Structural basis for (p)ppGpp-mediated inhibition of the GTPase RbgA

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Efficient adaptation to environmental changes is pivotal for all bacterial cells. Almost all bacterial species depend on the conserved stringent response system to prompt timely transcriptional and metabolic responses according to stress conditions and nutrient depletion. The stringent response relies on the stress-dependent synthesis of the second messenger nucleotides and alarmones (p)ppGpp, which pleiotropically target and reprogram processes that consume cellular resources, such as ribosome biogenesis. Here we show that (p)ppGpp acts on the ribosome biogenesis GTPase A (RbgA) of Gram-positive bacteria. Using X-ray crystallography, hydrogen–deuterium exchange MS (HDX-MS) and kinetic analysis, we demonstrate that the alarmones (p)ppGpp bind to RbgA in a manner similar to that of binding by GDP and GTP and thereby act as competitive inhibitors. Our structural analysis of Staphylococcus aureus RbgA bound to ppGpp and pppGpp at 1.8 and 1.65 Å resolution, respectively, suggested that the alarmones (p)ppGpp prevent the active GTPase conformation of RbgA by sterically blocking the association of its G2 motif via their 3'-pyrophosphate moieties. Taken together, our structural and biochemical characterization of RbgA in the context of the alarmone-mediated stringent response reveals how (p)ppGpp affects the function of RbgA and reprograms this GTPase to arrest the ribosomal large subunit.

Rapidly dividing bacterial cells depend on an effective translational machinery to maintain their fast growth rate. At the heart of this machinery, ribosomes translate mRNA into proteins. However, functional ribosomes have to be assembled in an efficient manner to meet the high demand on the translational machinery during cell proliferation. In Escherichia coli, ribosome assembly is estimated to take approximately 2 min with a corresponding assembly rate of 100,000 ribosomes/h (1). Bacterial ribosome biogenesis involves the initial transcription of a ∼5-kb primary rRNA transcript that is co-transcriptionally cleaved and modified to yield three mature rRNAs (23S, 16S, and 5S) that provide a platform for assembly of the large (50S) and small (30S) ribosomal subunits. Folding of the rRNA occurs co-transcriptionally and is accompanied by the hierarchically variable and block-wise incorporation of ∼50 ribosomal proteins (r-proteins) (2). The assembly process involves a set of ∼100 ribosome biogenesis factors to facilitate cleavage, modification, and chaperoning of intermediates in both the 50S and 30S biogenesis pathways (3). Therefore, ribosome biogenesis imposes a high metabolic load on bacterial cells and has to be precisely regulated during nutrient starvation to preserve cellular resources. In many bacterial species, ribosome biogenesis is regulated by the stringent response system that senses stress stimuli and signals the stress level via the pleiotropically acting nucleotide messenger alarmones (p)ppGpp (4, 5). Upon stress, such as restricted nutrient availability, RSH (RelA/SpoT homologue)-type proteins produce (p)ppGpp by transfer of pyrophosphate from ATP onto the 3'-OH moiety of GTP or GDP. Eventually, when environmental conditions ameliorate, (p)ppGpp is hydrolyzed by RSH-type hydrolases to retrieve GTP/GDP and consequently stress signaling declines. Alarmone-mediated regulation of ribosome biogenesis not only includes the repression of rRNA and r-protein gene transcription to shut down production of ribosomal components but may also involve the inhibition of ribosome biogenesis factors to block the assembly of ribosomal subunits (4, 6). In particular, the Staphylococcus aureus ribosome biogenesis associated GTPases RbgA, HfIX, Era, RsgA, and ObgE have been recently shown to be directly targeted by (p)ppGpp to suppress GTPase activity (6). It has been hypothesized that the (p)ppGpp-mediated GTPase activity suppression prevents the final ribosome subunit maturation step and might therefore arrest subunits before they engage as matured subunits in 70S formation and translation (6).

