Access to Ribosomal Protein Rpl25p by the Signal Recognition Particle Is Required for Efficient Cotranslational Translocation

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Submitted October 24, 2007; Revised April 10, 2008; Accepted April 18, 2008
Monitoring Editor: Reid Gilmore

Targeting of proteins to the endoplasmic reticulum (ER) occurs cotranslationally necessitating the interaction of the signal recognition particle (SRP) and the translocon with the ribosome. Biochemical and structural studies implicate ribosomal protein Rpl25p as a major ribosome interaction site for both these factors. Here we characterize an *RPL25GFP* fusion, which behaves as a dominant mutant leading to defects in co- but not posttranslational translocation in vivo. In these cells, ribosomes still interact with ER membrane and the translocon, but are defective in binding SRP. Overexpression of SRP can restore ribosome binding of SRP, but only partially rescues growth and translocation defects. Our results indicate that Rpl25p plays a critical role in the recruitment of SRP to the ribosome.

INTRODUCTION

The cotranslational mode of protein targeting is highly conserved from bacteria through to higher eukaryotes (Pool, 2005). Proteins that are destined to be secreted are synthesized with an N-terminal hydrophobic signal peptide, which is recognized by the signal recognition particle (SRP) as it emerges from the ribosome (Keenan *et al.*, 2001). SRP induces a transient retardation in protein synthesis (Siegel and Walter, 1985; Mason *et al.*, 2000) and, targets the ribosome-nascent chain (RNC) complex to the endoplasmic reticulum (ER) membrane, where it binds its cognate receptor, SRP receptor (SR; Gilmore *et al.*, 1982; Meyer *et al.*, 1982). SR mediates the transfer of the RNC complex from SRP to the translocation pore, formed by the Sec61p complex (Song *et al.*, 2000; Osborne *et al.*, 2005). This in turn releases the block in elongation, such that translation resumes with the nascent chain being translocated through the channel as it elongates.

The cotranslational nature of the targeting and translocation reaction necessitates contact between SRP, SR, and the ribosome. Cross-linking and cryo-electron microscopic (EM) studies have identified multiple contact sites between these targeting/translocation components and the ribosome. SRP makes six contacts with the ribosome: four located around the ribosomal exit site and two at the subunit interface (Pool *et al.*, 2002; Halic *et al.*, 2004; Terzi *et al.*, 2004), which repre-

sent the sites involved in signal sequence recognition and elongation arrest, respectively. The Sec61p complex interacts with the ribosome at between four and six contact points, again located at the ribosomal exit site (Beckmann *et al.*, 2001; Menetret *et al.*, 2005). There is considerable overlap in the two binding sites, and at least one of these contact sites, involving ribosomal protein Rpl25p (Rpl23a in higher eukaryotes) forms a major contact with both the Sec61p complex and SRP54, the signal-sequence binding component of SRP (Pool *et al.*, 2002). Indeed, biochemical and structural data suggest that SR plays an important role in repositioning SRP54 in the SRP-RNC complex such that Sec61p can gain access to Rpl25p (Jungnickel and Rapoport, 1995; Pool *et al.*, 2002; Halic *et al.*, 2006b).

Not only does Rpl25p contact SRP54 and Sec61, but several chaperones that interact with the nascent chain have been reported to bind to the ribosome via Rpl25, for example, the nascent polypeptide chain-associated complex (NAC; Wegrzyn *et al.*, 2006). In bacteria, L23, the homologue of Rpl25, has been shown to contact the bacterial homologues of SRP54 and Sec61p complex as well as the chaperone trigger factor (Kramer *et al.*, 2002; Gu *et al.*, 2003; Mitra *et al.*, 2005). This has led to the suggestion that Rpl25 forms a general factor-binding site for complexes, which interact with the nascent chain.

Our current understanding on the role of Rpl25 in protein translocation is derived exclusively from biochemical and structural studies performed in vitro. We therefore wanted to investigate the importance of Rpl25p in protein translocation in vivo using the genetically tractable yeast *Saccharomyces cerevisiae*. Translocation in yeast occurs via two pathways, the SRP-dependent pathway, as outlined above, and an SRP-independent pathway, which can function posttranslationally (Wilkinson *et al.*, 1997). In the SRP-dependent pathway, the Sec61p heterotrimer (Sec61p, Sbh1p, and Sss1p) is thought to function in a larger complex with Sec63p, Sec72p, and Sec71p (Jermy *et al.*, 2006). The same complex is involved in SRP-independent translocation, however, the presence of Sec62p is required additionally (De-shaies and Schekman, 1989; Panzner *et al.*, 1995). Properties

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E07-10-1074>) on April 30, 2008.

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Abbreviations used: CPY, carboxypeptidase Y; DPAP B, dipeptidyl aminopeptidase B; ER, endoplasmic reticulum; GFP, green fluorescent protein; NAC, nascent polypeptide chain-associated complex; RAC, ribosome-associated complex; RNC, ribosome-nascent chain; SRP, signal recognition particle; SR, SRP receptor.

