synthesis, but only after a considerable delay of 18–20 hr (data not shown). The accumulation of pre$F_1\beta$ thus preceded the first detectable increase in hsp70 levels by 10 hr. Second, while *sec61*, *sec62*, and *sec63* mutant cells also accumulate untranslocated precursor proteins in the cytoplasm, no pre$F_1\beta$ was detected when these cells were shifted to the nonpermissive temperature (Rothblatt et al., 1989).

Alternatively, it is possible that SRP is directly involved in mitochondrial protein import. This notion can be tested directly with available in vitro systems. Upon SRP depletion cells rapidly acquired a *rho*$^-$ phenotype (see Results; Felici et al., 1989). This loss of mitochondrial function could be a direct consequence of the impaired protein import.

Perspectives

The results presented here confirm in vivo the role of SRP in the early steps in protein secretion in eukaryotic cells. These experiments, however, also unveil unanticipated complexities such as the existence of redundant targeting pathways and the unexpected differential sensitivity of individual translocation substrates to disruption of the SRP-dependent pathway. Further complexities are introduced by the possibility that SRP may interact with sequences other than ER-directed signal sequences. The function of SRP may be more pleiotropic than previously appreciated, and perturbations of SRP may affect the maturation of proteins other than secretory or membrane proteins. Using yeast as the experimental organism will allow us to address these complexities in a system that is amenable to both in vivo and in vitro analyses. Through a comparison of results obtained in the yeast system with those obtained in the mammalian and prokaryotic systems, we hope to be able to discern which aspects of our models are unique to yeast, and which aspects are characteristic of a basic, evolutionarily conserved and ubiquitous mechanism by which nascent proteins are recognized and sorted by SRP.

Experimental Procedures

Strains, Antibodies, and General Methods

Yeast strains used in this study are listed in Table 1. Yeast media were as described in Sherman et al. (1986), except where noted. Yeast transformations were performed by the lithium acetate method (Ito et al., 1983) or by electroporation (Gene Pulser, Bio-Rad, Richmond, CA) using 0.2 cm cuvette cells and the following conditions: 600 V, 25 μF, 200 Ω. The following antisera were kindly provided by the indicated investigators: anti-DPAP-B (T. Stevens, Institute of Molecular Biology, University of Oregon), anti-Kar2p (M. Rose, Biology Department, Princeton University), anti-invertase, anti-prepro-α-factor, and anti-CPY (Randy Schekman, Division of Biochemistry and Molecular Biology, University of California at Berkeley), anti-$F_1\beta$ ATPase (M. P. Yaffe, Department of Biology, University of California, San Diego), and anti-hsp70 (Ab535) (E. A. Craig, Department of Physiological Chemistry, University of Wisconsin). Yeast genetic techniques were as described in Sherman et al. (1986). Recombinant DNA techniques were performed as described in Maniatis et al. (1982). SDS–PAGE was performed on 10%–15% SDS–polyacrylamide gradient gels, and all Western blots were done with ^{125}I-labeled goat anti-rabbit secondary antibodies except in Figure 5, where the Western blot was visualized by Enhanced Chemiluminesence (Amersham Corporation).

Preparation of α-SRP54p

Antiserum was raised against a fusion protein of glutathione-S-transferase, as the N-terminal domain, fused with the C-terminal domain of SRP54p from S. cerevisiae. The glutathione-S-transferase–SRP54p fusion protein was generated by subcloning a 1.7 kb SspI fragment from pSC54-01 (Hann et al., 1989) into the SmaI site of pGEX-3X (Smith and Johnson, 1988). The fusion construct encoded a 81 kd protein that contained SRP54p amino acid residues 45–541. The fusion protein expressed in E. coli was insoluble and therefore was solubilized in urea (Schloss et al., 1988) before purification by affinity chromatography on glutathione–agarose beads. Polyclonal rabbit antibodies to the fusion protein were prepared by BAbCO (Richmond, CA). For affinity purification, the antiserum was applied to an affinity column that was prepared by coupling the glutathione-S-transferase–SRP54p fusion protein to CNBr-activated Sepharose CL-4B (Pharmacia) at a concentration of 6 mg/ml according to the manufacturer's instructions. The resin was washed and the bound antibodies were eluted as described (Harlow and Lane, 1988). The

