Association of Protein Biogenesis Factors at the Yeast Ribosomal Tunnel Exit Is Affected by the Translational Status and Nascent Polypeptide Sequence*

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Ribosome-associated protein biogenesis factors (RPBs) act during a short but critical period of protein biogenesis. The action of RPBs starts as soon as a nascent polypeptide becomes accessible from the outside of the ribosome and ends upon termination of translation. In yeast, RPBs include the chaperones Ssb1/2 and ribosome-associated complex, signal recognition particle, nascent polypeptide-associated complex (NAC), the aminopeptidases Map1 and Map2, and the N-terminal acetyltransferase NatA. Here, we provide the first comprehensive analysis of RPB binding at the yeast ribosomal tunnel exit as a function of translational status and polypeptide sequence. We measured the ratios of RPBs to ribosomes in yeast cells and determined RPB occupation of translating and non-translating ribosomes. The combined results imply a requirement for dynamic and coordinated interactions at the tunnel exit. Exclusively, NAC was associated with the majority of ribosomes regardless of their translational status. All other RPBs occupied only ribosomal subpopulations, binding with increased apparent affinity to randomly translating ribosomes as compared with non-translating ones. Analysis of RPB interaction with homogeneous ribosome populations engaged in the translation of specific nascent polypeptides revealed that the affinities of Ssb1/2, NAC, and, as expected, signal recognition particle, were influenced by the amino acid sequence of the nascent polypeptide. Complementary cross-linking data suggest that not only affinity of RPBs to the ribosome but also positioning can be influenced in a nascent polypeptide-dependent manner.

Newly synthesized polypeptides exit the ribosome through a tunnel in the large ribosomal subunit. As soon as the polypeptides reach the tunnel exit, important decisions are required to direct subsequent steps of protein biogenesis. In all kingdoms of life a specific set of ribosome-associated protein biogenesis factors (termed RPBs2 hereafter) is mandatory for the process. However, RPBs differ significantly between bacterial and eukaryotic cells. Eubacteria possess trigger factor, a chaperone involved in cotranslational protein folding, which is restricted to eubacteria and signal recognition particle (SRP), a targeting factor involved in the translocation of membrane proteins with a hydrophobic signal-anchor sequence (1, 2). Consistent with the function of a general chaperone, trigger factor and ribosomes form 1:1 complexes whereas bacterial SRP, which is required for the biogenesis of only a subset of newly synthesized proteins, is present at ~1 molecule/100 ribosomes (1, 3). Notably, trigger factor and SRP bind to the same region close to the exit of the ribosomal tunnel (1, 2). The current view is that trigger factor and SRP can bind simultaneously to a single ribosome (1); however, it was suggested that only one at a time contacts a nascent polypeptide (4). According to this model, the decision-making process at the eubacterial tunnel exit would be straightforward: Whether trigger factor or SRP act on a nascent polypeptide depends on their relative affinities to the exposed stretches of amino acids (Refs. 4, 5 and references therein).

In eukaryotes, the situation is by far more complex and less well understood. In yeast a number of functionally diverse RPBs have been identified: Eukaryotic SRP (6), nascent polypeptide-associated complex (NAC) (7), the Hsp70 homolog Ssb1/2 (8), ribosome-associated complex (RAC) consisting of the Hsp40 zuotin (9) and the Hsp70 Ssz1 (10), two methionine aminopeptidases Map1 (11) and Map2 (Fig. 2), and the N-terminal acetyltransferase NatA (12) (for reviews see Refs. 2, 13–16). In a nutshell, SRP binds to signal sequences of endoplasmic reticulum (ER)-targeted proteins as they emerge from the ribosome and is essential for cotranslational translocation across the membrane (2, 13). The role of NAC is only partly understood; however, NAC displays some chaperone-like properties and might be involved in preventing mistargeting of proteins to the ER (17–19). Ssb1/2 and RAC are functionally interacting chaperones (20–23), Map1 and Map2 catalyze the essential removal of the initiator methionine from a specific set of nascent polypeptides (24), and finally, NatA is responsible for the cotranslational acetylation of N-terminal serine, alanine, thre-
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online, and glycine exposed after methionine cleavage. These modifications occur on the vast majority of newly synthesized polypeptides (12, 25).

For sterical considerations it is difficult to envisage that the full set of eukaryotic RPBs interacts simultaneously with one ribosome. In addition, the time frame for the action of RPBs on nascent polypeptides is only short. Logarithmically growing yeast cells translate with a speed of \( \sim 10 \) amino acids/second (26), and thus for the majority of polypeptides cotranslational actions have to be completed in significantly less than a minute. How the arrangement of RPBs at the ribosomal tunnel exit is functionally coordinated in time and space is one of the challenging questions. A prerequisite to understanding the dynamics is information about the interaction of RPBs with ribosomes as a function of translational status and polypeptide sequence. Although the problem is straightforward, the methods to pinpoint RPB dynamics are not. Analysis requires a uniform population of non-translating ribosomes, as well as defined ribosome nascent chain complexes (RNCs) in quantities that allow for immunodetection of RPBs. Moreover, the concentration of a significant number of proteins has to be analyzed in complex mixtures. We have developed the tools and have performed the first thorough investigation of RPB-ribosome interaction under physiological conditions. To that end, we have employed a homologous system in which all RPBs, ribosomes, and RNCs including nascent polypeptides were derived from yeast. Experimental conditions were chosen such that the ratios between different RPBs and between ribosomes and RNCs were the same as in intact cells. We regard this as important, as it was shown in the Escherichia coli system that the normal ratio of cytosolic components is critical for the delicate balance of nascent polypeptide interactions (5). The approach allowed us to study the interaction of the whole set of RPBs with non-translating ribosomes, randomly translating ribosomes, and specific RNCs under conditions that resemble, as closely as possible, an intact cell.