Table 1. Strains used in this study

Strain	Genotype	Reference
RS453	<i>MATα leu2 his3 trp1 ura3 ade2 his3</i>	Segref <i>et al.</i> (1997)
RPL25GFP	<i>MATα leu2 his3 trp1 ura3 ade2 rpl25::HIS3 [pYEplac112-RPL25GFP]</i>	Hurt <i>et al.</i> (1999)
Y1090	<i>MATα leu2 his3 trp1 ura3 ade2 rpl25::HIS3 [pYEplac195-RPL25GFP-ADE2-URA3]</i>	Hurt <i>et al.</i> (1999)
MPY6	<i>MATα leu2 his3 trp1 ura3 ade2 rpl25::HIS3 [pYCplac111-RPL25GFP(pMP233)]</i>	This study
MPY39	<i>RS453 [pYCplac111-RPL25GFP(pMP233)]</i>	This study
MPY69	<i>MATα leu2 his3 trp1 ura3 ade2 rpl25::HIS3 [pYCplac111-RPL25(pMP226)]</i>	This study
MPY75	<i>RS453 [pYCplac111-RPL25(pMP226)]</i>	This study
RSY455	<i>MATα leu2 trp1 ura3 his4 sec61-3</i>	Stirling <i>et al.</i> (1992)
CSY128	<i>MATα leu2 ade2 trp1 ura3 his3 sec65-1</i>	Stirling <i>et al.</i> (1992)
CSY221	<i>MATα leu2 ura3 his4 sec62-1</i>	Rothblatt <i>et al.</i> (1989)
BY4742	<i>MATα ura3Δ0 leu2Δ0 his3Δ0 lys2Δ0</i>	Winzeler <i>et al.</i> (1999)
Δ rpl39	<i>MATα ura3Δ0 leu2Δ0 his3Δ0 lys2Δ0 Δrpl39</i>	Winzeler <i>et al.</i> (1999)
Δ btt1	<i>MATα ura3Δ0 leu2Δ0 his3Δ0 lys2Δ0 Δbtt1</i>	Winzeler <i>et al.</i> (1999)
Δ egd1	<i>MATα ura3Δ0 leu2Δ0 his3Δ0 lys2Δ0 Δegd1</i>	Winzeler <i>et al.</i> (1999)
Δ egd2	<i>MATα ura3Δ0 leu2Δ0 his3Δ0 lys2Δ0 Δegd2</i>	Winzeler <i>et al.</i> (1999)
Δ zuo1	<i>MATα ura3Δ0 leu2Δ0 his3Δ0 lys2Δ0 Δzuo1</i>	Winzeler <i>et al.</i> (1999)

of the signal sequence largely determine which targeting pathway a particular substrate will use (Ng *et al.*, 1996).

We have made use of an RPL25GFP gene fusion, which still produces translation-competent ribosomes (Hurt *et al.*, 1999). Here we show that the RPL25GFP fusion behaves as a dominant mutant leading to defects in cotranslational protein targeting due to an inability of SRP to interact correctly with the ribosome.

MATERIALS AND METHODS

Plasmid Construction

The entire URA3 open reading frame (ORF) was amplified by PCR from YCplac33 using the primers 5'-GGAATTCATGACGAAAGCTACATATA-ACG-3' and 5'-TGGATTTCATATGT TAGTTTTGCTGGCCGCATC-3', which include flanking BspHI and NdeI sites, respectively. The resulting ~800-bp fragment was digested with these same enzymes and ligated into NcoI- and NdeI-digested pTPP (a gift from Peter Walter, University of California, San Francisco, San Francisco, CA), which contains the entire Pho8p ORF under the control of the PHO5 promoter in pRS314 (Chapman and Munro, 1994). The resulting plasmid, pMP211, encodes the first 82 residues of Pho8p followed by the complete URA3 ORF. pMP211 was digested with BglII, the released fragment, which includes the PHO8-URA3 cassette, and was ligated into pRS315 digested with BglII to yield pMP234.

To generate the Pho8p-Ura3p-myc fusion protein, the entire URA3 ORF was amplified by PCR from YCplac33 using the primers 5'-GGAATTCATGACGAAAGCTACATA TATAACG-3' and 5'-GGCCCTGCAGTATAGATCTCTCTCGCTTATTAGTTTTGTCGTTTGTGCTGGCCGCATC-3', which include flanking BspHI and PstI sites (underlined), respectively. The forward primer introduces the mutation K3N, which generates an N-glycosylation site, whereas the reverse primer adds a C-terminal Myc-tag. The product was digested with PstI and BspHI and ligated into pMP234 cut with the same enzymes, to give pMP219. The NUF2 3' untranslated region (UTR), including as a PstI-NotI fragment from plasmid pMS356 (Frey *et al.*, 2001) and ligated into pMP219 cut with the same enzymes to give pMP220.

pMP220 was digested with BglII, the released fragment, which includes the PHO8-URA3-myc cassette, and was ligated into pRS314 digested with BglII to yield pMP231.

The RPL25 gene, including 735-base pair 5' and 435-base pair 3' flanking sequence was amplified by PCR from genomic DNA prepared from strain W303 α using the following primers: 5'-CCCTAAGCTTGCTATTGAG-AGC-3' and 5'-GATGATCAATTGGGATCCCCATA TAAGCGAG-3', including flanking HindIII and MunI sites, respectively. The resulting ~2-kbp fragment was first cloned in to the TOPO II vector (Invitrogen, Paisley, United Kingdom), re-excised with HindIII and MunI, and ligated into YCplac111, which had been cut with HindIII and EcoRI, to generate YCplac111-RPL25 (pMP226).

