An in Vitro Assay Using Overexpressed Yeast SRP Demonstrates that Cotranslational Translocation Is Dependent upon the J-Domain of Sec63p†

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ABSTRACT: The signal recognition particle (SRP) is required for co-translational targeting of polypeptides to the endoplasmic reticulum (ER). Once at the membrane, the precursor interacts with a complex proteinaceous machinery that mediates its translocation across the bilayer. Genetic studies in yeast have identified a number of genes whose products are involved in this complex process. These mutants offer a potentially valuable resource with which to analyze the biochemical role played by each component in the pathway. However, such analyses have been hampered by the failure to reconstitute an efficient in vitro assay for SRP-dependent translocation. We report the construction of two multicopy vectors that allow overexpression of all seven gene products required to make SRP in the yeast *Saccharomyces cerevisiae*. The overexpressed subunits assemble into intact and functional SRP particles, and we further demonstrate that in vitro reconstitution of co-translational translocation is greatly enhanced using cytosol from the overexpression strain. We use this assay to demonstrate that Sec63p is required for co-translational translocation in vitro and specifically identify the "J-domain" of Sec63p as crucial for this pathway.

The signal recognition particle (SRP)[1] is a conserved ribonucleoprotein complex that targets nascent polypeptides to the endoplasmic reticulum (ER) (*1*). SRP recognizes and binds the hydrophobic signal sequence of a secretory precursor as it emerges from the ribosome and also interacts directly with the ribosome itself (*2*, *3*) to induce a transient translational arrest (*4*−*6*). The arrested complex is then targeted to the ER via the interaction of SRP with a membrane-bound receptor complex known as SRP-receptor (SR; *7*, *8*). This interaction stimulates release of the nascent chain into the Sec61p-containing translocon channel through which it is then co-translationally translocated across the ER membrane (*1*, *9*, *10*).

Yeast SRP comprises six polypeptide subunits (Srp14p, Srp21p, Sec65p, Srp54p, Srp68p, and Srp72p) plus the Scr1 RNA (*5*, *11*−*14*). Genetic studies have shown that all seven gene products are essential for SRP function. Indeed, loss of Srp14p, Srp21p, Srp68p, or Srp72p destabilizes the complex and leads to degradation of Scr1 RNA (*14*). Targeting to the bilayer requires both subunits of the SR-complex, namely, SRα and SRβ (*8*, *15*, *16*), whereas the translocation reaction further requires the Sec61-complex (comprising Sec61p, Sbh1p, and Sss1p; *17*−*19*) plus the polytopic membrane protein Sec63p (*17*, *20*, *21*) and the lumenal chaperone Kar2p (*21*). Proteins may also be targeted to the yeast ER via a posttranslational pathway which is independent of both SRP and SR. The precursors targeted via this pathway tend to be those with less hydrophobic signal sequences (*22*). This posttranslational pathway employs the same core translocon components (Sec61-complex, Sec63p, and Kar2p), plus three further membrane components (Sec62p, Sec71p, Sec72p) and two additional lumenal factors (Lhs1p and Sil1p; *23*).

The roles of the core translocon components are of considerable interest. Evidence suggests that the Sec61-complex forms an aqueous transmembrane channel through which all classes of precursor can pass en route to the ER lumen. The Sec63 protein includes a lumenal J-domain which interacts directly with Kar2p to stimulate its intrinsic ATPase activity. These two proteins, in combination with Lhs1p and Sil1p, are thought to combine to drive posttranslational translocation effectively by pulling precursors into the lumen (*23*−*26*). This driving force might not be required during co-translational translocation since, in this case, the ribosome is tightly bound to the cytosolic face of the Sec61-complex enabling the energy of translation to be coupled to translocation (*27*, *28*). Indeed, a mutation in the J-domain of Sec63p (*sec63*-1) which is defective for interaction with Kar2p results in a major in vivo defect in the translocation of posttranslationally translocated precursors, but remains competent for translocation of SRP-dependent precursors (*17*). A different role for Kar2p in the co-translational reaction has been suggested by studies of the mammalian homologue, BiP, which is required for gating the lumenal face of the translocon (*29*). While Sec63p is required for translocation of SRP-dependent precursors, its precise role in this reaction is unknown. Specifically, it is unclear whether Sec63p contributes to the Kar2p/BiP-dependent gating reaction.

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[1] SRP, signal recognition particle; ER, endoplasmic reticulum; SR, SRP receptor; DPAP B, dipeptidyl aminopeptidase B; PCR, polymerase chain reaction; ppαF, prepro-α-factor, precursor of mating pheromone; gpαF, glycosylated form of α-factor precursor; DHC-αF, α-factor with signal sequence replaced by the DPAP B signal/anchor domain; gDHC-αF, glycosylated form of DHC-αF; BiP, immunoglobulin heavy chain binding protein.