**EXPERIMENTAL PROCEDURES**

**Construction of Plasmids**—Genes encoding Rpl17a, Rpl39, Rps9a, Ascl, Srp54, Ard1, Nat1, Map1, Map2, and Ssb1 were amplified from yeast genomic DNA. In case of Asc1, Rps9a, and Ssb1, the single intron was removed prior to transfer to the expression vector. For expression in E. coli, all genes were fused to the N-terminal hexahistidin test (His\(_6\)) contained in pET28a (Novagen). Genes encoding Dap2 (yeast dipeptidyl aminopeptidase-B), ppa-factor (yeast prepro \( \alpha \)-factor), andPgk1 (yeast 3-phosphoglycerate kinase) were amplified from genomic DNA and were cloned into the transcription/translation vectors pSPUTK (Stratagene), pSP64, or pSP63 (Promega), resulting in plasmids pSPUTK-Dap2, pSPUTK-FLAG-Dap2, pSPUTK-Pgk1, pSPUTK-FLAG-E-Pgk1, pSPUTK-FLAG-E-ppa, pSPUTK-Dap2-E(2)K, pSP64-Pgk1-S(2)K, and pSP65-ppa-R(2)K. N-terminal FLAG tags (DYKDDDDK) and lysines were introduced via the forward primer as indicated. In the FLAG-tagged versions of Pgk1 and ppa-factor the first amino acid after the tag was converted to glutamate.

**Purification of His\(_6\)-tagged Standard Proteins**—Proteins were purified using nickel-nitrilotriacetic acid according to the manufacturer’s protocol for native or denatured protein purification, respectively (Qiagen). His\(_6\)-Rpl17a, His\(_6\)-Rpl39, His\(_6\)-Nat1, His\(_6\)-Map1, and His\(_6\)-Ssb1 were further purified by extraction from a preparative 10 or 16% (for Rpl39) Tris-Tricine gel (27). To that end, protein bands were cut, homogenized in 1× cathode buffer (0.1 M Tris-HCl, pH 8.25, 0.1 M Tricine, 0.1% SDS), and were finally precipitated by adding 2 volumes of ice-cold acetone. Pellets were solubilized in 50 mM Tris-HCl, pH 8.0, 8 M urea. His\(_6\)-Nat1 was insoluble in 8 M urea and was resolved in 20 mM Tris-HCl, pH 6.8, 1% SDS, 10% glycerol. Purification procedures for NAC and RAC have been reported elsewhere (10, 17).

**Determination of Protein Concentrations**—Protein concentrations were determined according to the manufacturers’ manuals, with bovine serum albumin as a standard by the Bradford assay (Bio-Rad), the BCA assay (Sigma), and the DC protein assay (Bio-Rad) or were calculated from absorption at 280 nm (supplemental Table S1).

**Antibodies and Immunoblotting Procedures**—Polyclonal antibodies were raised in rabbits (EUROGENTEC, Bel S. A.). Antibodies directed against the antigens Rpl17a, Rps9a, and Ssb1 also recognized the functionally redundant homologs Rpl17b (99% identical to Rpl17a), Rps9b (97% identical to Rps9a), and Ssb2 (99% identical to Ssb1), respectively. Concentrations throughout the study relate to the overall concentration of Rpl17a/Rpl17b (Rpl17), Rps9a/Rps9b (Rps9), and Ssb1/Ssb2 (Ssb1/2). Immunoblots were developed using ECL with horseradish peroxidase-conjugated goat anti-rabbit IgG (Pierce) as the secondary antibody or with 125I-labeled protein A (28). For ECL detection membranes were incubated for 1 min in 100 mM Tris-HCl, pH 8.6, either in the presence of 1× reagent (0.2 mM p-cumaric acid in Me\(_2\)SO, 1.2 mM luminol sodium salt in Me\(_2\)SO, 0.01% H\(_2\)O\(_2\)) in case of quantifications of purified RNCs, or with 0.5× reagent for all other immunoblots. Quantifications were performed using the AIDA ImageAnalyzer (Raytest).

**Quantification of Ribosomes and RPBs in Yeast Cells**—Total yeast extract was prepared by the method of Yaffe and Schatz (29) from log-phase (\( A_{600} = 0.7–1.2 \)) wild type yeast strain MH272-3fa (23) grown on YPD (1% yeast extract, 2% peptone, 2% glucose). Cell numbers were determined using a Neubauer improved counting chamber (Marienfeld). MH272–3fa of an \( A_{600} = 1 \) contained 4.48 × 10\(^7\) cells/ml. The concentration of ribosomes and RPBs/cell was calculated from the molar protein concentrations/ml divided by the cell number/ml.