YEplac112-RPL25GFP (Hurt *et al.*, 1999), a gift from Ed Hurt (Biochemie Zentrum, University of Heidelberg, Heidelberg, Germany), was digested with BglII, and the resulting fragment was ligated into YCplac111 digested with BglII to give YCplac111-RPL25GFP (pMP233).

Plasmid pMR12 (a gift from Colin Stirling, University of Manchester, Manchester, United Kingdom) comprises a fusion of carboxypeptidase Y (CPY) with URA3 in the vector YEpl351 (C. Stirling, personal communication).

To generate pMP270, the URA3 ORF and 3' UTR were amplified from pRS416 using primers 5'-AAAACTAGTATGTCGAAAGCTACATATAAG-G-3' and 5'-CCAAAGCGGCCGCCATTATTGACTATATTAATT-3' containing flanking SpeI and NotI sites, respectively. The fragment was digested with these enzymes and inserted into pRS315 cut with the same enzymes. The HMG1-SUC2 fusion (N-terminal 463 residues of Hmg1p together with the first 307 residues of mature invertase) was amplified by PCR from pCS5 (Stirling *et al.*, 1992) using primers 5'-ATCGCTGCAGCGTTAAATTAGATTATCGCCGC-3' and 5'-GCAACTAGTCATAAATGATCTCCATGGGTTAG-3', with flanking PstI and SpeI sites, respectively. The fragment was cloned via these sites in front of the URA3 fragment in pRS315.

Yeast Strains and Media

Yeast strains were grown either in YPD (rich) media (1% (wt/vol) yeast extract, 2% (wt/vol) peptone, 2% (wt/vol) glucose) or minimal media (0.67% [wt/vol] yeast nitrogen base, 2% [wt/vol] glucose) plus appropriate amino acid supplements. 5-Fluoro orotic acid (5-FOA; Melford Laboratories, Chesham, Ipswich, United Kingdom) was added to plates at a final concentration of 1 g $\cdot$ l⁻¹. Azetidine 2-carboxylic acid (AZC; Sigma, Poole, United Kingdom) was added to YPD medium at a final concentration of 1.0 g $\cdot$ l⁻¹.

The strains used in this study are detailed in Table 1. Δ rpl25 strains complemented with CEN-based plasmid derivatives of RPL25 (MPY6 and MPY 69) were generated by plasmid shuffling as follows: Y1090 α (Hurt *et al.*, 1999) was transformed with plasmid YCplac111-RPL25GFP (pMP233) or YCplac111-RPL25 (pMP226). The resulting transformants were then streaked on minimal media containing 5'-FOA and grown for 3 d at 30°C to select for loss of the YEplac195-RPL25GFP plasmid. Resulting colonies were restreaked on minimal medium lacking leucine and grown for 2 d at 30°C. Loss of YEplac195-RPL25GFP was verified by streaking on minimal medium lacking either adenine or uracil.

Polysome Analysis

Yeast cells were grown in complete media to an OD₆₀₀ of ~0.6 and then treated with 0.1 mg $\cdot$ ml⁻¹ cycloheximide for 10 min. Cells were then lysed, and extracts were analyzed by sucrose density gradient fractionation on 10–50% (wt/vol) sucrose gradients (Rueggsegger *et al.*, 2001) followed by fractionation and continuous monitoring of A_{260 nm} with an ISCO fractionator.

Subcellular Fractionation

Yeast cells were grown shaking in complete media to an OD₆₀₀ of ~0.6 and then treated with 0.1 mg $\cdot$ ml⁻¹ cycloheximide for 10 min. Cells were then fractionated exactly as described previously (Frey *et al.*, 2001). Alternatively, lysates were cleared of cellular debris by centrifugation at 1200 $\times$ g, and membranes were solubilized by the addition of 1% (wt/vol) CHAPS, in the presence of 500 mM KOAc. Insoluble material was removed by centrifugation for 20 min at 16,000 $\times$ g. The resulting supernatant was centrifuged for 1 h at 256,000 $\times$ g to generate a ribosome-enriched pellet and postribosomal supernatant.

Mass Spectrometry

Coomassie-stained bands were excised from gels, reduced, and then alkylated before digestion with trypsin. Digested samples were analyzed by liquid chromatography (LC)-tandem mass spectrometry on a Q-TOF Micro (Waters, Northwich, United Kingdom), and the resulting peptides were