Table 1. Yeast Strains Used in This Study

Strain	Genotype	Source
PWY101	*ade6 pep4-3 MAT***a**	Hansen et al., 1986
GY142	*ura3 his3 trp1 scr1::URA3 [rho] MAT***a** *(scr1-Δ1)*	Felici et al., 1989
TR1	*trp1/trp1 lys2/lys2 his3/his3 ura3/ura3 ade2/ade2 MAT***a**/*MATα*	Parker et al., 1988
TR3	*trp1 lys2 his3 ura3 ade2 MATα*	T. Simmons
FCY207	*trp1 lys2 his3 ura3 ade2 [rho$^-$] MAT***a**	F. Chang
BHY116	*trp1 lys2 his3 ura3 ade2 srp54::LYS2 [rho$^-$] MATα (srp54-Δ1)*	Hann et al., 1989
BHY104	BHY116 + *[pGalSRP54]*	This study
BHY112	*trp1 lys2 his3 ura3 ade2 scr1::HIS3 [rho] MATα (srp54-Δ1)*	This study
BHY135	*trp1 lys2 his3 ura3 ade2 srp54::LYS2 scr1::HIS3 [rho$^-$] MATα*	This study
BHY143	*trp1/trp1 lys2/lys2 his3/his3 ura3/ura3 ade2/ade2 (srp54-Δ1 scr1-Δ2) srp54::LYS2/SRP54 scr1::HIS3/SCR1 [rho$^-$] MAT***a**/*MATα*	This study
RSY529	*sec62-1 leu2-3,-112 his4 ura3 MATα*	Rothblatt et al., 1989
JRY151	*sec63-1 leu2-3,-112 ura3 pep4-3 MATα*	Rothblatt et al., 1989
MYY147	*mas1-1 leu2 his3 ura3 MAT***a**	Witte et al., 1988

affinity-purified antibodies were concentrated by ammonium sulfate precipitation, resuspended in PBS containing 50% glycerol and 0.02% NaN_3 as described (Walter and Blobel, 1983) at a concentration of 1.5 mg/ml, and stored at −20°C.

Preparation of Yeast Cell Extracts

Cell extracts from S. cerevisiae were prepared as described (Hansen et al., 1986) with the following modifications. Spheroplasts were lysed in lysis buffer (20 mM HEPES–KOH [pH 7.5], 0.1 M potassium acetate, 6 mM magnesium acetate, 2 mM dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM EDTA, 1 mM EGTA, 100 U/ml Trasylol, 2 mM vanadyl ribonucleoside complex [VRC], 100 U/ml human placental ribonuclease inhibitor, and 2 mg/ml each of pepstatin A, chymostatin, antipain, and leupeptin) either by ten strokes in a tight-fitting Dounce homogenizer or by agitation with zirconium oxide beads (Biospec Products, Bartlesville, OK) as described (Bernstein et al., 1985). The suspension was centrifuged at 27,000 × g for 15 min in a Beckman JS13 rotor. The supernatant was transferred to a new tube, and the centrifugation step was repeated. The cell extracts, containing 20–40 mg/ml protein, were frozen in 1 ml aliquots at −80°C.

Sucrose Gradient Centrifugation

Aliquots of cell extract (200–400 μl) were adjusted to 0.5 M potassium acetate, layered onto 13 ml 5%–20% sucrose gradients, and centrifuged for 15 hr at 40,000 rpm in a Beckman SW40 rotor at 4°C. In addition to sucrose, the gradient solutions contained 20 mM HEPES–KOH (pH 7.5), 0.5 M potassium acetate, 6 mM magnesium acetate, 1 mM DTT, 0.01% Nikkol detergent, 1 mM EDTA, 1 mM EGTA, 100 U/ml Trasylol, 0.5 mM PMSF, 1 mM VRC, and 100 U/ml human placental ribonuclease inhibitor. After centrifugation, 1 ml fractions were manually collected from the top of the gradient. The pellet was resuspended in 1 ml of water. Aliquots from each fraction were prepared for immunoprecipitation, RNA extraction, or SDS–PAGE, as described below.