**In Vitro Transcription and Translation**—Yeast translation extracts were prepared as previously described (30) from strain JK9–3dx (31). RNCs were generated as previously described (17). Templates for transcription reactions were generated by PCR using one of the following plasmids as a template: pSPUTK-Dap2, pSPUTK-FLAG-Dap2, pSPUTK-Pgk1, pSPUTK-FLAG-E-Pgk1, pSPUTK-FLAG-E-ppa, or pSPUTK-Dap2-E(2)K, pSP64-Pgk1-S(2)K, and pSP65-ppa-R(2)K. The concentration of nascent polypeptides was determined using a Neubauer improved counting chamber (Marienfeld).

**Cross-linking of Nascent Polypeptides to RPBs**—The homobifunctional cross-linker bis-(sulfosuccinimidyl)-suberate (BS\(^{3}\))

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was used for cross-linking reactions (spacer length, 1.14 nm; Pierce). Cross-linking reactions and immunoprecipitations under denaturing conditions were performed as previously described (20). aMap2 did not efficiently immunoprecipitate Map2. We have therefore not tested for Map2 cross-links. All other RPBs were tested (see “Results”).

**Purification of FLAG-tagged RNCs under Native Conditions**—For a typical experiment 75-μl translation reactions were performed at 20 °C for 80 min and were terminated by the addition of cycloheximide to a final concentration of 200 μg/ml. Translation reactions were then added to 40 μl of ANTI-FLAG® M2 affinity gel (aFLAG-beads; Sigma) resuspended in 500 μl of immunoprecipitation buffer (20 mM HEPES-KOH, pH 7.4, 150 mM potassium acetate acetate, 2 mM magnesium acetate, 50 μg/ml trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor mix: 1.25 μg/ml leupeptin, 0.75 μg/ml antipain, 0.25 μg/ml chymostatin, 0.25 μg/ml elastatin, 5 μg/ml pepstatin A). Native immunoprecipitation reactions were incubated for 4 h at 4 °C on a shaker. The beads were separated from the supernatant by centrifugation and were washed twice with 500 μl of ice-cold immunoprecipitation buffer. Immunoblotting confirmed that RPBs were not lost during the washes (data not shown). Washed aFLAG beads were incubated in SDS-PAGE sample buffer for 10 min at 95 °C, and aliquots and standard proteins were run on the same 10% Tris-Tricine gels. PAGE sample buffer for 10 min at 95 °C, and aliquots and standard proteins were run on the same 10% Tris-Tricine gels. Non-tagged versions of each nascent polypeptide were trans-

**Generation of Non-translating and Translating Ribosomes**—Efficient puromycin release requires conditions of high ionic strength (32), which interfere with ribosome association of RPBs. To prevent such release of RPBs from ribosomes we have made use of the observation that non-translating ribosomes are efficiently generated in vivo when glucose is removed from the growth medium (33). For the analysis of RPB interaction with randomly translating ribosomes and non-translating ribosomes, cultures of MH272-3fα were grown to an A600 of 0.4 at 30 °C on YPD, collected, and resuspended in YPD or in YP medium lacking glucose. Growth was resumed for 10 min at 30 °C. Cells were harvested in the presence of 100 μg/ml cycloheximide to stabilize translating ribosomes. Preparation of cell extract was carried out by glass bead disruption in 20 mM HEPES-KOH, pH 7.4, 100 mM potassium acetate acetate, 2 mM magnesium acetate, 100 μg/ml cycloheximide, 0.5 mM dithiothreitol as described (33). Of each lysate 10 A260 units were loaded onto a 10.8-ml 15–55% linear sucrose gradient and centrifuged for 2.5 h at 200,000 × g. Gradients were fractionated from top to bottom with a density gradient fractionator (Teledyne Isco, Inc.) monitoring A254.

**RESULTS**

**RPB Concentration in Yeast Cells**—Quantification of untagged RPBs and ribosomes in complex mixtures requires quantitative immunoblotting. We have heterologously expressed and puri-

**FIGURE 1. Quantification of RPBs and ribosomal proteins.** A, purified RPBs and ribosomal proteins were used as standard proteins. Each 1 μg of the purified protein was separated on a 10% Tris-Tricine gel followed by Coomasie staining. Heterodimeric RAC and NAC were purified from *Saccharomyces cerevisiae*. The Srp54 subunit of SRP and the Nat1 and Ard1 subunits of NatA, Map1, Map2, and Ssb1 were expressed as His6-tagged versions in *E. coli*. For the quantification of ribosomes two proteins of the small ribosomal subunit (Rps39 and Rps9a) and two proteins of the large subunit (RP17 and Rpl17a) were expressed as His6-tagged versions in *E. coli*. For details see “Experimental Procedures.” B, quantification via immunoblotting. Total cell extract corresponding to 0.6–2.4 × 10^10 cells of logarithmically growing wild type yeast was separated on 10% Tris-Tricine gels. Standard proteins were applied to the same gel and were analyzed by immunoblotting using antibodies specifically recognizing the proteins of interest. As an example, immunoblots for the quantification of Ssb1/2, Rpl17, and Srp54 are shown. Note that the purified, His6-tagged standard proteins have a slightly higher molecular mass. C, calibration curves. Densitometric analysis was performed to determine the range of linearity for each standard and to quantify protein concentrations in the total cell extract. A summary of the results is shown in Table 1 and in Fig. 3B.
dynamic cycling on and off ribosomes for all RPBs with the exception of NAC and Ssb1/2 (see below).