Figure 1. *RPL25GFP* cells have a specific defect in cotranslational translocation. (A) Yeast strains RS453 (wild-type), *sec61-3*, *RPL25GFP* (MPY6), and $\Delta rpl25::pRPL25$ (MPY69) carrying the *PHO8-URA3* reporter plasmid (pMP211) were plated in parallel on synthetic media lacking either tryptophan (-trp; selects for plasmid) or tryptophan and uracil (-trp -ura; selects for reporter) and grown for 2 d at 30°C. (B) Yeast strains W303 α (wild-type), *sec62-1*, *sec65-1*, and *RPL25GFP*, possessing the *CPY-URA3* reporter plasmid (pMR12), were plated on synthetic media lacking either leucine (-leu) or leucine and uracil (-leu -ura). (C) Wild-type (RS453), *RPL25GFP*, *sec65-1* and *sec61-3* transformed with pMP220 (*PHO8-URA3-myc*) were pulse-labeled for 5 min with [35 S]methionine at either 25 or 37°C and lysed, and then radiolabeled proteins were immunoprecipitated with α -myc antibodies and analyzed by SDS-PAGE and phosphorimaging. Positions of g-Pho8-Ura3p (membrane integrated) and Pho8p-Ura3p (cytosolic) are indicated. (D) Wild-type (RS453), *RPL25GFP*, and *sec61-3* cells were pulse-labeled as in C and immunoprecipitated with α -CPY antibodies. Positions of g-CPY (p1-luminal) and ppCPY (cytosolic) are indicated.

searched against the MSDB database using Mascot software (Matrix Science, Boston, MA).

Antibodies

Anti-myc (4A6) was from Upstate (Milton Keynes, United Kingdom), anti-Zwf1p was from Sigma. Antibodies against Rps3p, Sec61p, DPAP B, Srp72p, Ssa1p, and CPY have been described previously (Stirling *et al.*, 1992; Ogg *et al.*, 1998; Mason *et al.*, 2000; Frey *et al.*, 2001).

RESULTS

RPL25GFP Cells Have a Cotranslational Translocation Defect

In yeast, the *RPL25* gene is essential, as the corresponding gene product, Rpl25p, plays an essential role in 60S subunit assembly (Kooi *et al.*, 1994). Therefore the role of Rpl25p in protein targeting cannot be assessed by simple gene deletion. As an alternative approach, we made use of a yeast strain, which possesses an *RPL25-GFP* gene fusion as the sole copy of *RPL25*. This strain had previously been shown to be viable, and Rpl25-GFP is efficiently incorporated into functional ribosomes, but exhibits a marked growth defect (Hurt *et al.*, 1999). Considering the evidence implicating Rpl25p as a binding site for targeting and translocation factors, we wanted to investigate whether the growth defects of the *RPL25GFP* strain might arise as a consequence of defects in cotranslational protein targeting.

To monitor cotranslational translocation, we generated a reporter construct, composed of the entire *URA3* ORF fused to the N-terminus of the type II integral membrane protein, Pho8p, including its signal anchor sequence. The fusion protein was expressed from a medium-strength constitutive promoter from a CEN-based plasmid. Targeting of Pho8p occurs exclusively via the SRP-dependent targeting pathway (Ng *et al.*, 1996), and so we expected the Pho8p-Ura3p fusion protein to be inserted into the membrane in wild-type cells or in cells with defects in the posttranslational pathway, but to accumulate in the cytoplasm in cells with defects in the SRP-dependent pathway. In a wild-type ($\Delta ura3$) strain Pho8p-Ura3p is efficiently targeted to the ER such that the Ura3p moiety resides in the lumen and is unable to access its

cytosolic substrate. Hence these cells cannot grow in the absence of uracil (Figure 1A). In contrast, in a *sec61-3* strain, targeting is inefficient and Pho8p-Ura3p accumulates in the cytoplasm, and the cells can grow without uracil.

RPL25GFP cells, carrying the *PHO8-URA3* reporter, were able to grow in the absence of uracil (Figure 1A). In contrast, a $\Delta rpl25$ strain, possessing a plasmid copy of wild-type *RPL25*, and also the isogenic wild-type strain, were both Ura⁻. This suggests that *RPL25GFP* cells have a cotranslational translocation defect. Furthermore, cells possessing both a wild-type copy of *RPL25* and *RPL25GFP* also became Ura⁺ in the reporter assay (Supplemental Figure S1), indicating a partial dominance of the translocation phenotype induced by *RPL25GFP*.

A second reporter, comprising the first six transmembrane domains of Hmg1p fused to the mature domains of Suc2p and Ura3p, also led to a Ura⁺ phenotype in *RPL25GFP* but not wild-type cells (Supplemental Figure S2A). Hmg1p was previously shown to integrate in an SRP-dependent manner (Stirling *et al.*, 1992); hence this result confirms that SRP targeting is compromised in *RPL25GFP* cells.

To test if this translocation defect was specific to the cotranslational pathway, we used a posttranslational reporter based on CPY. Unlike *PHO8-URA3*, the *CPY-URA3* reporter did not lead to uracil prototrophy in *RPL25GFP* cells (Figure 1B). As expected, a *sec62-1* mutant carrying the *CPY-URA3* reporter became Ura⁺ (Ng *et al.*, 1996), indicating that the posttranslational translocation pathway is intact in *RPL25GFP* cells.