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Studies of protein translocation in yeast have mainly been approached by genetic means. Biochemical analysis of specific components has been hindered by the failure to reconstitute SRP-dependent translocation in vitro. Recently, an in vitro assay has been developed in which it was shown that the concentration of SRP was limiting in yeast cytosol (*5*). Here we report a system in which all seven subunits of yeast SRP are overexpressed in yeast cytosol. These subunits correctly assemble into a fully functional SRP complex competent for co-translational targeting to yeast membranes in an in vitro assay. This assay is extremely efficient and provides an ideal system for the biochemical analysis of the co-translational translocation reaction. Using this assay, we have analyzed a novel mutant allele of *sec63* and have shown that the J-domain is indeed required for the translocation of an SRP-dependent precursor. These results demonstrate that the functions of Sec63p and Kar2p must be coordinated within the core translocon.

EXPERIMENTAL PROCEDURES

Materials. DNA restriction and modification enzymes were purchased from Roche Molecular Biochemicals. [^{35}S]methionine was from NEN Life Science Products. Oligonucleotides were from MWG-Biotech AG. All other reagents were from Sigma, Roche Molecular Biochemicals, and Melford Labs (Suffolk, UK) at analytical grade. Site-directed mutagenesis was performed using the QuickChange Site-directed Mutagenesis kit from Stratagene according to the manufacturer's instructions.

Strains, Media, and Growth Conditions. Saccharomyces cerevisiae and *Escherichia coli* strains were grown as previously described (*30*). *MET3-SEC63* is an allele of *SEC63* whose expression is repressed in the presence of methionine. Repression was performed by addition of methionine essentially as described (*31*), except strains were grown for 7 h in YPD (2% peptone, 1% yeast extract, 2% glucose) if microsomes were to be used in in vitro translation/translocation assays. *MET3-SEC63* shut off prior to total yeast extract preparation was performed in minimal yeast medium (0.675% yeast nitrogen base, 2% glucose) + 0.2 mM methionine for 7 h. Strains used: MWY26 (*Mat***a** *pep4−3 his3 ura3 leu2 ade2*, this study), BYY5 (*Mat*α *ade2 his3 ura3 leu2 trp1 can1 kanMX4-P$_{MET3}$-SEC63*; *21*).

Plasmid Constructions. YEp-based multicopy plasmids (pMW295 and pMW299) designed to overexpress SRP subunits were constructed as follows. Plasmids p65-A6 and pCS47 (*13*) were used as the source for *SRP54* and *SEC65* respectively. The remaining genes were all amplified by PCR of wild-type genomic DNA, subcloned and sequenced (see Table 1 for all oligonucleotides used).

Construction of pMW295: An approximately 4 kb deletion was made in pCS47 (*13*) by digesting with *Hin*dIII plus *Xba*1, treating with Klenow fragment and religating, giving pMW226. *SRP21* was amplified with SRP21-1 and SRP21-2 and cloned into *Sac*1−*Bam*HI of pMW226, giving pMW253. *SRP72* was amplified with primers SRP72-1-long and SRP72-2-long and cloned into *Bam*HI of pBluescript SK+, giving pMW276. Finally, the *Bam*HI-fragment containing *SRP72* from pMW276 was cloned into *Bam*HI of pMW253, giving pMW295.

Construction of pMW299: *SRP14* was PCR amplified with primers SRP14-1 and SRP14-2, the 1269 bp PCR product digested with *Bam*HI and *Sac*1 and cloned into *Bam*HI−*Sac*1 of YEp351 giving pMW228. A 1422 bp fragment containing *SCR1* was amplified with primers SCR1-1 plus SCR1-2, digested with *Sal*1 and *Xba*1 and subcloned into *Sal*1−*Xba*1 of pBluescript-KS+ giving pMW238. The same *Xba*1−*Sal*1 fragment containing *SCR1* was subsequently cloned into *Xba*1−*Sal*1 of pMW228 giving pMW272. *SRP54* was cloned as a B*am*HI−*Xba*1 fragment from p65-A6 into *Bam*HI−*Xba*1 of pMW272 giving pMW293. *SRP68* was PCR amplified with primers SRP68-1 plus SRP68-2, the 2605 bp product cloned into *Bam*HI of pBluescript-KS+ giving pMW246. The same *Bam*HI fragment containing *SRP68* was finally cloned into *Bam*HI of pMW293, giving pMW299. SRP54 and SEC65 were subcloned from previously described plasmids (*13*) and hence not sequenced. DNA sequencing of all the remaining SRP genes to identify PCR-induced errors showed that *SRP21* and *SRP72* contains no changes to the wild-type sequence. *SRP14* contains the conservative change Glu48^{GAA} → Glu48^{GAG} and two T → C changes at positions −352 and −417 relative to the open reading frame. *SRP68* has an A insertion at position −25, a conservative Leu461^{TTA} → Leu461^{TTG}, and a T → G 18 nucleotides downstream from stop codon. *SCR1* contains an A → G change 612 nucleotides outside the RNA encoding sequence. These few changes to the corresponding wild-type sequences were all thought to be of no consequence to expression of the genes.