The 50S subunit ribosome biogenesis GTPase RbgA (Ribosome biogenesis GTPase A; also called YlqF) has been shown to be essential for growth in Bacillus subtilis (7). Depletion of RbgA leads to a reduction of 70S ribosomes resulting from an arrest of large subunit biogenesis at premature 45S particles that lack the ribosomal proteins L16, L27, L28, L33, L36, and L37 and might be incompetent in 70S formation (8–12). RbgA homologues (YlqF-related GTPase, YRG family) are evolution-
upon 60S subunit completion (GTPase-dependent release of the nuclear export adapter Nmd3 subunit maturation and was shown to be involved in the RbgA homologue Lsg1 acts late during the final ribosomal large subunit and a C-terminal ligand-dependent movement of the C-terminal domain, which hypothesized that the cpGTPase fold might allow a nucleotide k loop for k

Table 1
Crystallographic table
Statistics for the highest-resolution shell are shown in parentheses.

| Structure     | SaRbgA–GDP | SaRbgA–GMPPNP | SaRbgA–ppGpp | SaRbgA–pppGpp |
|---------------|------------|---------------|--------------|---------------|
| PDB code      | 6G0Z       | 6G12          | 6G14         | 6G15          |
| Data collection |            |               |              |               |
| Space group   | P 2 1 2 2 1 | P 2 1 2 2 1   | P 2 1 2 2 1  | P 2 1 2 2 1   |
| Cell dimensions |           |              |              |               |
| a (Å)         | 71.932     | 71.899        | 71.781       | 72.084        |
| b (Å)         | 77.812     | 77.71         | 74.512       | 78.467        |
| c (Å)         | 124.667    | 124.36        | 125.215      | 125.022       |
| α (°)         | 90.00      | 90.00         | 90.00        | 90.00         |
| β (°)         | 90.00      | 90.00         | 90.00        | 90.00         |
| γ (°)         | 90.00      | 90.00         | 90.00        | 90.00         |
| Energy (k)    | 0.97625    | 0.97903       | 0.97625      | 0.97625       |
| Resolution (Å) | 48.65–2.15 | 62.24–1.93 (2.00–1.93) | 47.93–1.80 (1.864–1.80) | 48.89–1.65 (1.71–1.65) |
| No. unique reflections | 38,760 (3822) | 52,769 (5238) | 62,299 (6048) | 85,889 (8495) |
| Rmerge (k)    | 0.041 (0.277) | 0.056 (0.332) | 0.106 (0.555) | 0.035 (0.989) |
| l/d of | 8.79 (2.45) | 6.36 (2.08) | 7.88 (1.74) | 24.70 (1.85) |
| Completeness (%) | 99.9 (99.9) | 99.2 (99.5) | 99.0 (97.5) | 100.0 (99.9) |
| Redundancy    | 2.0 (2.0)  | 2.0 (2.0)     | 4.2 (4.2)    | 7.4 (7.4)     |
| CC1/2         | 1.00 (0.88) | 1.00 (0.54)   | 1.00 (0.94)  | 1.00 (0.81)   |
| Refinement    |            |               |              |               |
| Resolution (Å) | 48.65–2.15 | 62.24–1.93 | 47.93–1.80 | 48.89–1.65 |
| Rwork/Rfree  | 0.041/0.277 | 0.056/0.332 | 0.106/0.555 | 0.035/0.989 |
| No. atoms     | 4989       | 5026          | 5020         | 5090          |
| Macromolecule | 4636       | 4636          | 4541         | 4611          |
| Ligand        | 56         | 64            | 72           | 80            |
| Water         | 297        | 326           | 407          | 399           |
| r.m.s.d.      |            |               |              |               |
| Bond lengths (Å) | 0.011     | 0.008         | 0.009        | 0.007         |
| Bond angles (°) | 1.30      | 1.12          | 1.19         | 1.11          |
| Ramachandran (%) | 98        | 98            | 99           | 97            |

Results

Structures of S. aureus RbgA bound to GDP and GMPPNP

Typically, GTPases undergo conformational rearrangements upon hydrolysis of GTP to GDP and the subsequent release of P

RbgA homologue Lsg1 acts late during the final ribosomal large subunit maturation and was shown to be involved in the GTase-dependent release of the nuclear export adapter Nmd3 upon 60S subunit completion (15–17). RbgA belongs to the TRAFAC (translation factor) GTase family and comprises a N-terminal Rossmann fold GTP-binding domain (G domain) and a C-terminal α-helical domain (18). The G domain features a K loop for K

Crystal structures of guanosine nucleotide-bound RbgA homologues of Thermotoga maritima (18) and B. subtilis (PDB code 1PUJ) are available and reveal the N-terminal GTase fold followed by an α-helical C-terminal putative RNA-binding domain. However, the molecular details of ribosome interaction and the mechanism of GTase activation for 50S maturation have remained enigmatic. Furthermore, the mechanism by which the stringent response alarmone (p)ppGpp blocks the GTase activation of RbgA to arrest the maturation of large ribosomal subunits is also unknown.