**Qualitative Analysis of RPB Interaction with Randomly Translating Ribosomes**—Association of RPBs with ribosomes and polysomes in total cell extract can be qualitatively demonstrated by sucrose density centrifugation (Fig. 2A). Resulting ribosome profiles have been previously employed to demonstrate ribosome association of Ssb1/2 (8, 18, 34–36), RAC (9, 18, 36), NAC (18, 37), and Map1 (11). Although the studies agree on ribosome association of RPBs, the extent varies significantly (e.g. association of zuotin in Refs. 9 and 36 or NAC in Refs. 37 and 18). The variability most likely reflects differences in extract preparation and buffer composition and complicates a comparative evaluation of existing data. Moreover, ribosome profiles of Map2 and yeast SRP have not previously been published. We have now revisited the issue and have analyzed in parallel the distribution of the complete set of RPBs in a polysome-rich extract (Fig. 2B). Gentle extract preparation and physiological salt concentrations revealed that the bulk of NAC, RAC, NatA, Map1, and Map2 was bound to polysomes and 80 S ribosomes. As reported previously, Ssb1/2 (8) was abundant also in the cytosolic fraction. SRP was the only other RPB detected in the cytosolic fraction in significant amounts (Fig. 2B). After release from ribosomes, RPBs colocalized with soluble cytosolic proteins, confirming that comigration in the profiles was due to ribosome association (data not shown).

**Quantitative Analysis of RPB Interaction with Non-translating and Randomly Translating Ribosomes**—The ratio of ribosomes to RPB in each fraction is a measure of how many ribosomes are occupied by a particular RPB. To analyze the effect of the general translational status on these interactions, we have determined the ratio between ribosomes and RPBs in polysomal fractions as well as in fractions containing non-translating ribosomes (Fig. 3A). On average, 88% of non-translating ribosomes were occupied by NAC, 19% by RAC, 15% by Ssb1/2, 2% by Map1, 2% by NatA, and 1% by Map2. SRP was not detected in fractions containing non-translating ribosomes. 89% of randomly translating ribosomes were occupied by NAC, 35% by RAC, 30% by Ssb1/2, 4% by Map1 and NatA, 2% by Map2, and 1% by SRP (Fig. 3B). In general, RPBs displayed a preference for translating ribosomes over non-translating ribosomes, which is consistent with their function. An exception was NAC, which occupied even non-translating ribosomes to a large extent. Please note that Ssb1/2, which approximately equals the number of ribosomes in total extract, occupied only about one third of ribosomes involved in translation (see also “Discussion”).

**RPB Interaction with RNCs Carrying Either Cytosolic or ER-targeted Nascent Polypeptides**—Polysomes carry an undefined mixture of nascent polypeptides with respect to amino acid sequence. To assess how RPB binding was affected in the presence of specific nascent polypeptides, we generated RNCs engaged in the translation of particular nascent polypeptides (Fig. 4). To that end, in vitro translation reactions were performed using truncated, stop codon-less mRNAs as a template. Under the conditions of the experiment, the bulk of nascent polypeptides remained quantitatively and firmly attached to ribosomes (Fig. S1A). To purify specific RNCs, nascent polypeptides were fused to an N-terminal FLAG tag.

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**TABLE 1**

Quantification of RPBs and ribosomes in a logarithmically growing yeast cell

Quantifications were performed as outlined in Fig. 1 and are derived from the analysis of at least three independently grown cultures. Protein/subunit per cell is the number of the respective molecule in a yeast cell. Oligomer per cell is an average of the number of subunits contained in one complex. RPBs per 100 ribosomes is the percentage of RPBs compared to ribosomes in a logarithmically growing yeast cell.