Table 1: Oligonucleotides Used in This Study

SCR1-1	GGGGTCGACTGATCAACTTAGCCAGGACA
SCR1−2	GGGTCTAGAACTCAGCTTTGCGGGCCTC
SEC65-a	CAAATTTCAAATCAGATTGTATGATGGC
SRP14−1	GGGGGATCCATCCACCCGTATTCAGA
SRP14−2	GGGGAGCTCAAGGAATTTCAAAAATAGTC-ATC
SRP21−1	GGGGAGCTCTCCCCTACCTTTTACAAGTC
SRP21−2	GGGGATCCACGACAGTCTTGAAACC
SRP54−1	GCTCTCCTATACTTCAGTCACTACCC
SRP54−2	GTTAGAGGGTAGAACCTTTTCCATGGC
SRP68−1	GGGGGATCCAAATATACGAATACTGCCTTG
SRP68−2	GGGGGATCCTAGATAACTCGGTTTTGAGCA
SRP72−1-long	GGGGGATCCAATTAAAGTATGACAGAATC-GTTTGAAGG
SRP72−2-long	GGGGGATCCTGGGAAGGACTGATGATGA-ATTCCC
Sec63djd1a	CGCTGCTACAAAATTAAGATCTCCTTATGA-AATCCTTGG
Sec63djd1b	CCAAGGATTTCATAAGGAGATCTTAATTTT-GTAGCAGCG
Sec63djd2a	GAAATACGGTCATAGATCTGGCCCACAATC-TACTTC
Sec63djd2b	GAAGTAGATTGTGGGCCAGATCTATGACCG-TATTTC

The wild-type *SEC63* gene in pJKR2 was made by cloning a genomic 3682 bp *Hin*dIII fragment from a library clone into *Hin*dIII of pRS316. The J-domain delete version of *SEC63* was made by introducing *Bgl*II sites into pJKR2 by site-directed mutagenesis using the oligonucleotides Sec63djd1a, Sec63djd1b, Sec63djd2a, and Sec63djd2b. pAJ8 was the made by excision of the *Bgl*II fragment encoding the J-domain resulting in residues Phe^{124}−Asp^{199} being deleted and replaced by an arginine and a serine residue.

Fractionation of SRP and Western Blot Analysis. Preparation and sucrose gradient fractionation of SRP from wild-type and the SRP overexpression strain was done as

previously described (*32*). Whole yeast cell extracts and preparation of yeast microsomes were done as described (*31*). Antibodies against Srp54p, Sec65p, Sec61p, Sec63p, DPAP B, and Kar2p have previously been described (*17*, *21*, *23*, *33*). Gal3p antibodies were kindly donated by Dr. Richard Reece (Manchester, UK).

In Vitro Translation/Translocation. Plasmid pEH3 (600 bp *Eco*RI−*Xba*I fragment from pDJ100 (*34*) in pGEM3Z) was used for in vitro transcription/translation of ppαF. pEH3 was linearized with *Xba*1 and transcribed with Ribomax (Promega) T7 RNA polymerase according to the manufacturer's instructions. pJD96 (*5*) encoding the D_{HC}-αF was linearized with *Xba*1 and transcribed with Ribomax SP6 RNA polymerase. Translation competent yeast cytosol was prepared as described (*35*) with the following modification: Cells were grown in yeast minimal medium (to keep selection for plasmids either with or without SRP genes) to OD_{600} = 0.5, spun down, and grown for a further 6 h in 4l YPD (final OD_{600} = 2.0) prior to harvesting. Translations in the presence of [^{35}S]methionine were carried out as described (*36*). Microsomes (50 OD_{280}/mL) were added to a final concentration of 10% at the start of the reaction ("co") or after terminating translation by addition of 0.4 mM cycloheximide ("post"). Digestion with proteinase K (250 μg/mL) was done with or without 0.5% Triton X-100 for 30 min on ice followed by TCA precipitation. Samples were analyzed by SDS−PAGE and phosphorimage analysis. Bands were quantified using the AIDA software (version 2.31).

RESULTS

To complement in vivo observations, we were keen to develop an efficient in vitro assay for protein translocation in yeast cytosol. Previous in vitro assays have performed well when monitoring posttranslational translocation; however, reconstitution of co-translational translocation in the yeast system has proven more demanding due to the limiting concentration of SRP in yeast cytosol. We therefore sought to engineer yeast cells to overexpress SRP.

Cloning of all SRP Genes. To overexpress SRP, we created two yeast multicopy vectors which together contained all the seven genes that encode SRP subunits (*SCR1*, *SEC65*, *SRP14*, *SRP21*, *SRP54*, *SRP68*, and *SRP72*). Cloned DNA fragments containing two of the genes (*SEC65* and *SRP54*) were already available, and the remaining SRP genes were amplified by PCR and cloned into appropriate vectors. After DNA sequencing, the individual genes were subcloned to generate the two final vectors for expression in yeast: pMW295 (*SEC65*, *SRP21*, *SRP72*, *URA3*) and pMW299 (*SCR1*, *SRP14*, *SRP54*, *SRP68*, *LEU2*) (Figure 1). Simultaneous selection for both plasmids on media lacking uracil and leucine allowed us to maintain both plasmids in the yeast strain MWY26. Verification that the presence of pMW295 and pMW299 causes increased amounts of SRP subunits is seen in Figure 2. Compared to the unchanged levels of Gal3p (control), the levels of Srp54p and Sec65p are markedly increased in cells that contain pMW295 and pMW299.

