RsgA releases RbfA from 30S ribosome during a late stage of ribosome biosynthesis

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RsgA is a 30S ribosomal subunit-binding GTPase with an unknown function, shortage of which impairs maturation of the 30S subunit. We identified multiple gain-of-function mutants of *Escherichia coli* *rbfA*, the gene for a ribosome-binding factor, that suppress defects in growth and maturation of the 30S subunit of an *rsgA*-null strain. These mutations promote spontaneous release of RbfA from the 30S subunit, indicating that cellular disorders upon depletion of RsgA are due to prolonged retention of RbfA on the 30S subunit. We also found that RsgA enhances release of RbfA from the mature 30S subunit in a GTP-dependent manner but not from a precursor form of the 30S subunit. These findings indicate that the function of RsgA is to release RbfA from the 30S subunit during a late stage of ribosome biosynthesis. This is the first example of the action of a GTPase on the bacterial ribosome assembly described at the molecular level.

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Introduction

In the course of ribosome biosynthesis, chains of events including transcription, stepwise cleavages of the primary transcript, modifications of ribosomal proteins and ribosomal RNAs, and assemblies of dozens of ribosomal proteins with rRNAs proceed coordinately.

It has been shown that bacterial rRNA and ribosomal proteins can be spontaneously assembled *in vitro* into translationally competent ribosomal subunits without additional factors (Traub and Nomura, 1968; Nomura and Erdmann, 1970; Nierhaus and Dohme, 1974). However, such an *in vitro* reconstitution of the ribosomal subunit requires nonphysiological conditions, namely, high Mg⁺⁺ concentrations and ionic strengths as well as long incubation times under

elevated temperatures, highlighting the significance of *trans*-acting assembly factors for efficient biosynthesis of the ribosome in cells. These factors should also be required for elaborate coordination with transcription, cleavages and modifications of rRNAs *in vivo*. Nevertheless, no bacterial factor with a definite role in the process of ribosome biosynthesis has been reported except for well-defined modification enzymes, RNases or components of the ribosome themselves, though dozens of putative *trans*-acting assembly factors for ribosome biogenesis have been proposed (Kaczanowska and Rydén-Aulin, 2007; Wilson and Nierhaus, 2007; Wilson, 2009).

GTPases comprise a large class in putative bacterial assembly factors for the ribosome (Karbstein, 2007; Britton, 2009), including Era (Inoue *et al*, 2003; Sharma *et al*, 2005), ObgE (Sato *et al*, 2005; Jiang *et al*, 2006), Der (Hwang and Inouye, 2006), RbgA (Matsuo *et al*, 2006; Uicker *et al*, 2006), YqeH (Loh *et al*, 2007; Uicker *et al*, 2007), YsxC (Wicker-Planquart *et al*, 2008) and RsgA (Daigle and Brown, 2004; Himeno *et al*, 2004). The involvement of GTPases seems reasonable, considering some thermodynamic barriers in the process of ribosome assembly *in vitro* (Held and Nomura, 1973). To date, none of the functions of these bacterial GTPases has been clarified, while the functions of some eukaryotic GTPases in ribosome biosynthesis have been characterized at the molecular level (Strunk and Karbstein, 2009; Kressler *et al*, 2010).

Our interest has been focused on RsgA (also known as YjeQ) as a key factor of bacterial ribosome biosynthesis. RsgA is a GTPase composed of an N-terminal OB-fold putatively involved in RNA binding, a central GTPase domain comprising circularly permuted GTPase motifs and a C-terminal zinc-binding domain (Levdikov *et al*, 2004; Shin *et al*, 2004; Nichols *et al*, 2007). RsgA of *Escherichia coli* has a faint intrinsic GTPase activity (Daigle *et al*, 2002), which is significantly enhanced by the 30S subunit of the ribosome (Daigle and Brown, 2004; Himeno *et al*, 2004). RsgA is stably bound to the A site of the 30S subunit in the presence of GDPNP (guanosine 5'-[β , γ imido]-triphosphate), an unhydrolyzable analogue of GTP, but not in the presence of GTP or GDP (Himeno *et al*, 2004), suggesting that RsgA binds to the ribosome in the GTP form and dissociates upon GTP hydrolysis. In *E. coli*, *rsgA* was initially reported as an essential gene (Arigoni *et al*, 1998) but was later shown to be nonessential for viability (Himeno *et al*, 2004). It has been shown that 17S RNA, a precursor of 16S rRNA with extra 115 and 33 nucleotides at the 5' and 3' ends, respectively (Young and Steitz, 1978), accumulates (Himeno *et al*, 2004) in an *rsgA*-null mutant of *E. coli* cells. It has also been shown that an *rsgA*-null mutant of *E. coli* (Himeno *et al*, 2004) as well as an orthologous *yloQ*-null mutant of *Bacillus subtilis* (Campbell *et al*, 2005) exhibits decrease in the ratio of the 70S ribosomes to the 50S and 30S subunits and reduction in growth rate. These disorders caused by RsgA depletion are partly suppressed by overexpression of other GTPases, Era or IF2, in *E. coli* (Campbell and Brown, 2008).

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RsgA and other 30S subunit-associated factors, RbfA, Era and RimM, have been categorized into a group of assembly factors for the 30S subunit, based on phenotypic similarities upon their depletions or some mutations and their genetic interactions (Wilson and Nierhaus, 2007; Connolly and Culver, 2009). However, the molecular basis for their roles in maturation processes as well as their genetic interactions has not yet been elucidated.

Here, we describe the functional interplay of RsgA and RbfA during maturation of the 30S subunit at both the genetic and molecular levels. RbfA is a small protein composed mainly of a single type II KH domain (Huang *et al*, 2003), initially identified as a multicopy suppressor of a cold-sensitive mutation (C23U) of 16S rRNA (Dammel and Noller, 1995). RbfA binds to the 30S subunit but not to the 50S subunit, the 70S ribosome or polysome (Dammel and Noller, 1995). It has been proposed that binding of RbfA destabilizes the 5' end helix of 16S rRNA in the 30S subunit (Dammel and Noller, 1995), which has been supported by a cryo-electron microscopic map of the 30S subunit in complex with RbfA (Datta *et al*, 2007). An *rbfA*-null mutant of *E. coli* shares similar phenotypes with an *rsgA*-null mutant, including accumulation of 17S RNA (Bylund *et al*, 1998), decrease in the 70S ribosomes relative to the 50S or 30S subunits and reduction in growth rate (Dammel and Noller, 1995), all of which are partly suppressed by overexpression of Era (Inoue *et al*, 2003, 2006). Increased expression of *rbfA* partly suppresses defects in growth and translation in a *rimM*-null mutant (Bylund *et al*, 1998, 2001).