| Protein/subunit | Protein/subunit per cell | Oligomer per cell | RPBs per 100 ribosomes |
|-----------------|--------------------------|------------------|------------------------|
| **Ribosome**    |                          |                  |                        |
| Rps9            | 2.2 × 10^3               | 3.15 × 10^5      |                        |
| Asc1            | 2.6 × 10^3               |                  |                        |
| Rpl39           | 3.9 × 10^3               |                  |                        |
| Rpl17           | 3.9 × 10^3               |                  |                        |
| Ssb1/2          | 2.80 × 10^3              |                  | 89.1                   |
| RAC             |                          |                  |                        |
| Szl1            | 6.71 × 10^3              | 8.61 × 10^4      | 27.3                   |
| Zuo1            | 1.05 × 10^3              |                  |                        |
| NAC             |                          |                  |                        |
| αNAC            | 3.91 × 10^3              |                  | 125                    |
| SRP             |                          |                  |                        |
| Srp54           | 7.85 × 10^3              |                  | 2.5                    |
| Map1            |                          |                  |                        |
| Map1            | 2.11 × 10^3              | 6.7              |                        |
| Map2            |                          |                  |                        |
| Map2            | 6.21 × 10^3              | 2.0              |                        |
| NatA            |                          |                  |                        |
| Nat1            | 7.66 × 10^3              | 7.63 × 10^3      | 2.4                    |
| Ard1            | 7.59 × 10^3              |                  |                        |
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Yeast Pgk1 (3-phosphoglycerate kinase 1) is a monomeric cytosolic protein (39), yeast ppo-factor is the precursor of the secreted pheromone α-factor (40), and yeast Dap2 is a vacuolar type II membrane protein (41). RNCS containing the N-terminal 87 amino acids of Pgk1, ppo-factor, or Dap2 as a nascent polypeptide were used for RPB binding and cross-linking experiments. For the purification of RNCs, nascent polypeptides were fused to an N-terminal FLAG tag (DYKDDDDK). The position of lysines (K) that provided primary amino groups for the cross-linking reactions is indicated. For cross-linking experiments untagged versions of the proteins were used in which the amino acid at position 2 was changed to a lysine. Helical regions of Pgk1, the N-terminal signal sequence of ppo-factor, and the signal anchor sequence of Dap2 are indicated.

Cross-linking of RPBs to Cytosolic and ER-targeted Nascent Polypeptides—The experiments described above revealed the extent to which each type of RNC attracted RPBs. Based on this information we now asked how recruitment correlated with nascent polypeptide interaction. To that end, RNCS were generated under the same conditions as in the RPB binding studies. After RNC isolation, RPB proximity to nascent polypeptides was assessed using a homobifunctional cross-linker reactive toward primary amino groups as represented by the amino terminal signal sequence of ppO-factor, or Dap2. Consistent with the exposure of the signal anchor sequence of Dap2, SRP was strongly enriched on Dap2-RNCs. Remarkably, ppo-factor, which also exposes a signal sequence, did not recruit more SRP to RNCs than Pgk1. Ssb1/2 and NAC were recruited 2-fold less efficiently to Dap2-RNCs compared with Pgk1-RNCs (Fig. 5C). In comparison to non-translating RNCS, Ssb1/2 affinity for Dap2-RNCs was of similar strength, whereas NAC interaction with Dap2-RNCs was significantly decreased.

Cross-linking Experiments—The experimental setup used was tested whether and how nascent polypeptides of 87-amino acid length affected RPB-ribosome interaction. The nascent polypeptide represented three specific protein biogenesis pathways: Pgk1 (39), a monomeric soluble protein localized to the cytosol; prepro-α-factor (ppo-factor), a precursor that matures into the secreted pheromone α-factor (40); and Dap2 (41), a type II membrane protein that is finally localized to the vacuole (Fig. 4). The data were evaluated under the assumption that a ≥2-fold difference in RPB binding reflected a significant change in affinity. As a result, the amount of RAC, Map1, and NatA bound to RNCs was not significantly affected by the sequence of the nascent polypeptide (Fig. 5C). With respect to the nascent polypeptide-modifying enzymes Map1 and NatA, one has to bear in mind that due to the experimental design neither of the nascent polypeptides represented a substrate (25). Additional experiments are on the way to determine how the affinity of the aminopeptidases and acetyltransferase are affected by substrate polypeptides. Binding of Ssb1/2, NAC, and SRP was modulated by the sequence of nascent polypeptides (Fig. 5C). The three RPBs distinguished between RNCs carrying nascent Pgk1, ppo-factor, or Dap2. Consistent with the exposure of the signal anchor sequence of Dap2, SRP was strongly enriched on Dap2-RNCs. Remarkably, ppo-factor, which also exposes a signal sequence, did not recruit more SRP to RNCs than Pgk1. Ssb1/2 and NAC were recruited 2-fold less efficiently to Dap2-RNCs compared with Pgk1-RNCs (Fig. 5C). In comparison to non-translating RNCS, Ssb1/2 affinity for Dap2-RNCs was of similar strength, whereas NAC interaction with Dap2-RNCs was significantly decreased.