Overexpressed SRP Subunits Assemble into Intact SRP Particles. In the absence of the gene products Srp14p, Srp21p, Srp68p, or Srp72p cells will lose the cytosolic ScR1 RNA (*14*) and the remaining ScR1 RNA remains largely nuclear (*37*). Sec65p (like Srp14p, Srp21p, Srp68p, and

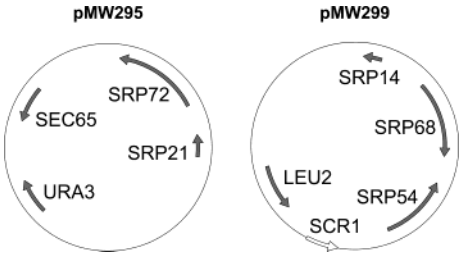

FIGURE 1: Plasmids for overexpression of yeast SRP. Schematic diagram of pMW295 and pMW299. Open reading frames are indicated with filled arrows, and RNA encoding sequence is indicated with open arrow. Simultaneous selection for both multicopy vectors (on medium lacking uracil and leucine) allows overexpression of all seven genes that encode RNA or proteins of the yeast SRP.

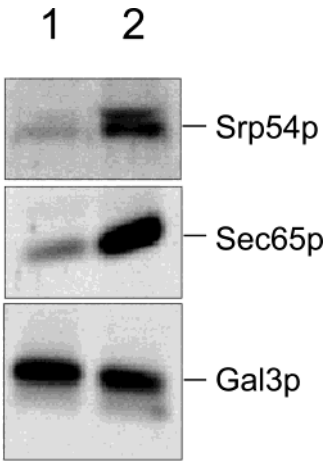

FIGURE 2: Transformation with SRP overexpression vectors causes significant increased levels of Srp54p and Sec65p. Western blot of total yeast extract from wild-type (MWY26 + YEp351 + YEp352, lane 1) and SRP overexpressor strain (MWY26 + pMW295 + pMW299, lane 2) probed with antibodies against Srp54p, Sec65p, and Gal3p.

Srp72p) assembles with ScR1 RNA into pre-SRP in the nucleus, whereas Srp54p is finally assembled with the pre-SRP in the cytosol after nuclear export (*37*, *38*). The integrity of SRP particles can be determined by applying preparations of SRP onto sucrose gradients. Co-sedimentation of Srp54p, Sec65p, and ScR1 RNA on sucrose gradients should only occur if fully assembled intact SRP particles are being analyzed. We therefore assayed assembly of intact SRP by fractionation on sucrose gradients. SRP prepared from cells transformed with only the control vectors YEp351 + YEp352 was loaded onto a 5−30% sucrose gradient. After centrifugation, fractions were analyzed for the presence of Srp54p and Sec65p. As seen in Figure 3 (upper panels) the two markers for the wild-type SRP co-sediment toward the lower half of the sucrose gradient. The same fractionation pattern is observed when the SRP overexpressing cells are analyzed (Figure 3, lower panels). The strongly increased signals suggest that cells that have been transformed with both pMW295 and pMW299 produce significantly higher amounts of intact SRP. Another observation to support this conclusion is that ScR1 RNA is overproduced and co-fractionates with the Sec65p/Srp54p marker proteins in MWY26 + pMW295 + pMW299 (data not shown).

Functional Overexpressed SRP Particles Improve Co-Translational Translocation in Vitro. Having established that

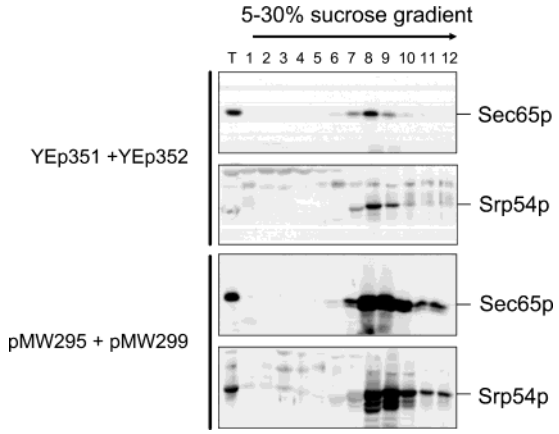

FIGURE 3: Overexpressed SRP subunits assemble into intact particles. SRP from MWY26 transformed with either YEp351 + YEp352 (control) or pMW295 + pMW299 (SRP overexpression) was analyzed on sucrose gradients. Fractions were subjected to Western blot analysis with anti-Sec65p and anti-Srp54p antibodies. The co-sedimentation of Sec65p and Srp54p in fractions 8−9 in the wild-type control (two upper panels) is mirrored in the gradient with overexpressed SRP (two lower panels).