In the present study, we demonstrate that multiple kinds of mutations in *rbfA* suppress defects in growth and maturation of the 30S subunit of an *rsgA*[−] strain. We also show that RsgA can promote release of RbfA from the 30S subunit. Our results suggest that this event occurs during a late stage of maturation of the 30S subunit when 17S pre-rRNA is processed into 16S rRNA. Our results also indicate that cellular disorders upon depletion of RsgA are the consequence of prolonged retention of RbfA on the ribosome. Interplay of RbfA and RsgA on the 30S subunit established in the present study would render a novel process involving RbfA, RsgA and possibly other putative assembly factors including RimM and Era, providing a framework to our knowledge of the entire processes of ribosome biosynthesis.

Results

Isolation and characterization of suppressor mutants of growth defect from an *rsgA*[−] strain

As previously described (Himeno *et al*, 2004), *E. coli* W3110Δ*rsgA* strain suffers from a growth defect. We isolated 29 independent mutant strains from W3110Δ*rsgA* in which growth is restored in LB medium. We constructed a DNA library in a multicopy plasmid from genomic DNA of QIG26, one of the growth-restored revertant strains. The library was introduced into W3110Δ*rsgA* and clones yielding large colonies on LB plates were selected. As a result, one clone, designated pUC26-6, with an insert of 2427 bp was obtained. DNA sequencing revealed that the insert is a part of the *metY* operon, which includes *rbfA* as a single full-length gene (Supplementary Figure 1A). The insert possessed a point mutation, G358A, which causes a substitution of asparagine for aspartic acid at position 120 of RbfA. Subsequent analysis

Table 1 Eight mutations that suppress *rsgA*[−] phenotypes, and the resulting alterations in RbfA

Mutation in <i>rbfA</i> gene ^a	Resulting alteration in RbfA protein ^b	Number of isolation	Representative strain ^c
G9A	R10H	2	QIG121
C89T	P30L	1	QIG107
G230A	G77D	9	QIG124
G252A	G84E	11	QIG4
A299G	D100G	1	QIG11
Deletions of C333 and C334	Frameshift at 111 ^d	3	QIG1
G358A	D120N	1	QIG26
G364T	Termination at 122	1	QIG15

^aNucleotide positions are those relative to the first nucleotide of the first codon of *rbfA*.

^bAmino acid positions are those relative to the first amino acid of RbfA. Wild-type RbfA is comprised of 133 amino acids. Mutations mapped to a tertiary structure are illustrated in Supplementary Figure 1B.

^cOne representative strain was chosen per each mutation for use in experiments for which results are shown in Figure 1A, E and F and in Supplementary Figure 2.

^dResults in truncated RbfA with 116 amino acids, which consists of the native N-terminal 110 and C-terminal frameshifted 6 amino acids.

(see Materials and methods) revealed that each of the 29 mutant strains possesses a single-point mutation somewhere within the coding region of *rbfA*. As summarized in Table 1, the 29 mutations include identical mutations, being converged into eight kinds (six missense mutations, one nonsense mutation and one frameshift mutation). The eight mutations are dispersed throughout the whole span of RbfA with no apparent bias in the primary (Table 1) or tertiary (Supplementary Figure 1B) structure.

Growth rates of eight kinds of nonredundantly chosen *rbfA* mutant strains as well as W3110, W3110Δ*rbfA* and W3110Δ*rsgA*Δ*rbfA* are shown in Figure 1A and Supplementary Figure 2A. It is noteworthy that W3110Δ*rsgA*Δ*rbfA*, W3110Δ*rsgA* and W3110Δ*rbfA* cells showed slow growth with similar rates, indicating that deletions of *rbfA* and *rsgA* have no additive effect on growth.

Each of the eight kinds of mutant *rbfA* was subcloned into a low-copy plasmid vector to confirm the responsibility for suppression of slow growth of W3110Δ*rsgA* cells (Figure 1B; Supplementary Figure 2B). W3110Δ*rbfA* into which each of the eight kinds of the plasmid was introduced exhibited a growth rate indistinguishable from that of W3110Δ*rbfA* harbouring plasmid carrying wild-type *rbfA* (Figure 1C; Supplementary Figure 2C).

Mutations in *rbfA* restore ribosome maturation

Another distinctive feature of *E. coli* *rsgA*[−] strains besides slow growth is their impairment in maturation of the ribosome. They have a decreased proportion of the 70S ribosomes to the 50S or 30S subunits and accumulate 17S RNA (Himeno *et al*, 2004). To see the effect of mutations in *rbfA* on the impaired maturation of the ribosome in *rsgA*-depleted cells, we examined the proportion of subunits and the composition of 17S/16S RNAs.

The ribosomes from W3110Δ*rsgA*-derived growth-restored mutant cells were compared with those from W3110, W3110Δ*rsgA*, W3110Δ*rbfA* and W3110Δ*rsgA*Δ*rbfA* cells by sucrose density gradient centrifugation. The ribosome

Figure 1 Mutations in *rbfA* suppress disorders of *rsgA*[−] cells. (A–C) Mutations in *rbfA* restore cell growth. Panels represent growth of (A) W3110, W3110Δ*rsgA* and W3110Δ*rsgA*-derived mutant strains, (B) W3110Δ*rsgA* strains harbouring plasmids carrying mutant *rbfA* and (C) W3110Δ*rbfA* strains harbouring plasmids carrying mutant *rbfA*. The effects of only two mutations are shown, and those of the remaining mutations are shown in Supplementary Figure 2. Doubling times were 0.9 h for W3110, 1.1–1.4 h for W3110Δ*rsgA*-derived mutant strains and 2.0 h for W3110Δ*rsgA*, W3110Δ*rbfA* and W3110Δ*rsgA*Δ*rbfA*. (D, E) Mutations in *rbfA* restore 70S ribosomes to an almost normal level. The lysate of 10 mg (wet weight) of cells was subjected to a sucrose density gradient centrifugation. The direction of sedimentation is right to left. Panels represent ribosome profiles of (D) W3110, W3110Δ*rsgA*, W3110Δ*rbfA* and W3110Δ*rsgA*Δ*rbfA*, and (E) growth-restored mutants derived from W3110Δ*rsgA*. (F) Mutations in *rbfA* suppress accumulation of 17S RNA. Total RNA from log-phase cells of each strain was analysed by 1.5% agarose gel electrophoresis. The bands of 23S, 17S and 16S RNAs are indicated with arrows.

profiles of the growth-restored mutants, which were almost the same, were more similar to that of the wild-type than that of the *rsgA*[−] strains (Figure 1D and E). Both W3110Δ*rsgA* and W3110Δ*rbfA* had a decreased level of 70S ribosomes and accumulated free subunits (Figure 1D), being consistent with previous reports (Dammel and Noller, 1995; Himeno *et al*, 2004). W3110Δ*rsgA*Δ*rbfA* showed almost the same ribosome profile as that of W3110Δ*rsgA* or W3110Δ*rbfA*, indicating that deletions of *rbfA* and *rsgA* have no additive effect on subunit assembly into the 70S ribosome.