Additional Experiments—The experiments described above revealed the extent to which each type of RNC attracted RPBs. Based on this information we now asked how recruitment correlated with nascent polypeptide interaction. To that end, RNCS were generated under the same conditions as in the RPB binding studies. After RNC isolation, RPB proximity to nascent polypeptides was assessed using a homobifunctional cross-linker reactive toward primary amino groups as represented by the α-amino group of lysines and the Nε-amino group of polypeptides (Fig. 4). RAC and Map1 did not form cross-links to any of the nascent polypeptides (data not shown). Nascent Dap2 formed an efficient cross-link to SRP and a weak cross-link to NAC, but no cross-link to Ssb1/2 and NatA. NascentPgk1 and ppoα-factor formed cross-links to NAC, Ssb1/2, and NatA, but not to SRP.
The absence of a cross-link between nascent ppα-factor and SRP differs from previous results demonstrating an efficient cross-link between yeast ppα-factor and mammalian SRP (42). Introduction of an additional lysine at position 5 (ppα-S5K) (42) did not alter the cross-linking pattern of ppα-factor (supplemental Fig. S2). We conclude that the signal sequence of yeast ppα-factor does not attract yeast SRP to RNCs (Fig. 5C) nor does it interact with SRP (Fig. 6). In fact, RPBs were either in close proximity to nascent Pgk1 and ppα-factor (Ssb1/2, Nat1) or nascent Dap2 (SRP). Only NAC formed cross-links to all three nascent polypeptides; consistent with its less efficient binding to Dap2-RNCs, NAC cross-links to nascent Dap2 were weaker than to nascent Pgk1 or ppα-factor (Fig. 6).

DISCUSSION

The Ratio of RPBs Versus Ribosomes; Dynamics at the Tunnel Exit—Prior to this study, quantitative immunoblotting had been applied to only few RPBs. The best documented example was Ssb1/2, for which a cellular ratio of 3 ± 2 molecules/ribosome was reported (43). We now find that Ssb1/2 is expressed at lower concentrations, approximately equimolar to ribosomes. An elegant high throughput study had previously determined cellular expression levels by epitope tagging the open reading frames of yeast, such that the fusion proteins were expressed under control of their natural promoters (44). In this study, levels of ribosomal proteins were highly variable, and for some RPBs, e.g. Srp54, the expression levels were not determined. However, expression levels of most RPBs, including Ssb1/2, are in excellent agreement. With the exception of Ssb1/2 and NAC, which will be discussed below, RPBs are expressed at substoichiometric levels compared with ribosomes. This shortage strongly suggests that the interaction with ribosomes cannot be static but

FIGURE 5. Quantification of RPBs on RNCs engaged in the translation of specific nascent polypeptides. A yeast translation extract was programmed with truncated mRNA encoding the N-terminal 87 amino acids of Pgk1 (Pgk1–87), ppα-factor (ppα–87), or Dap2 (Dap2–87) fused to an N-terminal FLAG tag (+FLAG) or without a tag (−FLAG) (see Fig. 4 and “Experimental Procedures”). RNCs carrying FLAG-tagged nascent polypeptides were isolated by native immunoprecipitation using αFLAG-covered beads. RNCs carrying the same nascent polypeptide but lacking the tag served as a control in parallel reactions. Aliquots of the material recovered on αFLAG beads and standard proteins (Fig. 1) were applied to the same Tris-Tricine gel and were subsequently analyzed by immunoblotting. Signals obtained from non-tagged RNCs were subtracted as a background from the signals derived from FLAG-tagged RNCs. Quantification was performed as described in Fig. 1. As examples Rps9a and SRP (A) and Rps9a, αNAC, Sz1, and Zuo1 (B) are shown. C, occupation of RNCs with RPBs. The occupation of Pgk1-RNCs, ppα-RNCs, and Dap2-RNCs by RPBs is given in percent. Error bars indicate the S.D.
requires cycling of RPBs. SRP affinity is known to be modulated by substrates containing signal sequences (Ref. 45 and references within). What affects ribosome binding in the case of the other RPBs is not understood. For example, RAC binding was not significantly influenced by the specific nascent polypeptides tested in the course of this study (Fig. 5C); however, translation in general seemed to exert a positive effect on RAC binding (Fig. 3B). As RAC acts in concert with Ssb1/2 (20–22), these two RPBs very likely associate with a single ribosome, at least transiently. However, our data do not favor a model where RAC is recruited preferentially to ribosome-Ssb1/2 complexes: Pgk1-RNCs, which carried significantly more Ssb1/2 than non-translating ribosomes, did not carry more RAC. Moreover, RAC is also quantitatively associated with ribosomes obtained from a yeast strain lacking Ssb1/2 (23). In this context it is also interesting to recall that yeast can tolerate very low cellular RAC concentrations without much effect (22). Although details remain to be established, the combined data suggest that RAC binds to ribosomes with high affinity but also in a highly dynamic fashion that ensures its action on a large number of ribosomes.

It is a unique property of Ssb1/2 that it behaves like an integral component of the translating ribosomal particle (43). This stable association led to the plausible model that Ssb1/2 becomes a static component of the ribosome when translation starts and cycles between two rounds of translation (43). It was therefore unexpected to find that only a fraction of translating ribosomes carried Ssb1/2 (Figs. 3B and 5C). The result would be consistent with a model where only some polypeptides are synthesized on ribosomes stably occupied by Ssb1/2. Alternatively, the interaction of Ssb1/2 with translating ribosomes might be more dynamic than anticipated. Release may be coupled to a specific step of the elongation cycle. In this case, tight binding of Ssb1/2 to RNCs, as observed after breaking cells or in vitro, may reflect the absence of ongoing translation.