To confirm these results, we monitored translocation directly by pulse-labeling cells with [35 S]methionine and immunoprecipitating with antibodies to specific substrates. Translocation was monitored by changes in SDS-PAGE migration due to the addition of N-linked glycans and signal-sequence cleavage upon exposure of the substrates to the ER lumen. In wild-type cells, a Pho8p-Ura3p-myc fusion, engineered with a single N-glycosylation site, was almost completely glycosylated, indicating efficient integration (Figure 1C; Wilkinson *et al.*, 2006). In contrast, in the *RPL25GFP* strain, a considerable amount of nonglycosylated (nontrans-

Figure 2. 60S subunit biogenesis defects do not affect cotranslational translocation. (A) Extracts from MPY69 ($\Delta rpl25[pRPL25]$), $RPL25GFP$ (MPY6) and $\Delta rpl39$ cells were centrifuged on 10–50% linear sucrose gradients and fractionated with continuous monitoring of A_{254nm} . Positions of 40S, 60S, 80S, and polysomes are indicated along with the presence of halfmers (*). (B) BY4742 (wild-type), $\Delta rpl39$, and $RPL25GFP$ cells, carrying the $PHO8-URA3$ reporter plasmid (pMP234), were plated on synthetic media lacking either leucine (-leu) or leucine and uracil (-leu -ura).

located) precursor accumulated, as observed in SRP mutant (*sec65-1*) and *sec61-3* strains (Figure 1C). Furthermore, accumulation of nonglycosylated (nontranslocated) Hmg1-Suc2-Ura3p was also detected under steady-state conditions in $RPL25GFP$ cells (Supplemental Figure S2B).

In both wild-type and $RPL25GFP$ cells, only the signal-sequence-cleaved and glycosylated (p1) form of CPY was observed (Figure 1D). In contrast, a *sec61-3* strain revealed considerable accumulation of unprocessed, and hence non-translocated, CPY. These results confirm that $RPL25GFP$ strains have a specific defect in co- but not posttranslational translocation.

Defects in 60S Subunit Biogenesis Do Not Account for Cotranslational Translocation Phenotypes

Considering the central role that the L23 family of ribosomal proteins plays in ribosome assembly (Kooi *et al.*, 1994), we were concerned that the $RPL25GFP$ cells might have defects in 60S assembly and that this might indirectly lead to translocation defects. Polysome analysis of wild-type and $RPL25GFP$ strains revealed a minor defect in 60S subunit assembly as indicated by the presence of halfmers, small shoulders to the 80S and polysomal peaks. These are the

Figure 3. Compromised NAC function does not lead to cotranslational targeting defects. (A) Yeast strains BY4742 (wild-type), $\Delta egd1$, $\Delta egd2$, $\Delta btt1$, and $RPL25-GFP$, all transformed with the $PHO8-URA3$ reporter plasmid (pMP234), were streaked in parallel on synthetic media lacking either leucine (-leu; selects for plasmid) or leucine and uracil (-leu -ura; selects for reporter) and incubated for 2 and 4 d, respectively, at 30°C. (B) Yeast strains BY4742 (wild-type), $\Delta egd1$, $\Delta egd2$, $\Delta btt1$, $\Delta zuo1$, and $RPL25-GFP$, all transformed with the $PHO8-URA3-myc$ reporter plasmid (pMP220), were pulse labeled for 5 min with [^{35}S]methionine at either 30°C and lysed, and then radiolabeled proteins were immunoprecipitated with α -myc antibodies and analyzed by SDS-PAGE and phosphorimaging. Positions of g-Pho8-Ura3p (membrane integrated) and Pho8p-Ura3p (cytosolic) are indicated.

result of recruitment of the small subunit to the mRNA, but subsequent failure of a 60S subunit to join, because of a lack of 60S subunits, leaving the 40S subunit stalled at the AUG codon. This mild 60S biogenesis defect may explain the slightly lower labeling efficiency of Pho8-Ura3p in the pulse assays and may also contribute to the slower growth phenotype.

To test if the biogenesis defect could indirectly compromise translocation, we made use of a $\Delta rpl39$ strain, which is viable but is known to have a 60S subunit biogenesis defect (Sachs and Davis, 1990). This was confirmed by the significant degree of halfmer formation shown in the polysome profile of $\Delta rpl39$ cells. We therefore tested $\Delta rpl39$ strains using the reporter assays; unlike $RPL25GFP$ strain, neither the $PHO8-URA3$ (Figure 2B) nor $CPY-URA3$ (not shown) reporter led to growth in the absence of uracil. This indicates that a subunit biogenesis defect is unlikely to be the explanation of the cotranslational translocation defect in the $RPL25GFP$ strain.

Loss of NAC Function Does Not Rationalize $RPL25GFP$ Translocation Defect

A recent report identified Rpl25p as a component of the docking site for the nascent polypeptide chain-associated complex (NAC; Wegrzyn *et al.*, 2006). Experiments in mammalian systems have implicated a potential role for NAC in modulating ER protein targeting (Wiedmann *et al.*, 1994). We therefore wanted to exclude the possibility that the Pho8p translocation defects in a $RPL25GFP$ strain may be due to disruption of NAC function. Hence, we again used the reporter assay to assess whether strains deleted for components of NAC resulted in translocation defects. Unlike, $RPL25GFP$, deletion of any of the components of NAC ($\Delta egd1$, $\Delta egd2$, and $\Delta btt1$) failed to produce a Ura^+ phenotype in the $PHO8-URA3$ reporter assay (Figure 3A). To exclude the possibility that a translocation phenotype was masked due to misfolding of the Ura3p reporter as a conse-