Next, the composition of ribosomal RNAs from cells was checked by electrophoresis on an agarose gel (Figure 1F). A considerable level of 17S RNA was observed in W3110Δ*rsgA* and W3110Δ*rbfA* as previously reported (Bylund *et al*, 1998; Himeno *et al*, 2004). W3110Δ*rsgA*Δ*rbfA*, W3110Δ*rsgA* and W3110Δ*rbfA* had almost the same proportions of 17S RNA/16S rRNA (2.28 ± 0.08 , quantified from the band intensity), reconfirming that inactivations of *rbfA* and *rsgA* have no additive effect. In the growth-restored mutants, the level of 17S RNA relative to that of 16S rRNA was significantly decreased ($17S/16S = 0.30 \pm 0.06$).

Reduced level of RbfA bound to 30S subunits in *rsgA*-suppressing mutant cells

We examined the levels of the eight RbfA mutants bound to the 30S subunits *in vivo*. The fractions of sucrose density gradient centrifugation were precipitated with acetone, and RbfA was detected by western immunoblotting. Each of the *rsgA*-suppressing mutant cells had a lower level of RbfA in the 30S fraction (relative to that in the soluble fractions at the top of sedimentation) than did wild-type cells (Figure 2A). The decrease in the level of RbfA was further confirmed by normalizing the amount of the 30S fractions in terms of A₂₆₀ units, which showed that cells of each mutant had a lower level of RbfA in the 30S fraction than did either

wild-type or *rsgA*[−] cells (Figure 2B). All mutations that suppress growth defect of an *rsgA*[−] strain were found exclusively in the coding region of the *rbfA* gene (Table I), and they conferred reduced levels of RbfA in the 30S fractions (Figure 2). This might lead to the idea that the increased level of RbfA free from the 30S subunits is responsible for suppression of *rsgA*[−] phenotypes. However, this is not supported by another observation that overexpression of *rbfA*, which would increase the level of RbfA outside the ribosome, does not suppress growth of an *rsgA*[−] strain (Campbell and Brown, 2008). We also overexpressed *rbfA* in *rsgA*[−] cells to confirm that it restores neither growth (Supplementary Figure 3A) nor ribosome profile (Supplementary Figure 3B) of the *rsgA*[−] strain. Furthermore, none of the 29 growth-restored revertant strains we obtained had a mutation that might elevate the level of RbfA in the cell, such as that around the promoter region of the *rbfA* gene. Thus, the reduction in the level of RbfA bound to the ribosome rather than the increase in the level of RbfA outside the ribosome would be involved in suppression of *rsgA*[−] phenotypes. However, complete loss of RbfA bound to the ribosome would not lead to suppression of *rsgA*[−] phenotypes, as indicated by the fact that W3110Δ*rsgA*Δ*rbfA* sustains *rsgA*[−] phenotypes (Figure 1A, D and F). Interestingly, a high level of forced overexpression of *rbfA* led to growth arrest in *rsgA*[−] cells, but not in wild-type cells (Supplementary Figure 3C), indicating that the significance of RsgA increases with increase in the cellular level of RbfA.

RbfA promotes association of RsgA with the 30S subunit

We prepared recombinant proteins to investigate the possible effect of RbfA protein on the GTP hydrolytic activity of RsgA *in vitro*.

Figure 2 Localization of RbfA in cells of wild-type, *rsgA*[−] and *rsgA*-suppressing mutants detected by anti-RbfA antibody. **(A)** Localization of RbfA in the 30S fraction and other fractions. The lysate of 100 mg (wet weight) of log-phase cells was subjected to a sucrose density gradient centrifugation and divided into 20 fractions after sedimentation. One-third of each fraction was precipitated with acetone and separated by SDS-PAGE. RbfA was immunochemically detected. The 70S, 50S and 30S fractions of sedimentations are indicated. **(B)** RbfA in the 30S fraction. The lysate of 10 mg (wet weight) of cells was subjected to a sucrose density gradient centrifugation. 0.15 A₂₆₀ units of the 30S fraction was precipitated with acetone followed by SDS-PAGE. RbfA was detected by western immunoblotting.

As shown in Figure 3A, the addition of purified RbfA increased the 30S subunit-dependent GTPase activity of RsgA, while no increase in the 30S subunit-independent GTPase activity was detected. Dose-dependent enhancement by RbfA reached a plateau (about two-fold) at a concentration of 200 nM (four-fold concentration of the 30S subunits). This suggests a functional interaction between RbfA and RsgA on the 30S subunit.

Figure 3 RbfA promotes association of RsgA with the 30S subunit. **(A)** Hydrolysis of GTP (2.5 mM) by RsgA (300 nM) with or without the 30S subunits (50 nM) was monitored with increasing concentrations of RbfA. The ratio of the initial velocity (V_0) to that without RbfA is indicated by the right vertical axis. The experiment was repeated three times and the average values with s.d. are presented. **(B)** Hydrolysis of GTP (2.5 mM) by RsgA (300 nM) with or without RbfA (six-fold concentration each of that of the 30S subunits) was monitored with increasing concentrations of the 30S subunits. The ratio of the initial velocity (V_0) to that with the 30S subunits (50 nM) in the absence of RbfA is indicated by the right vertical axis. The experiment was repeated three times and the average values with s.d. are presented. P -values calculated by unpaired t -test with Welch correction between V_0 with and without RbfA were 0.02, 0.006, 0.1, 0.45 and 0.86 at 50, 150, 450, 1350 and 4050 nM of the 30S subunits, respectively. **(C)** Complex formation between RsgA and the 30S subunit monitored by FCS. Two nM of RsgA randomly labelled with TAMRA (RsgA*) was incubated for 60 min at room temperature with 1 mM GDPNP and various concentrations of the 30S subunits in the presence or absence of RbfA (10-fold concentration each of that of the 30S subunits). Diffusion time measured using an MF20 system (Olympus) was plotted against concentration of the 30S subunits. Diffusion times of control reactions with RsgA* and various concentrations of RbfA in the absence of the 30S subunits were also measured. The measurement was repeated five times and the average value with s.d. is presented. The difference in diffusion time of RsgA in the presence of the 30S subunits with versus without RbfA was significant at 1–16 nM of the 30S subunits ($P < 0.004$; unpaired t -test with Welch correction).