Here we present high-resolution X-ray crystal structures of S. aureus RbgA in complex with GDP, GMPPNP, ppGpp, and pppGpp. Dynamic and kinetic analysis shows that the alarmones (p)ppGpp act as competitive GTase inhibitors of RbgA. Comparison of ribosome-free RbgA with the ribosome-associated GTase active state of the eukaryotic RbgA homologue Lsg1 suggests how RbgA GTase activation is triggered at the large subunit and inhibited by (p)ppGpp. Taken together, our structural and biochemical analyses of RbgA reveal how the GTase active conformation is suppressed by (p)ppGpp to arrest large ribosomal subunits during the stringent response.

The abbreviations used are: GMPPNP, guanosine-5′-[(β,γ)-imido]triphosphate; r.m.s.d., root-mean-square deviation; HDX, hydrogen–deuterium exchange; MST, microscale thermophoresis; SEC, size-exclusion chromatography; HDMS, high-definition MS; PTC, peptidyl-transferase center; MPD, 2-methyl-2,4-pentanediol.
Figure 1. Crystal structures of S. aureus RbgA bound to GDP and GTP. 

A, amino acid sequence of RbgA illustrating the domain arrangement (N-terminal G domain and C-terminal RNA-binding domain). G motifs are highlighted in turquoise and labeled according to the identity (G1–G5). 

B and C, crystal structure of RbgA (gray cartoon representation) in complex with GDP (B) and GMPPNP (C) (yellow stick representation) in two 180° rotated views. Secondary structure elements are labeled according to their identity, and N and C indicate the respective termini. The G1–G5 motifs are colored turquoise and are labeled accordingly. 

D and E, detailed view on the GTPase active sites with the accommodated nucleotide GDP (D) and GMPPNP (E). Coloring is as in B. G motif and adjacent site chains are shown in stick representation and are labeled according to their identity.
GTpase active site of RbgA (Fig. S1, A and B). The guanine base is accommodated by stacking interactions of lysine 88 and lysine 59 and distinguished by hydrogen-bonding interactions of aspartate 86 and 61 and asparagine 58 of the G5 and G4 GTpase motifs (Fig. 1, D and E). Amino acids 129–134 of the G1 motif contribute to coordination of the α-, β-, and γ-phosphate moieties via hydrogen bonding and salt-bridge interactions (Fig. 1, D and E). The γ-phosphate of GMPNP is furthermore surrounded by the nonpolar side chains of proline 129 and isoleucine 175 of the G1 and G3 motifs and is less well-defined in the electron density map than the α- and β-phosphate moieties (Fig. S1B). Thus, RbgA coordinates its GDP and GTP nucleotides similar to the T. maritima (18) and B. subtilis (PDB code 1PUJ) RbgA homologues. Despite the different nucleotide content, no significant structural differences between the GDP and GMPNP-bound state could be observed. The root-mean-square deviation (r.m.s.d.) between both structures and the individual monomers in the unit cell was below 0.2 Å (Table S1 and Fig. S2, A–E). This observation is substantiated by the fact that both states of RbgA crystallized under different crystallization conditions but in the same space group with identical cell dimensions (Table 1).