Figure 4. *RPL25GFP* cells have defects in SRP-ribosome binding. (A) Cell extracts from wild-type and *RPL25GFP* cells (1 OD₆₀₀) were solubilized with 1% CHAPS under high-salt (500 mM KOAc) conditions. After removal of unsolubilized material, ribosomes were isolated by centrifugation at 200 000 × *g*, and the total, pellet, and supernatant fractions were then analyzed by Western blot with Sec61p, Srp72p, and Rps3p antisera. (B) Ribosomal fractions from wild-type, *RPL25GFP*, and *sec65-1* cells shifted to 37°C for 3 h, were prepared as in A; equivalent amounts of total extracts and ribosomal pellets were analyzed by Western blot with Sec61p and Rps3p antisera.

quence of the loss of NAC, we also monitored translocation of the Pho8-Ura3p fusion directly by pulse labeling (Figure 3B). In contrast to *RPL25GFP* cells, all of the NAC mutants displayed translocation efficiencies similar to wild-type cells.

RPL25GFP Mutant Affects Ribosome Association of SRP But Not the Sec61p Complex

We next wanted to assess whether the presence of the C-terminal GFP moiety affected the interaction of ribosomes with components of the targeting and translocation machinery. We therefore grew wild-type and *RPL25GFP* cells to midlog phase and then lysed them in the presence of the mild detergent CHAPS and high-salt conditions that preserve the interaction of programmed ribosomes with the translocon (Panzner *et al.*, 1995). The resulting extracts were used to prepare a ribosomal fraction and postribosomal supernatant by ultracentrifugation. Blotting for the SRP component Srp72p revealed a considerable proportion of the protein associated with ribosomes in wild-type cells (Figure 4A). However, in the *RPL25GFP* strain there was a substantial reduction of Srp72p in the ribosome-bound fraction. In contrast, ribosome association of Sec61p was only modestly reduced in *RPL25GFP* strains compared with wild type. To test if this reduction in association of Sec61p is a consequence of the compromised SRP function, we assessed the association of Sec61p with ribosomes in a *sec65-1* strain shifted to the nonpermissive temperature for 3 h (Figure 4B). This led to a greater reduction in Sec61p association with the ribosome, suggesting that loss of SRP binding is likely the

prime cause of the reduction in Sec61p ribosome-association in the *RPL25GFP* strain.

Taken together, these results suggest that in *RPL25GFP* cells, SRP fails to bind efficiently to ribosomes and is the major cause of the translocation defect.

RPL25-GFP Mutant Shows Up-Regulation of Stress-induced Chaperones

While analyzing the *RPL25GFP* strain, we noticed that a number of proteins with masses of 60–100 kDa were more abundant in total protein extracts (Figure 5A). These changes were not due to 60S biogenesis defects because they were not observed with *Δrpl39*. Four of these proteins were identified using mass spectrometry as Hsp104p, Sti1p, Ssa1/2p, and Hsp60p. Up-regulation of Ssa1/2p in the *RPL25GFP* strain was confirmed by Western blot (Figure 5B). All these proteins fall into the class of stress-induced chaperones and possess HSF elements in their promoter regions, which mediate their activation upon the accumulation of unfolded cytosolic proteins (Sorger, 1991; Albanese *et al.*, 2006).

This effect is strikingly similar to the adaptation process that occurs upon SRP depletion, where the very same chaperones are up-regulated (Arnold and Wittup, 1994; Mutka and Walter, 2001). Hence, the fact that *RPL25GFP* cells exhibit a similar up-regulation of these chaperones is consistent with the disruption of cotranslational targeting and SRP-ribosome association.

Disruption of the ribosome-associated chaperone complex (RAC) also leads to heat-shock chaperone induction (Al-

Figure 5. *RPL25GFP* cells show up-regulation of heat-shock chaperones. (A) Total cell extracts, prepared from wild-type (*RS453*), $\Delta rpl39$, and *RPL25GFP* cells were analyzed by SDS-PAGE and stained with Coomassie brilliant blue. Indicated bands (*) were excised and identified by mass spectrometry as follows: Hsp104p (accession no. P31539; 26 matching peptides), Sti1p (P15705; 18 peptides), Ssa1p (P10591; 28 peptides), Ssa2p (P10592; 31 peptides), and Hsp60p (P19882; 21 peptides). (B) Extracts from wild-type (*RS453*) and *RPL25GFP* cells were analyzed by Western blot with Ssa1p, Zwf1p, and Rps3p antibodies. (C) Fivefold serial dilutions of *RPL25-GFP*, $\Delta zuo1$ strains and isogenic wild-type strains (*RS453* and *BY4742*, respectively) were spotted on to YPD or YPD supplemented with 1 mg $\cdot$ ml $^{-1}$ AZC and incubated for 2 and 3 d, respectively, at 30°C.

banèse *et al.*, 2006), so it is feasible a disruption of RAC activity by Rpl25GFP could rationalize chaperone up-regulation. To test if RAC function is compromised, we assessed whether *RPL25GFP* cells are sensitive to the proline analogue AZC, a hallmark of mutants lacking RAC components (Albanèse *et al.*, 2006). In contrast to a $\Delta zuo1$ mutant, *RPL25GFP* cells were not sensitive to AZC, suggesting RAC function is not grossly disrupted in *RPL25GFP* cells (Figure 5C). Conversely, $\Delta zuo1$ mutants do not show cotranslational targeting defects (Figure 3B).