Next, we examined the effect of RbfA on GTPase activity of RsgA at various concentrations of the 30S subunits. Hydrolysis of GTP by RsgA with or without RbfA (six-fold concentrations of that of the 30S subunits) was monitored with increasing concentrations of the 30S subunits. Maximum effect (about two-fold) was observed in the presence of 50 nM of the 30S subunits (Figure 3B). The effect was decreased with increased concentrations of the 30S subunits, and little or no effect was observed in the presence of $\geq 1 \mu\text{M}$ of the 30S subunits (Figure 3B). This indicates that RbfA stimulates association of RsgA with the 30S subunit rather than the catalytic step of GTP hydrolysis on the 30S subunit.

Enhancement of affinity of RsgA to the 30S subunit by the addition of RbfA was also examined using fluorescence correlation spectroscopy (FCS). This system monitors photons emitted by fluorescent particles crossing a confocal volume irradiated by a focused laser beam. Stochastic analysis of fluctuations of fluorescent intensity calculates the diffusion time, the average time for the particle to cross the confocal volume, which positively correlates with the mass of the particle. RsgA randomly labelled with 5-carboxytetramethylrhodamine (TAMRA) was incubated with GDPNP and various concentrations of 30S subunits in the presence or absence of RbfA. As shown in Figure 3C, the diffusion time of RsgA, which reflects complex formation, increased with increasing concentrations of the 30S subunits. In the presence of RbfA, an approximately three-fold lower concentration of 30S subunits was sufficient for complex formation with RsgA. This confirms that RbfA increases the affinity of RsgA to the 30S subunit.

RsgA dissociates RbfA from the 30S subunit

The above-described results indicate that the reduced level of RbfA in the 30S fraction (Figure 2) correlates with the suppression of *rsgA*[−] phenotypes (Figure 1). One of the simplest explanations for this correlation is that RsgA helps dissociation of RbfA from the 30S subunits in some step of ribosome maturation, which is required for optimal growth, and *rsgA*-suppressing mutations provide RbfA with the ability to dissociate without RsgA. We therefore examined the effect of RsgA on stability of the complex of RbfA and the 30S subunits.

The 30S subunits were incubated with an excess amount of RbfA. After incubation, unbound RbfA were washed out by centrifugal ultrafiltration with a 100-kDa molecular weight cutoff. Upon incubation, a considerable amount of RbfA successfully bound to the 30S subunits and was retained during ultrafiltration (Figure 4).

Then the complex of the 30S subunits with RbfA that remained on the filter was further incubated with various concentrations of RsgA in the absence or presence of guanine nucleotides. As expected, RsgA dissociated RbfA from the 30S subunit in a GTP-dependent manner (Figure 4A). Dissociation was not observed in the presence of an RsgA mutant (T250A) lacking GTPase activity (Hase *et al*, 2009). Dissociation also occurred in the presence of ATP but required about a 10-fold higher concentration than that of GTP (Supplementary Figure 4), in agreement with the results of a previous study showing that RsgA hydrolyzes ATP with K_m higher than that for GTP (Daigle *et al*, 2002). RsgA efficiently dissociated RbfA from the 30S subunit in the presence of GTP

Figure 4 Dissociation of RbfA from the 30S subunit *in vitro*. The 30S subunits (50 nM) with an excess of RbfA (500 nM) were incubated for 30 min at 37°C. The mixture was subjected to centrifugal ultrafiltration using Microcon YM-100 (Millipore) to obtain the complex of RbfA and the 30S subunits. The complex of RbfA and 30S subunits was incubated under indicated conditions. After incubation, RbfA that remained on the 30S subunits was obtained by ultrafiltration using YM-100, followed by precipitation with acetone. The precipitate was separated by SDS-PAGE and RbfA was immunochemically detected. (A) RsgA dissociates RbfA from the 30S subunits. The complex of RbfA with the 30S subunits was incubated for 30 min at 37°C with or without 500 nM of wild-type or T240A mutant of RsgA in the presence or absence of GTP or ATP. (B) Enzymatic dissociation of RbfA from the 30S subunits by RsgA. The complex of the 30S subunit and RbfA was incubated with 5 nM, 50 nM or 500 nM of RsgA in the absence (–) or presence of guanine nucleotide (GDP, GTP or GDPNP) for 30 min at 37°C.

but not in the presence of GDP during incubation (Figure 4B). This is consistent with the results of previous studies showing that the GTP form but not the GDP form of RsgA stably binds to the 30S subunit (Daigle and Brown, 2004; Himeno *et al*, 2004). Dissociation efficiently occurred even in the presence of a 1/10 molar ratio of RsgA to the complex of RbfA and the 30S subunit, suggesting enzymatic reaction of RsgA. Dissociation of RbfA was also seen in the presence of GDPNP, an unhydrolyzable analogue of GTP, in the place of GTP.

Binding properties of RbfA and RsgA to mature or immature 30S subunits

We have reported that the 30S subunits rich in 17S RNA, which are prepared from the 30S fraction from RsgA-depleted cells, inefficiently enhance the GTPase activity of RsgA, while the 30S subunits rich in 16S rRNA, which are prepared from the 70S fraction from the same cells, enhance the GTPase activity as efficiently as do the 30S subunits from wild-type cells (Himeno *et al*, 2004). On the other hand, RbfA appears to efficiently bind to the 30S subunits, both from the 30S fraction from RsgA-depleted cells (Figure 2) rich in 17S RNA (Figure 1F) and from the 70S fraction from wild-type cells (Figure 4) rich in 16S rRNA. Accordingly, there would be a difference between RsgA and RbfA in preference of the degree of maturation of the 30S subunit.

To study such a preference, we prepared the 30S subunits rich in 16S rRNA from the 70S fraction (mature 30S subunits) and the 30S subunits rich in 17S RNA from the 30S fraction

Figure 5 Preference for mature or immature 30S subunit by RsgA or RbfA. **(A)** Complex formation between RsgA and mature or immature 30S subunits monitored by FCS. Two nM of TAMRA-labelled RsgA (RsgA*) with 1 mM GDPNP incubated for 60 min at room temperature with various concentrations of the 30S subunits in the presence or absence of RbfA. Diffusion time was measured using an MF20 and is plotted against concentration of the 30S subunits. The measurement was repeated five times and the average value with s.d. is presented. **(B)** Complex formation between RbfA and mature or immature 30S subunit monitored by FCS. Two nM of TAMRA-labelled RbfA (RbfA*) was incubated for 60 min at room temperature with various concentrations of the 30S subunits. Diffusion time was measured using an MF20 and is plotted against concentration of the 30S subunits. The measurement was repeated five times and the average value with standard deviation is presented. **(C)** Stability of the complex of RbfA and mature or immature 30S subunit. A measure of 50 nM of mature or immature 30S subunits was incubated with RbfA (500 nM) for 37°C. Washed complex of RbfA and mature or immature 30S subunit retained by ultrafiltration using YM-100 was diluted to 50 nM and incubated for 30, 60 or 120 min in the presence or absence of RsgA (50 nM) with GTP (1 mM) at 37°C. After incubation, ribosome-bound RbfA retained by ultrafiltration using YM-100 was separated by SDS-PAGE and immunochemically detected.