Our data also suggest that Ssb1/2 may adopt different conformations on the ribosome or, alternatively, possess more than one ribosomal binding site. This would explain how Ssb1/2 that was bound with similar efficiency to ppa-RNCs (24% occupation) and Dap2-RNCs (17% occupation) formed an efficient cross-link to nascent ppa-factory but not to nascent Dap2. Because ppa-factor contains only a single lysine at position 2 whereas Dap2 contains a lysine at the same position plus additional lysines (Fig. 4), we do not favor the possibility that differences in the availability of primary amino groups account for the lack of a Dap2 cross-link. Interestingly, the same applies to NatA that was bound equally well to all RNCs but formed a cross-link only to nascent Pgk1 and ppa but not to nascent Dap2. The failure of nascent Dap2 to cross-link to Ssb1/2 as well as to NatA was not confined to a specific length of the nascent polypeptide but was also observed for shorter and longer versions of Dap2. As cross-linking is suited to reveal even short-lived interactions, it seems unlikely that ribosome-bound Ssb1/2 or NatA are even transiently close to nascent Dap2. It will be interesting to identify the sequence attributes of nascent polypeptides that seemingly affect RPB positioning on the ribosome. Experiments are on the way to determine whether it is a general feature of Ssb1/2 and NatA to discriminate SRP substrates.

The affinity of yeast SRP increased from non-translating ribosomes to ppa-RNCs ≡ Pgk1-RNCs to Dap2-RNCs. In a previous study fluorescence techniques have been employed to determine affinities of mammalian SRP to wheat germ ribosomes and RNCs at equilibrium (45). In this experimental system the affinity increased from SRP-non-translating ribosomes to SRP-RNCs lacking signal sequences to various SRP-RNCs bearing a signal sequence (45). Thus, our data are in good agreement with respect to the general preferences of SRP and confirm that SRP distinguishes not only between RNCs bearing a signal sequence or not but also between non-translating and translating ribosomes (45). The complete lack of interaction between SRP and the signal sequence of ppa-factory was surprising, particularly in light of the earlier data (42). However, it confirms work of Walter and co-workers (46), who have shown in vivo that Dap2 requires SRP to be translocated to the ER whereas ppa-factor does not. Our data support the idea that the affinity of SRP for a signal sequence determines whether an ER-targeted protein enters the SRP-dependent, cotranslational or the SRP-independent, posttranslational pathway (46, 47). Interestingly, it was recently found in the E. coli system that proteins containing signal-anchor sequences are selected for cotranslational targeting by SRP at an early stage during biogenesis, whereas nascent secretory proteins were not (5).

SRP and NAC displayed inverse affinity for the RNCs analyzed in the course of this study (Fig. 5C). The observation is consistent with a previous model suggesting that SRP competes with NAC for the same binding site on the ribosome (48). Recent studies have confirmed that SRP and NAC indeed inter-

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act with the same ribosomal protein at the tunnel exit (18, 49, 50). There is evidence, however, that SRP and NAC can simultaneously occupy a single ribosome (18), and our data do not exclude this possibility. The experimental system described in this report shall facilitate future experiments to address these fundamental questions.