transformation of MWY26 with pMW295 + pMW299 gives rise to increased amounts of intact SRP particles, we determined the efficiency of in vitro translocation using cytosol from wild-type cells and cytosol from cells that overexpress SRP. To do this, we chose two forms of αF, differing only in their signal sequences. It is widely accepted that ppαF translocates independently of SRP, whereas the same precursor with its signal sequence replaced by the hydrophobic core of the DPAP B signal sequence depends on SRP for translocation (*22*). We found that both these constructs could be translated in vitro, and that the rates of translation remained similar in cytosols from both wild-type cells and cells overexpressing SRP. Translocation of these precursors into yeast microsomes can be monitored by the appearance of glycosylated forms which migrate more slowly. The relative levels of glycosylated/unglycosylated forms can be used as a measurement of the translocation efficiency. In doing so, we found that whereas translocation of ppαF is only slightly better in the SRP overexpression cytosol (Figure 4A, lanes 3 and 8), translocation of D_{HC}-αF is significantly improved (compare lanes 5 and 10). We have quantified the translocation efficiency and found that overexpression of SRP resulted in an average 49% efficiency of translocation, corresponding to a 3-fold increase in efficiency when compared to wild-type extracts (16%) (Figure 4B).

In vitro translation/translocation reactions can be performed as either "co-" or "post-" translational translocation reactions. In "co" reactions precursors are being translated in the presence of yeast microsomes and can as such be translocated co-translationally. It is of course not possible to prevent posttranslational translocation from taking place in a "co" reaction. In a "post" reaction on the other hand, translation is terminated by addition of cycloheximide prior to the addition of microsomes. In this case, the only option is therefore posttranslational translocation. In our in vitro assay, it is important to establish how the different precursors behave. As seen in Figure 4C, the triply glycosylated form of αF (gpαF) can be seen in both "co" and "post" reactions, whereas gD_{HC}-αF is only seen in a "co" reaction. As opposed to the precursors, the glycosylated forms are protected against

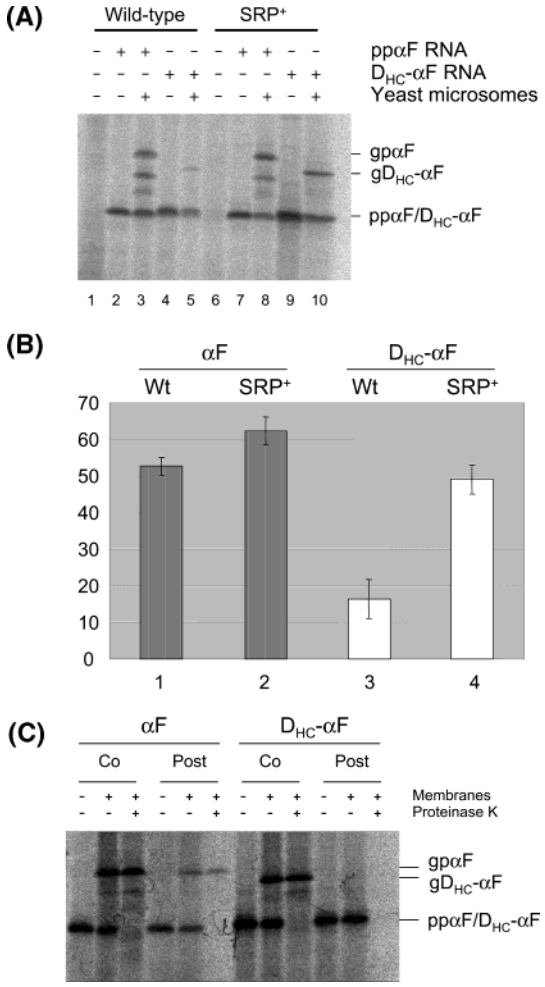

FIGURE 4: Co-translational translocation in vitro is enhanced by overexpression of SRP. (A) Yeast cytosol from either wild-type (MWY26 + Yep351 + Yep352, lanes 1−5) or SRP overexpressor strain ("SRP+": MWY26 + pMW295 + pMW299, lanes 6−10) was used for in vitro translation/translocation reactions. In vitro transcribed RNAs encoding either ppαF or D_{HC}-αF were used as templates and microsomes from MWY26 were included from the start of the reaction ("co", see Experimental Procedures). The glycosylated forms (gpαF and gD_{HC}-αF) are seen only in reactions containing yeast microsomes. A significant increase in translocation of D_{HC}-αF is seen in SRP overexpressor cytosol. (B) Quantification of translocation in several in vitro translation/translocation assays. αF RNA (grey bars) or D_{HC}-αF RNA (white bars) was translated/translocated into microsomes from MWY26 in cytosol from wild-type ("Wt": MWY26 + YEp351 + YEp352, bars 1 and 3) or SRP overexpressor (MWY26 + pMW295 + pMW299, bars 2 and 4). Translocation efficiencies for these four combinations of RNA/cytosol were (mean values): 52.6, 62.3, 16.4, and 49.1%. Each bar represents the mean from at least four independent experiments (error bars are standard error of the mean). (C) D_{HC}-αF is co-translationally translocated. "Co" or "Post" (see Experimental Procedures) translation/translocation reactions were carried out in SRP overexpression cytosol using wild-type microsomes. As expected, only the glycosylated (translocated) forms are protected against proteinase K degradation. Whereas ppαF is translocated in both "Co" and "Post" reactions, D_{HC}-αF is strictly co-translationally translocated.

proteinase K degradation, which indicates that they have been completely translocated into the microsomes. Hence, whenever we observe gD_{HC}-αF we are measuring a genuine co-translational translocation event. Finally, we repeatedly found higher levels of ppαF translocation in "co" relative to "post" reactions (see Discussion).