(immature 30S subunits) from W3110 Δ rsgA Δ rbfA cells. The RNA composition and sedimentation profile of each type of 30S subunit are presented in Supplementary Figures 5A and B, respectively. The GTPase activity of RsgA was enhanced by

the addition of mature 30S subunits, while little enhancement was observed in the presence of immature 30S subunits (Supplementary Figure 5C), which is analogous to previous results obtained by using the 30S subunits from W3110 Δ rsgA cells (Himeno *et al*, 2004).

The equilibrium affinity of RsgA or RbfA to each type of 30S subunit was then measured using FCS. RsgA-GDPNP required about a four-fold higher concentration of immature 30S subunits than that of mature 30S subunits to form a complex (Figure 5A). In contrast, RbfA required similar concentrations of mature and immature 30S subunits to form a complex (Figure 5B). Thus, RsgA has a significant preference for mature subunits over immature 30S subunits, while RbfA does not. In addition, RbfA did not promote association of RsgA with immature 30S subunits (Figure 5A).

Next, we examined the stability of the complex of RbfA and mature or immature 30S subunits. The complex of the 30S subunits and RbfA prepared by ultrafiltration was incubated in the absence and presence of RsgA with GTP. Aliquots of the reaction mixture were taken at various time points and RbfA that remained on the 30S subunit was obtained by ultrafiltration. The results are shown in Figure 5C. During incubation, RbfA gradually dissociated from mature 30S subunits (with a half-life of the complex of $\sim$ 60 min, estimated from the band intensities), while RbfA in complex with immature 30S subunits was relatively stable (with a half-life of $\geq$ 120 min). It was also found that RsgA accelerated the dissociation of RbfA from mature 30S subunits (the half-life of the complex decreased to $<$ 30 min) but not from immature 30S subunits (the half-life of the complex remained $\geq$ 120 min), reconfirming the preference of RsgA for mature 30S subunits over immature 30S subunits. We also examined the stability of the complex of mature or immature 30S subunits and rsgA-suppressing mutant forms of RbfA. From eight such RbfA mutants (Table I), we chose RbfA^{R10H} (RbfA from QIG121) and RbfA^{D120N} (RbfA from QIG26). Both of them dissociated from mature 30S subunit at rates comparable to that of dissociation of wild-type RbfA in the presence of RsgA (with a half-life of the complex of $<$ 30 min). In contrast, these mutants hardly dissociated from immature 30S subunit (with a half-life of the complex of $\geq$ 120 min).

Discussion

We found a novel genetic interaction between *rsgA* and *rbfA*; 29 independent mutations that confer improved growth on W3110 Δ rsgA strain (Figure 1A; Supplementary Figure 2A) occurred exclusively in *rbfA* (Table I). These mutations allow RbfA to release itself from the 30S subunit without the help of RsgA (Figures 2 and 5C) and lead to the suppression of impaired maturation of the ribosome due to the absence of RsgA (Figure 1E and F). We also found that RsgA-GTP enhanced release of RbfA from the 30S subunit *in vitro* (Figure 4) and that RsgA preferentially targets the 30S subunit in complex with RbfA rather than the free 30S subunit (Figure 3). All of these results indicate that the function of RsgA is to release RbfA from the 30S subunit.

We further determined the binding properties of RsgA and RbfA to mature and immature 30S subunits *in vitro* (Figure 5). As shown in Figure 5B, RbfA bound to both immature 30S subunit containing 17 RNA and mature 30S subunit containing 16S rRNA with a similar equilibrium

Figure 6 Schematic diagram of the involvement of RbfA and RsgA in the course of ribosome biosynthesis. Arrows with solid lines represent the processes established by this study. Arrows with dashed lines represent presumable processes.

affinity. In contrast, RsgA preferred mature 30S subunit to immature 30S subunit in terms of both enhancement of GTP hydrolytic activity (Supplementary Figure 5C) and equilibrium affinity (Figure 5A). These results suggest that RbfA binds to a pre-30S subunit and dissociates from nearly or completely matured 30S with the assistance of RsgA. Consistently, it was found that RbfA stays on immature 30S subunit with higher stability than that on mature 30S subunit in the absence of RsgA (Figure 5C). Furthermore, RsgA had little or no effect on the stability of the complex of RbfA and immature 30S subunit, while it accelerated the release of RbfA from mature 30S subunit (Figure 5C).

Based on these results, we propose the order of events in ribosome biosynthesis involving RbfA and RsgA as illustrated in Figure 6: (1) RbfA binds to the pre-30S subunit possibly containing 17S RNA, (2) maturation proceeds so that the pre-30S subunit is accessible by RsgA, (3) RsgA-GTP binds the 30S subunit to hydrolyze GTP and concomitantly RbfA dissociates from the 30S subunit and (4) RsgA-GDP dissociates from the 30S subunit. The timing of the action of RsgA in this scheme seems inconsistent with the fact that 17S RNA accumulates in *rsgA*⁻ strains. However, the accumulation of 17S RNA in *rsgA*⁻ cells could be explained as a consequence of impaired protein synthesis due to shortage of mature ribosomes in *rsgA*⁻ cells, considering that some kind of disturbance in protein synthesis, such as that arising from starvation of an amino acid (Dagley *et al*, 1963; Neidhardt and Eidlic, 1963) or exposure to a 50S subunit-targeting antibiotic, puromycin (Dagley *et al*, 1962; Hosokawa and Nomura, 1965), erythromycin (Siibak *et al*, 2009) or chloramphenicol (Lowry and Dahlberg, 1971; Himeno *et al*,

2004; Siibak *et al*, 2009), has been indicated to induce accumulation of 17S RNA.

The present results indicate that the interplay of RbfA and RsgA occurs during maturation of the 30S subunit. Yet, we cannot rule out the possibility that RbfA and RsgA also target mature 30S subunits during a cellular event other than ribosome biosynthesis, in consideration of the results shown in Figure 5. The presence of a substantial amount of RbfA in the 30S fraction from wild-type cells (Xia *et al*, 2003), which contain only a small amount of immature 30S subunits, might also support this possibility.