Structures of ppGpp- and pppGpp-bound RbgA

The alarmones (p)ppGpp inhibit the GTpase activity of RbgA, yet the underlying molecular mechanism has remained unknown (6). Therefore, we determined the crystal structures of RbgA bound to ppGpp and pppGpp at 1.8 and 1.65 Å resolution, respectively (Fig. 2, A and B, and Table 1). Within the unbiased electron density maps, we could unambiguously identify either ppGpp or pppGpp (Fig. S1, C and D). The GDP and GTP moieties of ppGpp and pppGpp, respectively, associate to the active site in an identical fashion as their native nucleotide counterparts. The 3′-pyrophosphate moieties of both alarmones point away from the active site toward the solvent and seem to be stabilized only by the ε-aminogroup of lysine 88 of the G5 motif (Fig. 2, A and B). However, lysine 88 is not conserved among RbgA homologues arguing against a substantial role of this residue for the coordination of (p)ppGpp to RbgA (Fig. S3). This is further supported by comparable binding constants of RbgA for GDP, GTP, ppGpp, and pppGpp as determined by microscale thermophoresis (MST) (Fig. 2C and Fig. S4, A–D). Structural comparison of the alarmon-bound states of RbgA with its GDP/GMPNP-bound states revealed no significant structural differences as indicated by the low r.m.s.d. (< 0.3 Å) (Table S1 and Fig. S2, A–E). These findings suggest that the alarmones do not alter the overall conformation of RbgA.

RbgA binds nucleotides in the absence of magnesium

Our inspection of the electron density maps of the presented structures did not show electron density for magnesium, although magnesium was present in the final size-exclusion buffer at a concentration of 20 mM. The absence of magnesium is also true for the crystal structures observed for the T. maritima RbgA in complex with GDP, GTP, and GMPNP (PDB codes 3CNN, 3CNO, and 3CNL (18)). Because the Mg²⁺ ion co-factor is essential for catalysis, we reasoned that our structures and the previously reported structure of the T. maritima homologue may not represent the GTpase active conformation of RbgA. In canonical GTpases, binding of Mg⁡₂⁺ in a tetragonal bipyramidal coordination sphere is facilitated by the G1, G2, and G3 motifs. Serine/threonine of the G5 motif GXXXGK(S/T) (P loop) forms a direct contact, whereas aspartate of the G3 motif DXXG forms a water-mediated contact to the Mg⁡₂⁺, which is required for tight binding of the co-factor (22). The coordination sphere is completed by a contact via the conserved threonine of the G2 motif. However, our crystal structures revealed that although the G1 motif is in a position capable of interacting with a properly placed Mg⁡₂⁺ ion, the G2 (switch I) and G3 (switch II) motifs are positioned in a manner apparently not allowing interaction with the Mg⁡₂⁺ ion (Fig. 3A). Comparison of our structure and the T. maritima RbgA structure with the GTP-bound B. subtilis RbgA suggested that a G3 rearrangement would be required for GTP and Mg⁡₂⁺ co-factor binding (Fig. 3, B and C, and Fig. S5). It is noteworthy that the G3 motif is directly connected to the putative C-terminal RNA-binding domain via a linker and rearrangement of the C-terminal domain upon contact with the large subunit might allow proper positioning of the G3 motif and GTP and Mg⁡₂⁺ co-factor binding or vice versa. Mutation of the conserved phenylalanine at position 180 to alanine in the G3 linker region has been shown to be lethal for B. subtilis RbgA in

Figure 2. Crystal structures of S. aureus RbgA bound to ppGpp and pppGpp. A and B, left panels, crystal structure of RbgA (gray cartoon representation) in complex with ppGpp (A) and pppGpp (B) (yellow stick representation). Secondary structure elements are labeled according to their identity, and N indicates the N terminus. The G1–G5 motifs are labeled accordingly. Right panels, close-up on the GTP binding site. Lys-88 (blue stick representation) of the G5 motif is in close proximity to the ε-phosphate moiety. C, binding and dissociation constants for GTP, GDP, ppGpp, or pppGpp and RbgA as determined by MST. Error bars represent the standard deviation of the calculated Kₘ or Kₜ values based on the fitting of the respective experimental data (Fig. S4).
vivo, underlining the importance of this region (21). In summary, displacement of the G2 and G3 motifs in absence of the proper RNA contact site might result in co-factor release and GTPase suppression.