Deletion of Sec63p J-Domain Affects Co-Translational Translocation in Vivo. We have previously shown that Sec63p and Kar2p are required for co-translational translocation in vivo *(21)*. These two proteins have been shown to interact as DnaJ/DnaK-like partners via the J-domain of Sec63p. This interaction may therefore be important for the function of these proteins in co-translational translocation. A single point mutation in the Sec63p J-domain (*sec63-1*) has been described (*17*), which has a major defect in posttranslational translocation and only a slight defect in co-translational translocation. It has been shown that Sec63-1p has a lower affinity for Kar2p in vitro (*39*). Given these facts, we wanted to address whether the J-domain was required for Sec63p's function in co-translational translocation. To do this, we transformed BYY5 (containing a genomic *MET3-SEC63* allele) with plasmids expressing either wild-type Sec63p (pJKR2) or a deletion derivative lacking the J-domain, Sec63p-ΔJ (pAJ8, Figure 5A). In the presence of methionine, expression of the wild-type Sec63p is repressed and the phenotype associated with the altered protein can be determined. We found that cells are unable to survive when they only express Sec63p-ΔJ (Figure 5B). As expected, the BYY5 + pAJ8 cells show a substantial accumulation of ppαF after 7 h in the presence of methionine (data not shown). Interestingly, judging from the steady-state levels of preDPAP B, these cells were also defective in translocation of this co-translationally translocated precursor under the same conditions (Figure 5C). This suggests that the J-domain is required for co-translational translocation in vivo.

Sec63p J-Domain Required for Co-Translational Translocation in Vitro. To verify these observations, we used microsomes from cells expressing Sec63p-ΔJ in the in vitro translocation assay described above. Translocation of both ppαF and D_{HC}-αF into BYY5 + pAJ8 shut off microsomes was severely affected (Figure 6A, lanes 4 and 8), whereas the corresponding wild-type control microsomes (BYY5 + pJKR2, Figure 6A, lanes 3 and 7) were only slightly less efficient than wild-type microsomes (Figure 6A, lanes 2 and 6). Western blot analysis confirmed that the shut off of *MET3-SEC63* was effective and that Sec63p-ΔJ was the major species in these microsomes (Figure 6B). The Western blot analysis also confirmed that the size, expression level, and membrane association was as expected. The level of Sec63p in microsomes from BYY5 + pJKR2 (Figure 6B, lane 2) is slightly higher than in the wild-type (lane1), which may account for the slightly reduced efficiency of in vitro import into these microsomes since it has previously been shown that overexpression of Sec63p creates a slight dominant defect in the translocation of prepro-α-factor in vivo (*37*, *38*). We also found that BYY5 + pJKR2 cells had a minor defect in ppαF translocation in vivo (data not shown). Finally, the mutant membranes might be non-specifically disrupted, leading to the observed general defects in translocation. To eliminate this possibility, we examined membrane integrity by incubating the microsomes with proteinase K. As expected, the untranslocated precursor of Kar2p (pre-Kar2p) was exposed on the outside of the microsomes, whereas the mature Kar2p was found to be protected against degradation by degradation proteinase K unless membranes were first solubilized by addition of 0.5% Triton X-100 (Figure 6C). This indicates that the (BYY5 + pAJ8) microsomes are intact. Crucially, we also found that