It has been shown that binding of RsgA-GTP to the 30S subunit is considerably inhibited by aminoglycosides that bind to the A site nucleotides of 16S rRNA (Himeno *et al*, 2004). In a cryo-EM map of the *Thermus thermophilus* 30S-RbfA complex, RbfA makes contact with the mRNA-binding cleft of the A and P sites, drastically altering the conformation around helix 44 of 16S rRNA (Datta *et al*, 2007). Consistently, RsgA-GDPNP alters chemical reactivities of several A site nucleotides, as well as other nucleotides around the P site and helix 44, presumably reflecting a conformational change in the 30S subunit (Kimura *et al*, 2008). The present study revealed preferential binding of RsgA-GTP to the 30S subunit in complex with RbfA (Figure 3). Thus, it is possible that RsgA-GTP prefers a distorted conformation around helix 44 of the 30S subunit formed by RbfA. Information on detailed structure of immature 30S subunit containing 17S RNA, which is currently unavailable, would provide an explanation for the differential-binding affinity of RsgA-GTP to mature and immature 30S subunits (Figure 5A) and the differential stability of the complex of RbfA with immature and mature 30S subunits (Figure 5C).

The interplay of RsgA and RbfA represents the first example of the participation of a bacterial GTPase in ribosome biosynthesis described at the molecular level. Meanwhile, functions of several eukaryotic GTPases in ribosome biosynthesis have been studied in detail at the molecular level (Senger *et al*, 2001; Hedges *et al*, 2005; Karbstein *et al*, 2005). Among these eukaryotic GTPases, *Saccharomyces cerevisiae* Efl1p (Senger *et al*, 2001) would be an interesting instance. It has been indicated that Efl1p releases Tif6p, a ribosome-binding factor, from the pre-60S subunit at a stage of maturation of the ribosome. Moreover, defects in cell growth and ribosome maturation due to depletion of Efl1p are suppressed by a mutation in Tif6p that weakens interaction with the ribosome. The relationship between Efl1p and Tif6p on the large subunit of the ribosome thus seems to be parallel to that between RsgA and RbfA on the 30S subunit described in the present report. However, overexpression of Tif6p suppresses defects due to depletion of Efl1p, while overexpression of RbfA does not suppress defects in cell growth (Supplementary Figures 3A and C; Campbell and Brown, 2008) and subunit assembly of the ribosome (Supplementary Figure 3B) due to depletion of RsgA. Therefore, recycling of Tif6p into the next pre-ribosome is likely to be a rate-limiting step during the ribosome maturation process in *S. cerevisiae*, while release of RbfA from the ribosome rather than recycling of RbfA to the next ribosome is likely to be a rate-limiting step during the ribosome maturation process in *E. coli*.

Both Era and RimM associate with the 30S subunit and depletions or some mutations of them lead to accumulation of 17S RNA, so that they are regarded as putative assembly

factors for the 30S subunit. *era* and *rimM* have genetic interactions with *rbfA* and *rsgA*: overexpression of *era* suppresses defects in growth and ribosome maturation of an *rbfA*-null mutant (Inoue *et al*, 2003, 2006); overexpression of *era* suppresses defects in growth and ribosome maturation of an *rsgA*-null mutant (Campbell and Brown, 2008); and increased expression of *rbfA* suppresses defects in growth and translation in a *rimM*-null mutant (Bylund *et al*, 1998, 2001). It has been assumed that these genetic interactions reflect concerted or complementary roles of the putative assembly factors, including RsgA, RbfA, Era and RimM, in ribosome biogenesis. However, the roles of these putative assembly factors other than RsgA, as well as the order of their participation, in biosynthesis of the 30S subunit remain uncertain. Unveiling them would be crucial to bridge the gap between our knowledge of ribosome assembly derived from *in vitro* reconstitution experiments and of actual ribosome biosynthesis in living cells. Incidentally, the function of RsgA indicated by the present study could reasonably connect the genetic interaction between *era* and *rbfA* with another interaction between *era* and *rsgA*; overproduction of Era, which bypasses the requirement of RbfA for optimal growth and ribosome maturation, might also bypass the requirement for RsgA, the releasing factor for RbfA, and consequently would suppress the defective phenotypes.

As shown by the present results, *E. coli* RbfA can easily acquire the capability to efficiently dissociate itself from the 30S subunit without the help of RsgA, which is competent for efficient maturation of the 30S subunit, even via a single-point mutation that is not restricted to a particular site (Table I; Supplementary Figure 1B). Similar events, in which an *rsgA*-dependent *rbfA* becomes converted into an *rsgA*-independent *rbfA* with a concomitant loss of *rsgA*, might have occurred also in evolutionary processes. In fact, *rsgA* seems phylogenetically less conserved than *rbfA*; to give an example, in a current version of COG (<http://www.ncbi.nlm.nih.gov/cog/>), a database comprised of clusters of orthologous groups of proteins, which has been delineated by comparing protein sequences encoded in complete genomes (Tatusov *et al*, 1997, 2003), the genes for COG1162, a group composed of orthologs of RsgA, are lacking in 10 bacterial genomes, while the genes for COG0858, composed of orthologs of RbfA, are conserved in all of the 50 bacterial genomes (Supplementary Table I).

The present results indicate that RbfA of *E. coli* requires RsgA to fulfill its role in the 30S subunit maturation process, which would explain pleiotropic phenotypes upon depletion of RsgA in *E. coli* (Arigoni *et al*, 1998; Himeno *et al*, 2004; Inoue *et al*, 2007; Campbell and Brown, 2008; Hase *et al*, 2009) and possibly also in other bacteria (Campbell *et al*, 2005, 2006; Cladière *et al*, 2006; Hunt *et al*, 2006; Absalon *et al*, 2008). However, the molecular basis for the role of RbfA itself in maturation of the 30S subunit remains equivocal. Mangiarotti *et al* (1975) found an activity in the 30S fraction, which facilitates *in vitro* assembly of ribosomal proteins with pre-16S rRNA but not with mature 16S rRNA into the 30S particle at a low temperature (0°C). Based on this observation, they predicted a factor for biosynthesis of the 30S subunit which binds to the pre-30S subunit containing pre-16S rRNA and dissociates from the 30S subunit containing mature 16S rRNA. RbfA, as revealed by the present study, would be such a factor. It has also been proposed that RbfA

prevents the pre-30S subunit from prematurely initiating translation by masking the mRNA path and shifting the anti-Shine–Dalgarno sequence of 16S rRNA on the basis of a cryo-EM structure of the 30S subunit in complex with RbfA (Datta *et al*, 2007). If this is the case, release of RbfA by RsgA might occur at the last step of maturation of the 30S subunit, which would allow the start of translation cycles, that is, RsgA might work as the finishing factor of maturation of the 30S subunit.