Overexpression of SRP Partially Rescues Growth and Translocation Phenotypes

If the presence of the GFP moiety reduces the affinity of SRP for the ribosome, we reasoned that overexpression of SRP may be able to suppress the growth defects of the *RPL25GFP* strain. We therefore transformed wild-type and *RPL25GFP* cells with the multicopy plasmids pMW295 and pMW299, which lead to overexpression of complete SRP (Willer *et al.*, 2003).

In wild-type cells, there was no difference in growth between cells overexpressing SRP and cells transformed with the equivalent empty vectors (Figure 6, A and B). However, overexpression of SRP led to a marked increase in growth rate of the *RPL25GFP* strain compared with the vector control. This indicates that the growth defect of the *RPL25GFP* strain is, at least in part, due to defects in translocation and in particular SRP ribosome binding.

We next assessed the level of association of SRP with the ribosome in the *RPL25GFP* strain in the presence of SRP overexpression. In the absence of overexpression, association of SRP with the ribosome was barely detectable (Figures

Figure 6. SRP overexpression partially rescues *RPL25GFP* phenotypes. (A) Wild-type (*RS453*) and *RPL25GFP* cells transformed with pMW295 and pMW299 (+SRP $\uparrow$) or pRS425 and pRS426, were grown to early log phase in minimal media lacking leucine and uracil (-leu -ura) and diluted to an OD₆₀₀ of 0.1, and then fivefold serial dilutions were spotted on -leu -ura plates and grown for 3 d at 30°C. (B) The same strains as in A were grown at 30°C in liquid minimal media lacking leucine and uracil to midlog phase, diluted in the same media to an OD₆₀₀ of 0.1, and then growth monitored over time. (C) Ribosome pellet fractions (prepared as in Figure 4A) from wild-type (*RS435*) and *RPL25GFP* cells, both transformed with pRS425 and pRS426, as well as *RPL25GFP* cells transformed with pMW295 and pMW299 (+SRP $\uparrow$), were analyzed by Western blot with Srp72p and Rps3p antisera. (D) The same strains as in C were pulse-labeled with [35 S]methionine (as in Figure 1C) and immunoprecipitated with anti-DPAP B antibodies. g-DPAP B, glycosylated DPAP B; DPAP B, nonglycosylated DPAP B.

4A and 6C). Overexpression of SRP restored ribosome binding to levels similar to that of wild-type cells (Figure 6C). To test the effect of overexpression on translocation directly, we monitored processing of the endogenous SRP-dependent substrate DPAP B by pulse labeling (Figure 6D). The *RPL25GFP* strain revealed a marked accumulation of nonglycosylated DPAP B, which was completely absent in the wild-type strain. Overexpression of SRP in *RPL25GFP* cells led to a significant rescue of the translocation phenotype. However, it is noteworthy that a proportion of unprocessed DPAP B was still observed. Therefore, as with the growth defect, the rescue of the translocation defect is incomplete.

DISCUSSION

Here we have shown that the addition of GFP to the C-terminus of Rpl25p results in a specific defect in cotranslational translocation. Analysis of reporter constructs composed of fusions of Ura3p to either Pho8p or the first six TMDs of Hmg1p both reveal a translocation defect as does the endogenous substrate DPAP B. All three of these proteins are well-characterized precursors, which exclusively utilize the SRP-dependent targeting pathway (Stirling *et al.*, 1992; Ng *et al.*, 1996). In contrast, a CPY-Ura3p fusion, which utilizes the posttranslational pathway (Ng *et al.*, 1996) translocates normally in this strain.

Perhaps surprisingly, considering the critical role of the L23 family of proteins in ribosome assembly, *RPL25GFP*

cells have only a very minor defect in 60S subunit biogenesis. It is therefore unlikely that a defect in subunit biogenesis indirectly causes compromised translocation. This is borne out by the fact that $\Delta rpl39$ cells, which have a well-characterized 60S subunit defect (Sachs and Davis, 1989) also translocate SRP-dependent substrates normally. Interestingly, Rpl39p is one of the three proteins that line the inside of the ribosomal exit tunnel (Nissen *et al.*, 2000), and recent data have indicated a function for these ribosomal proteins in recognizing features of the nascent chain during membrane protein integration (Woolhead *et al.*, 2004). However, our data suggest that, at least for integration of a single spanning type II membrane protein such as Pho8p, recognition events involving Rpl39p are not essential.

The simplest explanation for the translocation defect we observe in *RPL25GFP* cells is that the GFP moiety occludes Rpl25p such that targeting factors are sterically hindered from accessing the protein. The fact that a small FLAG tag added to the C-terminus of Rpl25p leads to no impairment of growth indicates that a free C-terminus is not essential for function (Inada *et al.*, 2002). Our data indicate that there is a clear defect in SRP interaction with the ribosome in the *RPL25GFP* strain. Cryo-EM studies indicate that mammalian SRP makes six contacts with the ribosome; however, the major contact is centered on Rpl25p and involves the tip of the N-domain of SRP54 (Pool *et al.*, 2002; Halic *et al.*, 2004). Furthermore, this interaction is conserved in the bacterial SRP-ribosome complex (Halic *et al.*, 2006a; Schaffitzel *et al.*, 2006) and so is also highly likely to occur in the analogous yeast complex. We presume that the presence of GFP destabilizes this interaction and this has a major impact on SRP ribosome binding. Overexpression of SRP is able to restore SRP binding to close to wild-type levels, and this suggests that the GFP moiety may diminish the affinity of SRP for the ribosome but does not abolish it completely. It is most likely that at elevated cellular concentrations of SRP, the remaining contacts are sufficient to support binding. The fact that SRP overexpression also significantly improves growth of the *RPL25GFP* strain indicates that the translocation defect is the prime cause of the slow growth phenotype. Moreover, if defective ribosome binding via Rpl25p of alternate nascent chain-interacting factors such as chaperones was the cause of the growth phenotype, one might well expect that SRP overexpression would exacerbate rather than relieve the growth defect.