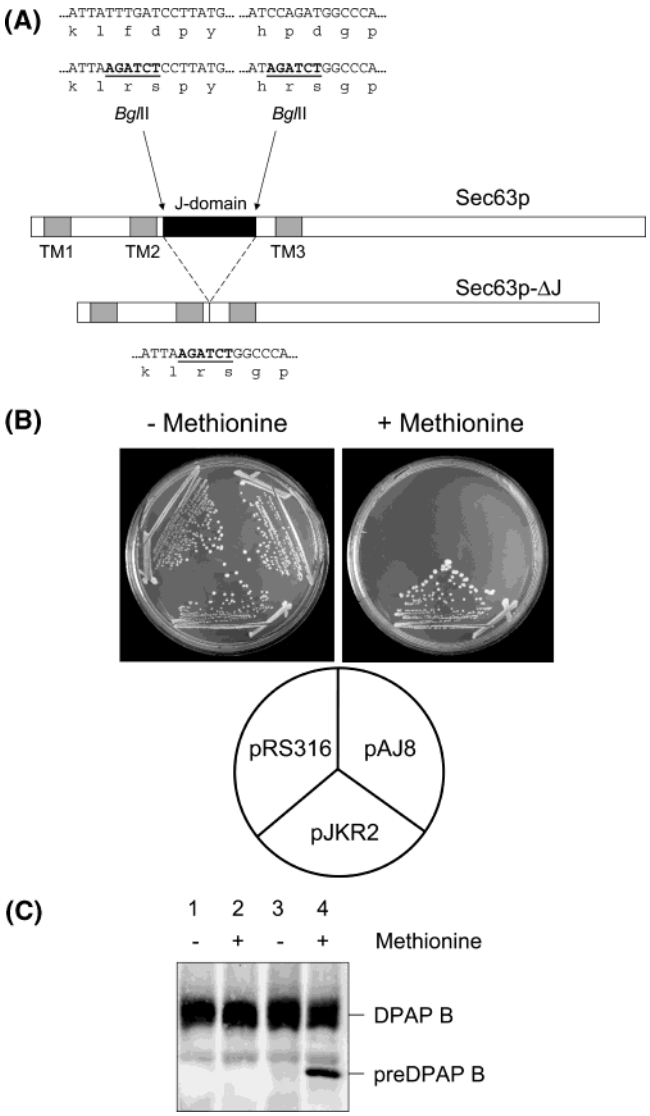

FIGURE 5: J-domain of Sec63p is essential for viability and in vivo co-translational translocation. (A) Schematic diagram of the wild-type and J-domain delete forms of Sec63p. The J-domain of Sec63p (black box) is situated between transmembrane domains 2 and 3 (grey boxes). Site-directed mutagenesis was used to introduce two *Bgl*II restriction sites into the coding sequence of the wild-type *SEC63* gene on a yeast single copy plasmid (pJKR2). Deletion of the DNA fragment between the two *Bgl*II sites results in a plasmid (pAJ8) that allows expression of a form of Sec63p which lacks the J-domain (Sec63p-ΔJ). The DNA sequence before and after introduction of *Bgl*II sites and after excision of the J-domain is shown. (B) The J-domain of Sec63p is essential for viability. Yeast strain BYY5 was transformed with pRS316 (vector control), pJKR2 (wild-type *SEC63*), or pAJ8 (J-domain delete *SEC63*) and grown in the absence of methionine. Expression of wild-type Sec63p from the *MET3-SEC63* allele of BYY5 was suppressed by streaking the cells onto medium containing methionine. Cells that had been transformed with pAJ8 were unable to grow in the absence of wild-type Sec63p expression. (C) Total yeast extracts from BYY5 (+ pJKR2, lanes 1−2; + pAJ8, lanes 3−4) grown for 7 h in yeast minimal medium + 0.2 mM methionine were subjected to Western blot analysis. Probing with anti-DPAP B antibodies show that the wild-type Sec63p (lanes 1−2) functionally complements the *MET3-SEC63* shut off, but Sec63p-ΔJ alone (lanes 3−4) causes accumulation of significant amounts of preDPAP B.

Sec63ΔJp was still able to assemble into the heptameric SEC-complex (data not shown), from which we conclude that deletion of the J-domain did not result in any gross perturbation of the protein's topology. We therefore conclude