Materials and methods

Bacterial strains

We used W3110, an *E. coli* K12 derivative, as a wild-type strain. W3110Δ*rsgA* is identical to W3110Δ*yjeQ* previously described (Himeno *et al*, 2004), a W3110 derivative in which the *rsgA* gene is disrupted by a kanamycin-resistant gene. QIG1, QIG4, QIG11, QIG15, QIG26, QIG107, QIG110, QIG121 and QIG128, derivatives of W3110Δ*rsgA* with restored growth, were isolated in the present study. W3110Δ*rbfA* is a W3110 derivative in which the *rbfA* gene is replaced by an in-frame scar encoding a short peptide-encoding gene using a Red recombinase system (Datsenko and Wanner, 2000) with a set of primers, 5'-AAAACGCGTTAATGTCGCGACCGCGACGACGAGGACGACTCATTAGTGTGTAGCTGGAGCTGCTTCG-3' and 5'-TTTAAAAAGGGGCTAACAGCCCTTTTGTTCAGGAGAATTTATTA TGATTCGGGGATCCGTCGACC-3', in combination with the standard P1 transduction. W3110Δ*rsgA*Δ*rbfA* was constructed by transferring the kanamycin-resistant marker of W3110Δ*rsgA* into W3110Δ*rbfA* via P1 transduction. For expression of recombinant RsgA and RbfA, we used *E. coli* BL21(DE3) (Promega Corporation). Cultures of constructed strains were grown at 37°C in LB medium with agitation; ampicillin (50 µg/ml) was added to the medium for strains harbouring a plasmid with an ampicillin-resistant marker (pUC26-2, pMW118, pMW*rsgA*, pMW*rbfA*⁺ or its variants and pGEM*rbfA* or its variants), and chloramphenicol (10 µg/ml) was added to the medium for strains harbouring a plasmid with a chloramphenicol-resistant marker (pCA24N- and pCA*rbfA*).

Isolation of mutant strains with restored growth from an *rsgA* disruptant

Single colonies of W3110Δ*rsgA* were separately inoculated into liquid LB media, grown overnight and spread on LB agar plates. A drop of saturated solution of a mutagen, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, was spotted at the centre of each plate, which was incubated overnight at 37°C. One large colony that formed around the mutagen was isolated from each plate. A total of 29 mutant strains were obtained.

Identification of the sites of mutations

A DNA library in a high-copy number plasmid vector, pUC18, was constructed from genomic DNA of QIG26 strain partially digested with a restriction enzyme, *Sau3A*I. The library was introduced into W3110Δ*rsgA* and screened for clones that suppress growth deficiency, yielding a large colony. One clone, designated pUC26-2, was obtained and re-introduced into W3110Δ*rsgA* to confirm that it possesses activity for suppression of the growth defect. Subsequent DNA sequencing revealed that pUC26-2 has an insert, 2427 bp in length, which includes a 3' part of *infB*, full-length *rbfA* and a 5' part of *truB* (Supplementary Figure 1A). The entire nucleotide sequence of the insert was the same as the counterpart of a wild-type *E. coli* strain, MG1655 (GenBank™ U00096.2), with the exception of position 358 of *rbfA*, which is G in the clone instead of A in the wild type. We confirmed that chromosomal DNA of QIG26 has the same mutation in *rbfA* and that of the parental strain W3110Δ*rsgA* does not. The chromosomal *rbfA* gene from each of the 28 mutant strains besides QIG26 was subjected to direct sequencing and was revealed to possess one of the eight kinds of point mutations including G358A. All eight kinds of mutant *rbfA* genes were subcloned into a low-copy number plasmid vector, pMW118, and confirmed to possess activity to suppress the growth defect of W3110Δ*rsgA* strain.

Plasmids

pMWrsGA, which is identical to pMW119yeQ previously described (Himeno *et al*, 2004), is a low-copy number plasmid clone containing a wild-type *rsgA* gene. pMWrbfA⁺ is a low-copy number plasmid with wild-type *rbfA*, which contains an *EcoRI*–*BamHI* fragment of wild-type *rbfA* gene amplified by PCR using primers 5'-ATCGAAGAATTTCGAAATCATCGAGATCC-3' and 5'-CCCTGAGGATCCAGCAACAAAACGC-3' at the multiple cloning site of plasmid vector pMW118 (Nippon Gene Co, Ltd). pMWrbfA1, pMWrbfA4, pMWrbfA11, pMWrbfA15, pMWrbfA26, pMWrbfA107, pMWrbfA110, pMWrbfA121 and pMWrbfA128 are variants of pMWrbfA⁺ that contain point mutations of the *rbfA* gene of QIG1, QIG4, QIG11, QIG15, QIG26, QIG107, QIG110, QIG121 and QIG128, respectively, instead of wild-type *rbfA*; each of them contains an *EcoRI*–*BamHI* fragment of PCR-amplified *rbfA* gene of the corresponding strain at the multiple cloning site of pMW118. pGEMrbfA⁺ is a plasmid for overproduction of RbfA, which contains a *HindIII*–*XbaI* fragment of wild-type *rbfA* amplified by PCR using primers 5'-TTTTCTAGAAGGAGAATTTATTATGCGCAAAG-3' and 5'-TTTGCATGCGTCGACGACTCATTAGTCTCC-3' at the multiple cloning site located downstream of the T7 promoter of plasmid vector pGEMEX-2 (Promega Corporation). pGEMrbfA26 and pGEMrbfA121 are variants of pGEMrbfA⁺ that contain PCR-amplified mutant *rbfA* genes of QIG26 and QIG121, respectively. The absence of unintended mutations in each insert was confirmed by DNA sequencing.

High-copy number plasmids, pCArbfA and pCA24N⁻, are clones of ASKA library GFP(-), a comprehensive *E. coli* K12 ORF plasmid library (Kitagawa *et al*, 2005). pCArbfA is a clone having the *rbfA* gene at the cloning site under the control of the IPTG-inducible T5-*lac* promoter. pCA24N⁻ is a control plasmid having no *E. coli* gene at the cloning site, which has been produced by removing the GFP gene from pCA24N (Kitagawa *et al*, 2005). We have confirmed that W3110Δ*rbfA* cells harbouring pCArbfA exhibited restored growth and ribosome profile indistinguishable from those of W3110 cells harbouring pCA24N⁻ when they were cultivated at 37°C in LB medium containing 10 µg/ml chloramphenicol in the absence of IPTG. We have also confirmed that the level of RbfA in the S100 fraction of W3110Δ*rsgA* cells cultivated at 37°C in LB medium containing 10 µg/ml chloramphenicol in the absence of IPTG was elevated when pCArbfA was introduced into the cells.