Native Immunoprecipitations

IgG from crude serum was bound to protein A–Sepharose CL-4B (Sigma) in immunoprecipitation buffer (IB) containing 0.5 M potassium acetate, 50 mM Tris–HCl (pH 7.5), 6 mM magnesium acetate, and 0.5% Nonidet P-40 detergent. Cell extract (100 μl) or gradient fractions (200 μl) were diluted at 4°C in 8 vol of IB^+ (IB plus 1 mM EDTA, 1 mM EGTA, 0.5 mM PMSF, 100 U/ml Trasylol, 2 mM VRC, and 1 mg/ml each of pepstatin A, chymostatin, antipain, and leupeptin) and preadsorbed with 50 μl of Sepharose CL-4B with mixing for 20 min. After removal of the Sepharose beads, the supernatants were mixed at 4°C for 30 min with 30–50 μl of antibody–protein A–Sepharose resin. The beads were pelleted by a brief centrifugation, washed once with IB^+ and three times with IB, and prepared for protein or RNA analysis.

Preparation of RNA

Yeast cell extracts or immunoprecipitation pellets were incubated for 60 min at 55°C with a solution containing 1% SDS, 10 mM EDTA, and 100 μg/ml proteinase K. The samples were extracted twice with phenol–$CHCl_3$ and, for samples to be pCp labeled, once with $CHCl_3$. The RNA was ethanol precipitated at −20°C for 2 hr. After centrifugation for 15 min, the pellet was washed with 80% ethanol at −20°C and resuspended in 5–10 μl of water. End labeling of the RNA with [^{32}P]pCp was performed as described (Siegel and Walter, 1988). RNA was analyzed on 50% urea–6% polyacrylamide gels. For Northern blot analysis, the RNA fragments were transferred to Gene Screen Plus (New England Nuclear, Boston, MA) and probed with a ^{32}P-labeled oligonucleotide (18-mer) complementary to nucleotides 490–507 in *SCR1* (Felici et al., 1989). Hybridization conditions were as described below.

Disruption of *SCR1*

The entire *SCR1* gene, except for the first 14 nucleotides, was deleted from the chromosome and replaced with the *HIS3* gene. Using the polymerase chain reaction (PCR), the genomic flanking regions on either side of *SCR1* were amplified from pSR6.21 (Felici et al., 1989) to generate a 248 nucleotide fragment (5′FR) corresponding to the 5′ flanking region (nucleotides −235 to +13; numbering according to Felici et al. [1989]) and a 454 nucleotide fragment (3′FR) corresponding to the 3′ flanking region (+541 to +995). The "inner" oligonucleotides, i.e., those immediately flanking the *SCR1* gene, were designed to create restriction sites for BamHI (5′FR) and EcoRI (3′FR). Each PCR product was cut with the appropriate restriction enzyme and, in a three-part ligation reaction, a 1.2 kb EcoRI–BamHI fragment containing the *HIS3* marker was ligated between the 5′FR and 3′FR. The product of the ligation reaction was used as template in a second round of PCR, which used as primers the two "outer" oligonucleotides used in the original PCR reactions. The major product from this reaction was a 1.9 kb fragment which, when digested with EcoRI or BamHI, gave rise to fragments of the predicted size. The 1.9 kb fragment was purified from an agarose gel and used as template in an additional round of PCR. This reaction yielded a single band at 1.9 kb, which was ethanol precipitated and used directly to transform TR1 cells by the lithium acetate method (Ito et al., 1983) in a one-step gene replacement (Orr-Weaver et al., 1981). After selecting for growth on His plates, surviving colonies were sporulated. Tetrad dissection was performed and the daughter cells were examined as described below.

To confirm that the *SCR1* gene was indeed deleted by site-specific recombination, Southern blot analysis was performed as follows. DNA was prepared (Davis et al., 1980) from the wild-type parent strain, the transformed, HIS^+ parent diploid strain, and the surviving daughter spores from a single tetrad (two his^- and one HIS^+), and digested with either EcoRI or ClaI. The resulting fragments were separated by electrophoresis in a 0.8% agarose gel and transferred to Gene Screen (New England Nuclear, Boston, MA). The blot was probed with a ^{32}P-labeled oligonucleotide corresponding to nucleotides 1124–1149 (roughly 600 nucleotides past the 3′ end of *SCR1* and outside of the region used for the gene disruption). Hybridization was performed as described (Church and Gilbert, 1984) at moderate stringency (42°C in 30% formamide, 7% SDS, 200 mM $NaPO_4$ [pH 7.5], 300 mM NaCl, 1 mM EDTA).