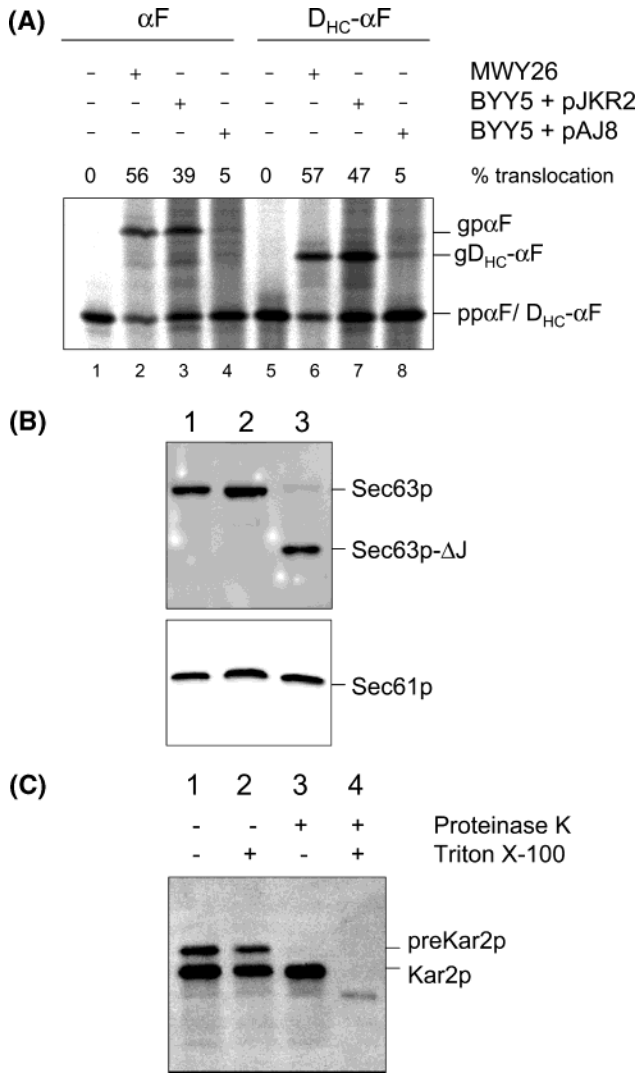

FIGURE 6: (A) In vitro defect in co-translational translocation into microsomes containing J-domain deleted Sec63p. In vitro transcription/translocation reactions ("Co" reactions in SRP overexpression cytosol, see Experimental Procedures) using RNA encoding ppαF (lanes 1−4) or D$_{HC}$-αF (lanes 5−8) and yeast microsomes prepared from cells that have been grown in YPD for 7 h. Microsomes are from MWY26 (lanes 2 and 6), BYY5 + pJKR2 (lanes 3 and 7) or BYY5 + pAJ8 (lanes 4 and 8). Control reactions without microsomes are seen in lanes 1 and 5. A significant defect in translocation of both ppαF and D$_{HC}$-αF into microsomes containing only Sec63p-ΔJ was observed (lanes 4 and 8). (B) Western blot analysis with anti-Sec63p antibodies of the microsomes used in the in vitro import assay (MWY26 (lane 1), BYY5 + pJKR2 (lane 2), BYY5 + pAJ8 (lane3)). Sec61p loading control in lower panel. (C) Microsomes from BYY5 + pAJ8 after 7 h shut off of *MET3-SEC63* in YPD were mock treated (lane 1), or treated with Triton X-100 (lanes 2 and 4) and Proteinase K (lanes 3 and 4). Western blot analysis with anti-Kar2p antibodies shows that the precursor form is exposed to and mature Kar2p is protected from degradation by proteinase K in the absence of detergent.

that the J-domain of Sec63p is required for co-translational translocation in vivo and in vitro.

DISCUSSION

By complementing in vivo observations with corresponding in vitro assays, it is often possible to address questions about different aspects of a particular cellular function. Yeast is well established as an experimental organism, and historically, in vivo studies have generated a large amount of information. Sophisticated screens to isolate mutants defective in translocation into the ER have identified many gene products required for these processes. Some aspects of the interactions between these proteins have been analyzed by biochemical means, and in vitro reconstitution of translocation has also been described. Specifically, translocation into yeast microsomes (*35*) or proteoliposomes (*40*, *41*) has successfully demonstrated the requirement for both the trimeric Sec61 complex, the tetrameric Sec63-complex, and Kar2p for translocation of ppαF in vitro.

We have created two multicopy yeast vectors (pMW295 and pMW299) that contain all the genes required to encode SRP subunits. Cells that have been simultaneously transformed with these two vectors overexpress intact and functional SRP. Overexpression of SRP was found to have no effect on the growth of the cells. Previous reports have shown that addition of 100 nM purified SRP could boost translocation efficiency of D$_{HC}$-αF in wild-type cytosol 1.5-fold to a final value of 25% (*5*). This corresponds well with our efficiency of co-translational translocation efficiency in wild-type cytosol of 16.4%. Purification of SRP is technically demanding, hence our vector-based strategy. In cytosol made from cells that overexpress SRP, we found that the efficiency of co-translational translocation of D$_{HC}$-αF was 49%, representing a 3× increase compared to the maximum observed when using wild-type levels of SRP. We also observed a slight increase in translocation of ppαF from 52.6 to 62.3%. As mentioned, ppαF is generally believed to translocate in a strictly SRP-independent posttranslational fashion. However, examination of the literature clearly shows that a small proportion of this particular precursor is translocated via the SRP-dependent pathway. For example, ppαF accumulates in *sec65-1* mutant cells (*22*), and it can also be cross-linked to SRP54p (*42*). The observed increase in the translocation efficiency of ppαF using overexpressed SRP supports this interpretation. We also found ppαF to translocate more efficiently in "co-" relative to "post-" reactions (see Figure 4C). However, we cannot exclude the possibility that much of this translocation is in fact of completed polypeptide chains whose translocation may become less efficient if precursors are permitted time to fold before microsomes are added for the "post" reaction. Nonetheless, our assay clearly provides a simple and very efficient assay for SRP-dependent co-translational translocation in vitro. If the membrane-bound SRP-receptor is a limiting factor, the efficiency can possibly be even further improved by overexpressing SRP receptor in the cells from which the microsomes are made. With this in vitro assay, it may now be possible to design experiments to investigate aspects of translocation that have not been revealed by in vivo experiments.

We have previously shown both Sec63p and Kar2p to be involved in co-translational translocation (*21*). The mammalian homologue of Kar2p, BiP, has been identified as a gating factor acting at the lumenal face of the translocon (*29*). Our data clearly demonstrate a role for the Sec63p J-domain in co-translational translocation. Given the well characterized interactions between the J-domain and Kar2p, we propose that Sec63p regulates translocon gating through the recruitment and activation of Kar2p.

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