Assay of GTPase activity

Reaction was carried out at 37°C in 20 µl of 50 mM Tris–HCl (pH 7.5), 200 mM KCl, 5 mM MgCl₂, 1 mM DTT, 2.5 mM unlabelled GTP, α-[³²P] GTP and 300 nM RsgA with indicated amounts of RbfA and 30S subunits. After 30 or 60 min of incubation, the reaction was stopped by adding 50 µl of 20 mM EDTA. It was then spotted on a PEI cellulose sheet for thin-layer chromatography. After development using 0.75 M KH₂PO₄ as a solvent, the radioactivities of the spots of GTP and GDP were monitored using a Bio-Image-Analyzer FLA3000 (Fuji Film). The GTPase activity continued to increase linearly until 60 min, and the initial velocity was calculated from GTP consumption at 30 min.

Preparations of ribosomal subunits

Mature and immature 30S subunits were prepared from 70S and 30S fractions, respectively, separated from crude ribosomes as follows. The buffers used were buffer A, which contains 10 mM Tris–HCl (pH 7.8), 10 mM MgCl₂, 60 mM NH₄Cl and 1 mM DTT, buffer B, which is the same as buffer A but with 0.1 mM MgCl₂, and buffer C, which is the same as buffer A but with 10% (v/v) glycerol. Crude ribosomes were prepared from cell lysate by successive centrifugations as previously described (Himeno *et al*, 2004). The crude ribosomes were layered on 5–20% sucrose density gradient in buffer A and centrifuged at 25 000 r.p.m. for 5 h with a P28S rotor (Hitachi). The 70S and 30S fractions of the gradient were collected and pelleted by centrifugation at 80 000 r.p.m. for 60 min with an RP80AT rotor (Hitachi). The pellet of the 30S fraction was suspended in buffer C and stocked as immature 30S subunits at –80°C. The pellet of the 70S fraction was suspended in buffer B, layered on 5–20% sucrose density gradient in buffer B, and centrifuged at 25 000 r.p.m. for 8 h with P28S. The 30S fraction of the gradient was collected and pelleted by centrifugation at 80 000 r.p.m. for 60 min with RP80AT. The resulting pellet was suspended in buffer C and stocked as mature 30S subunits at –80°C. The mature and immature 30S subunits prepared from W3110Δ*rsgA*Δ*rbfA* cells were used in the experiment for which

results are shown in Figure 5. The mature 30S subunits prepared from W3110 cells were used in other *in vitro* experiments. Concentration of mature or immature 30S subunit was determined with the conversion factor of 72 pmol to 1 A₂₆₀ unit.

Sedimentation profile

The extract from 10 mg (wet weight) of log-phase cells was loaded on 5–20% (w/v) linear sucrose density gradient containing 10 mM Tris–HCl (pH 7.8), 10 mM MgCl₂, 60 mM NH₄Cl and 1 mM DTT, and it was centrifuged at 36 000 r.p.m. using a P40ST rotor (Hitachi) for 3 h at 4°C. The gradient was divided into 20 fractions with monitoring of A₂₆₀ during fractionation. One-third of each fraction was subjected to precipitation with acetone followed by SDS–PAGE. RbfA was detected by western immunoblotting with an antibody raised against RbfA.

Preparation of RsgA

RsgA was prepared from *E. coli* BL21(DE3) strain (Promega Corporation) engineered for overexpression of recombinant RsgA as previously described (Himeno *et al*, 2004).

Preparation of RbfA or its variants

pGEMrbfA⁺, pGEMrbfA26 or pGEMrbfA126 was introduced into *E. coli* BL21(DE3). Cells were grown to an OD₆₀₀ of ~0.5 and then overexpression of RbfA was induced with 1 mM IPTG for 3 h. Cells suspended in a buffer containing 20 mM sodium phosphate (pH 5.8), 50 mM KCl and 1 mM DTT were lysed by sonication. The lysate was clarified twice by centrifugations at 10 000 and 100 000 g. The cleared lysate was loaded on an SP-Sepharose column and eluted by a linear gradient of 50–300 mM of KCl, and the fractions containing RbfA were collected. The buffer was exchanged for stock buffer containing 10 mM Tris–HCl (pH 7.8), 10 mM MgCl₂, 60 mM NH₄Cl, 1 mM DTT and 50% glycerol (v/v).

Ultrafiltration assay

The 30S subunits (50 nM) with an excess of RbfA (500 nM) were incubated for 30 min at 37°C in 250 µl of a buffer containing 10 mM Tris–HCl (pH 7.8), 150 mM NH₄Cl, 7 mM MgCl₂ and 1 mM DTT. The reaction mixture was loaded onto Microcon YM-100 (Millipore) and centrifuged for 10 min at 10 000 g. The filter was washed twice with 200 µl of the same buffer by centrifugation for 5 min at 10 000 g. Then 200 µl of the same buffer was added to the filter to recover the complex of the 30S subunits with RbfA by brief centrifugation with an inverted filter cup. Indicated amounts of RsgA and guanine nucleotide in 10 µl of the same buffer were mixed with 40 µl of the complex of the 30S subunits and RbfA. After incubation for the indicated period at 37°C, the reaction mixture was loaded onto YM-100 and centrifuged for 3 min at 10 000 g. The filter was washed twice with 100 µl of the same buffer by centrifugation for 5 min at 10 000 g. Then 200 µl of the same buffer was added to the filter to recover the complex of the 30S subunits with RbfA by brief centrifugation with an inverted filter cup. The resulting solution was subjected to precipitation with acetone followed by SDS–PAGE. RbfA was detected by western immunoblotting with an antibody raised against RbfA.

FCS measurement

RsgA or RbfA was labelled with TAMRA using the Protein Labeling Kit MF-543PX (Olympus) according to the manufacturer's instructions. Indicated amounts of 30S subunits, labelled or unlabelled RsgA or RbfA and nucleotides in 50 µl of solution containing 10 mM Tris–HCl (pH 7.8), 10 mM MgCl₂, 60 mM NH₄Cl, 1 mM DTT and 0.05% Tween 20 were incubated for 60 min at room temperature. The reaction mixture was applied to a glass-bottomed microplate and it was set in MF20 (Olympus). The measurement was performed using the FCS method with excitation wavelength at 543 nm and laser power of 100 µW. Data acquisition time was 5 s per measurement.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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