Overexpression of SRP, although able to restore binding of SRP to the ribosome, is not sufficient to completely rescue growth and translocation phenotypes. This may indicate that Rpl25p could play additional roles in the translocation reaction further to recruitment of SRP, for example, in triggering conformational changes in SRP54 that are known to occur upon ribosome binding and are thought to prime it for signal sequence and SR interaction (Bacher *et al.*, 1996; Halic *et al.*, 2004; Wild *et al.*, 2004).

RPL25GFP cells show a marked up-regulation of cytosolic stress-inducible chaperones such as Ssa1/2p and Hsp104p. This is strikingly reminiscent of the adaptation process that occurs upon depletion of SRP components (Arnold and Wittrup, 1994; Mutka and Walter, 2001). Hence this result is in good agreement with the observed disruption of the SRP-ribosome interaction. Induction of these chaperones most likely helps prevent aggregation in the cytosol of nontranslocated cotranslational precursors and/or helps redirect these precursors to the posttranslational pathway. Indeed the *RPL25GFP* strain may have undergone a similar adaptation as SRP deletion strains, allowing continued growth in the absence of efficient SRP-dependent targeting.

Disruption of the ribosome-associated chaperone complex, RAC, is also known to trigger induction of stress-inducible chaperones (Albanèse *et al.*, 2006). The RAC complex binds to the ribosome and modulates the interaction of Ssb1/2p with newly synthesized nascent chains (Pfund *et al.*, 1998; Gautschi *et al.*, 2001, 2002). Thus, it is quite possible that RAC may bind to the ribosome via Rpl25p and a disruption of this interaction could inhibit RAC function leading to an alternative explanation for the chaperone induction. However, we think this scenario is unlikely for two reasons: First, we show that *RPL25GFP* cells do not display similar phenotypes as mutants in RAC components, e.g., sensitivity to AZC (Figure 5C). Second, it has been recently reported that ribosome association of the RAC Hsp70 Ssb1/2p is normal in *RPL25GFP* cells (Wegrzyn *et al.*, 2006).

Ribosome binding of another chaperone, NAC, has also been shown to be disrupted in the *RPL25GFP* strain (Wegrzyn *et al.*, 2006). It was therefore important to verify that the observed targeting defects are not triggered indirectly by a defect in ribosome-binding of NAC. Using our reporter assays, we were unable to detect any defect in cotranslational targeting in strains deleted for NAC components, also in agreement with previous reports (Reimann *et al.*, 1999). Hence although NAC has been proposed to play a role in the fidelity of targeting, we could find no defects in SRP targeting in the absence of NAC. It therefore seems very unlikely the *RPL25GFP* translocation phenotypes are mediated via NAC.

Association of ribosomes with the Sec61p complex, the major ER ribosome receptor (Kalies *et al.*, 1994), appeared not to be strongly affected by the presence of the GFP moiety. The modest decrease in Sec61p binding is likely a consequence of the defect in SRP function, as a *sec65-1* mutant also induces a similar effect.

The fact that SRP and Sec61p appear differentially sensitive to the presence of the GFP moiety suggests the two complexes may interact distinctly with Rpl25p. A similar scenario is known to occur in bacteria where SRP and Trigger Factor can apparently both bind the Rpl25p homologue, L23, simultaneously, indicating distinct binding sites (Buskiewicz *et al.*, 2004). Alternatively, Rpl25p may be less critical for binding the Sec61p complex to the ribosome, and other contacts, involving rRNA and other ribosomal proteins, may be able to compensate (Beckmann *et al.*, 2001; Menetret *et al.*, 2005). Indeed biochemical studies implicate rRNA elements in translocon binding (Prinz *et al.*, 2000).

In conclusion our results indicate that access to universal ribosomal adaptor protein, Rpl25p, is critical for SRP binding and that this interaction is important for cotranslational ER protein targeting in vivo.

ACKNOWLEDGMENTS

We are grateful to Randy Schekman (University of California, Berkeley, CA), Peter Walter, Colin Stirling, Jeremy Brown (University of Newcastle, Newcastle, United Kingdom), Bernd Bukau (Zentrum für Molekulare Biologie, University of Heidelberg, Heidelberg, Germany), and Ed Hurt for the generous gift of strains, plasmids, and antibodies. Biomolecular Analysis Core Facility (University of Manchester) provided support for protein identification. We thank Colin Stirling and Martin Lowe for critical comments on the manuscript. This work was supported by a grant from the Biotechnology and Biological Sciences Research Council.

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