In Vitro Translation of SRP54p and Construction of pGalSRP54

By PCR mutagenesis, an XbaI site was introduced into pSC54-01 (Hann et al., 1989) 32 nucleotides upstream of the *SRP54* initiation ATG to create pSC54-X1. From this plasmid, a 2.3 kb XbaI–SalI fragment was subcloned into the same sites of pGEM2 (Promega Biotec, Madison, WI) to create pSC54-SP6, which was used for in vitro transcription with SP6 RNA polymerase as described (Hansen et al., 1986). In vitro translation of SRP54 was performed in a yeast cell-free translation system (Hansen et al., 1986). From pSC54-SP6 a 2.2 kb BamHI fragment was subcloned into the BamHI site of pTS161 (*URA3-CEN*) such that the gene was under control of the *GAL1* promoter to create pGalSRP54. Diploid TR1 cells in which one chromosomal *SRP54* gene had been disrupted (*SRP54::LYS2*) (Hann et al., 1989) were transformed with pGalSRP54 and, after sporulation and tetrad dissection, cells were isolated that were $MAT\alpha$ LYS^+ URA^+. This strain was named BHY104.

GAL1 Shutoff, Pulse Labeling, and Immunoprecipitations After SDS Denaturation

Wild-type TR3 or BHY104 cells were grown at 30°C to log phase in minimal medium containing appropriate supplements and 2% galactose. At 0 hr, dextrose was added to 2%. The cells were periodically diluted with prewarmed medium so that the OD_{600} remained between 0.05 and 1.0. At various time points, 3–5 OD_{600} units of cells was harvested and resuspended in the same medium at a density of 1 OD_{600} unit per ml. The cells were pulse labeled for 7 min with 50 μCi of [^{35}S]methionine per OD_{600} unit (Amersham, 1000 Ci/mmol). Cell growth was arrested by the addition of 1 vol of ice-cold 20 mM NaN_3. The cells were chilled on ice for 5 min and then pelleted by centrifugation for 5 min at 5000 × g. The cells were resuspended in 0.5 ml of TCA buffer (20 mM Tris–HCl [pH 8.0], 50 mM ammonium acetate, and 2 mM EDTA) and transferred to a chilled 2 ml tube containing 1 ml of zirconium oxide beads and 0.5 ml of 20% (w/v) TCA. With cooling on ice between cycles, the cells were lysed by two sequential 30 s agitations in a Mini-Bead Beater (Biospec Products, Bartlesville, OK). The supernatant was transferred to a clean tube, and the beads were washed with 500 μl of a 1:1 mixture of TCA buffer and 20% TCA. The pooled supernatant and wash solutions were centrifuged for 5 min in a microfuge, the supernatant was discarded, and the pellet was resuspended at 40 μl per OD_{600} unit in TCA resuspension buffer (3% SDS, 100 mM Tris–HCl [pH 11.0], 3 mM DTT) as described (Krieg et al., 1989). Immunoprecipitations were performed after addition of nonionic deter-

gent as described (Krieg et al., 1989). For the immunoprecipitation of invertase, cells were preincubated for 15 min in derepressing media (synthetic medium as described above but with 0.1% dextrose) before labeling. *srp54-Δ1*, *scr1-Δ2*, and *srp54-Δ1 scr1-Δ2* cells were grown to log phase at 30°C in minimal medium, labeled for 7 min, and prepared as described above. *sec62-1* and *sec63-1* cells were preincubated for 1 hr at 37°C before labeling. Tunicamycin-treated cells were preincubated for 15 min in medium containing 10 μg/ml tunicamycin before labeling.

Protease Protection and Pulse-Chase Analysis

Protease protection experiments were done essentially as described (Deshaies and Schekman, 1990). Metabolically labeled cell extracts were prepared as described by Bernstein et al. (1985). Proteinase K (0.5 mg/ml) digests were done for 20 min on ice and stopped with 10 mM PMSF. In mock digests, the proteinase K was preincubated with PMSF and added after the 20 min incubation. For pulse-chase analysis, cells (12 OD_{600} units) were pulse labeled as described above, except that after 2 min unlabeled L-methionine was added to a final concentration of 2.5 mM. At various time points, 2 OD_{600} units of cells was removed and added to an equal volume of ice-cold 20 mM NaN_3. The cells were harvested and processed for immunoprecipitation as described above.

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