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REFERENCES

1. Maier, T., Ferbitz, L., Deuerling, E., and Ban, N. (2005) Curr. Opin. Struct. Biol. 15, 204–212
2. Pool, M. R. (2005) Mol. Membr. Biol. 22, 3–15
3. Jensen, C. G., and Pedersen, S. (1994) J. Bacteriol. 176, 7148–7154
4. Eisen, G., Moser, M., Schäfer, U., Beck, K., and Müller, M. (2006) J. Biol. Chem. 281, 7172–7179
5. Ullers, R. S., Houben, E. N., Brunner, J., Oudega, B., Harms, N., and Lurink, J. (2006) J. Biol. Chem. 281, 13999–14005
6. Hann, B. C., and Walter, P. (1991) Cell 67, 131–144
7. George, R., Beddoo, T., Landl, K., and Lithgow, T. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 2296–2301
8. Nelson, R. J., Ziegelhoffer, T., Nicolet, C., Werner-Washburne, M., and Craig, E. A. (1992) Cell 71, 97–105
9. Yan, W., Schilke, B., Pfund, C., Walter, W., Kim, S., and Craig, E. A. (1998) EMBO J. 17, 4809–4817
10. Gautschi, M., Lilie, H., Fünfschilling, U., Mun, A., Ross, S., Lithgow, T., Rücknagel, P., and Rospert, S. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 3762–3767
11. Vetro, J. A., and Chang, Y. H. (2002) J. Cell Biochem. 85, 678–688
12. Gautschi, M., Just, S., Mun, A., Ross, S., Rücknagel, P., Dubaquie, Y., Ehrenhofer-Murray, A., and Rospert, S. (2003) Mol. Cell Biol. 23, 7403–7414
13. Wild, K., Halic, M., Sinning, I., and Beckmann, R. (2004) Nat. Struct. Mol. Biol. 11, 1049–1053
14. Rospert, S., Dubaquie, Y., and Gautschi, M. (2002) Cell Mol. Life Sci. 59, 1632–1639
15. Wegrzyn, R. D., and Deuerling, E. (2005) Cell Mol. Life Sci., 62, 2727–2738
16. Rospert, S., Gautschi, M., Ralkwasa, M., and Raue, U. (2005) in Protein Folding Handbook (Buchner, J., and Kieflhaber, T., eds) Vol. II, pp. 429–458, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany
17. Fünfschilling, U., and Rospert, S. (1999) Mol. Biol. Cell 10, 3289–3299
18. Grollath, M., Schwarz, J. P., Bottcher, U. M., Bracher, A., Hartl, F. U., and Siegers, K. (2006) EMBO Rep. 7, 78–84
19. Reimann, B., Bradsher, J., Franke, J., Hartmann, E., Wiedmann, M., Prehn, S., and Wiedmann, B. (1999) Yeast 15, 397–407
20. Gautschi, M., Mun, A., Ross, S., and Rospert, S. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 4209–4214
21. Huang, P., Gautschi, M., Walter, W., Rospert, S., and Craig, E. A. (2005) Nat. Struct. Mol. Biol. 12, 497–504
22. Hundley, H., Eisenman, H., Walter, W., Evans, T., Hotokezaka, Y., Wiedmann, M., and Craig, E. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 4203–4208
23. Ralkwasa, M., and Rospert, S. (2004) Mol. Cell Biol. 24, 9186–9197
24. Li, X., and Chang, Y. H. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 12357–12361
25. Polevoda, B., and Shaman, F. (2003) Biochem. Biophys. Res. Commun. 308, 1–11
26. Bonven, B., and Gullow, K. (1979) Mol. Gen. Genet. 170, 225–230
27. Schägger, H., and von Jagow, G. (1987) Anal. Biochem. 166, 368–379
28. Haid, A., and Suisa, M. (1983) Methods Enzymol. 96, 192–205
29. Yaffe, M. P., and Schatz, G. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 4819–4823
30. Garcia, P. D., Hansen, W., and Walter, P. (1991) Methods Enzymol. 194, 675–682
31. Heitmann, J., Movva, N. R., Hiestand, P. C., and Hall, M. N. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 1948–1952
32. Blobel, G., and Sabatini, D. (1971) Proc. Natl. Acad. Sci. U. S. A. 68, 390–394
33. Ashe, M. P., De Long, S. K., and Sachs, A. B. (2000) Mol. Biol. Cell 11, 833–848
34. Horton, L. E., James, P., Craig, E. A., and Hensold, J. O. (2001) J. Biol. Chem. 276, 14426–14433
35. Siegers, K., Bolter, B., Schwarz, J. P., Bottcher, U. M., Guha, S., and Hartl, F. U. (2003) EMBO J. 22, 5230–5240
36. Albanese, V., Yam, A. Y., Baughman, J., Parnot, C., and Frydman, J. (2006) Cell 124, 75–88
37. George, R., Walsh, P., Beddoo, T., and Lithgow, T. (2002) FEBS Lett. 516, 213–216
38. Beckmann, R., Spahn, C. M., Eswar, N., Helmers, J., Penczek, P. A., Sali, A., Frank, J., and Blobel, G. (2001) Cell 107, 361–372
39. Watson, H. C., Walker, N. P., Shaw, P. J., Bryant, T. N., Wendell, P. L., Fothergill, L. A., Perkins, R. E., Conroy, S. C., Dobson, M. J., Tuite, M. F., Kingsman, A. J., and Kingsman, S. M. (1982) EMBO J. 1, 1635–1640
40. Caplan, S., and Kurjan, J. (1991) Genetics 127, 299–307
41. Roberts, C. J., Pohlig, G., Rothman, J. H., and Stevens, T. H. (1989) J. Cell Biol. 108, 1363–1373
42. Plath, K., and Rapoport, T. A. (2000) J. Cell Biol. 151, 167–178
43. Pfund, C., Lopez-Hoyo, N., Ziegelhoffer, T., Schilke, B. A., Lopez-Buesa, P., Walter, W. A., Wiedmann, M., and Craig, E. A. (1998) EMBO J. 17, 3981–3989
44. Ghaemmaghami, S., Huh, W. K., Bower, K., Howson, R. W., Belle, A., Dephoure, O., O’Shea, E. K., and Weissman, J. S. (2003) Nature 425, 737–741
45. Flanagan, J. I., Chen, J. C., Miao, Y., Shao, Y., Lin, J., Bock, P. E., and Johnson, A. E. (2003) J. Biol. Chem. 278, 18628–18637
46. Ng, D. T., Brown, J. D., and Walter, P. (1996) J. Cell Biol. 134, 269–278
47. Martoglio, B., and Dobberstein, B. (1998) Trends Cell Biol. 8, 410–415
48. Powers, T., and Walter, P. (1996) Curr. Biol. 6, 331–338
49. Pool, M. R., Stumm, J., Fulga, T. A., Sinning, I., and Dobberstein, B. (2002) Science 297, 1345–1348
50. Wegrzyn, R. D., Hofmann, D., Merz, F., Nikolay, R., Rauch, T., Graf, C., and Deuerling, E. (2005) J. Biol. Chem. 280, 2847–2857