Conformational dynamics of RbgA

As suggested by our crystal structures of RbgA, the conformation of RbgA might not necessarily be dictated by the identity of the bound nucleotide. However, crystal-packing contacts might indirectly affect the configuration of the G motifs by impacting the domain orientation of the N-terminal G domain and the adjacent C-terminal RNA-binding domain (Fig. S6). To investigate the role of GDP, GMPNP, ppGpp, and (p)ppGpp on the conformational dynamics of RbgA in solution, we performed hydrogen–deuterium exchange MS (HDX-MS). The HDX-MS analysis revealed that GDP, GMPNP, and (p)ppGpp bind into the canonical guanosine nucleotide-binding site of RbgA (Fig. 4A). In particular, we observed a reduction in HDX of the nucleotide-bound RbgA when compared with apo-RbgA in the regions that contain the G motifs, demonstrating that the guanosine-binding site becomes stabilized upon nucleotide binding. It is noteworthy that we identified pronounced differences in HDX for GMPNP compared with GDP and (p)ppGpp. Four regions (R1–R4) that contain the G4, G5, G1, and G3 motifs were less protected from HDX in complex with GMPNP (Fig. 4, A and C), suggesting that GMPNP-bound RbgA might exist in a different conformation than observed for the GDP- or (p)ppGpp-bound states. It is important to note that HDX-MS yields a time-averaged snapshot of multiple states and hence, different rates in ligand association and dissociation might affect the observed HDX. Interestingly, we also observed a difference in HDX between the 5'-diphosphate (GDP and ppGpp) and 5'-triphosphate (GTP and pppGpp) nucleotides in region 4, which includes the G3 motif (switch II). The increased protection of region 4 observed for the 5'-diphosphate nucleotides shows that the G3 motif is less stabilized in presence of the 5'-triphosphate nucleotides and is indicative for a conformational change in this region. This observation supports the idea that the G3 motif rearranges upon association of the 5'-triphosphate nucleotides (compare with Fig. 3 and Fig. S5). It is noteworthy that the G3 motif is directly connected to the RNA-binding domain of RbgA and consequently, the identity of the nucleotide bound might change the relative domain orientation of RbgA. However, we did not observe a difference in HDX for the C-terminal RNA-binding domain of RbgA. Summed up, the analysis by HDX-MS revealed that (p)ppGpp binds to the GDP/GTP binding site of RbgA as also observed in our crystal structures. Furthermore, the analysis showed that RbgA adopts different conformations in solution depending on the identity of the nucleotide, which is in contrast to the virtually identical conformation observed in the crystal structures and suggests that crystal packing might have influenced the conformation observed in our structures.

RbgA GTPase inhibition by (p)ppGpp

Comparison of the crystal structure of alarmone-bound S. aureus RbgA, B. subtilis RbgA (PDB code 1PUJ), and the cryo-EM structure of ribosome associated S. cerevisiae Lsg1...
observed for GDP/GTP. Hence (p)ppGpp might act as a competitive inhibitor of GTP hydrolysis. To test this idea, we performed a kinetic analysis of RbgA’s GTPase activity. We determined the initial velocity of GTP hydrolysis by RbgA at different GTP concentrations in presence of ppGpp or pppGpp with or without purified 50S ribosomal subunits. Visualization of the initial velocities of GTP hydrolysis in the Lineweaver–Burk diagram (1/initial velocity versus 1/GTP) revealed that increasing concentrations of ppGpp or pppGpp result in a strong shift of the x intercept toward the coordinate origin, while the y intercept does not change considerably, which is characteristic for a competitive mode of inhibition (Fig. 6B). Conversion of the x and y intercepts from the Lineweaver–Burk diagram into 

\[ K_m \text{ and } V_{\text{max}} \] values substantiates this notion. The apparent 

\[ K_m \] values increase in a dose-dependent manner with (p)ppGpp, whereas the maximum velocities remain largely unchanged, suggesting that (p)ppGpp acts as a competitive inhibitor (Table S2). Given the caveats of the Lineweaver–Burk representation, we also plotted the initial velocities of GTP hydrolysis by RbgA against the concentration of GTP (v/S characteristic, Fig. S7). Although 

\[ V_{\text{max}} \] was not reached, fitting of the data according to the equation 

\[ v = \frac{V_{\text{max}} S}{K_m + S} \] resulted in a curve convincingly represented by our data. The derived values for 

\[ K_m \] and 

\[ V_{\text{max}} \] provide evidence for a strictly competitive inhibition as evidenced by 

\[ K_m \] values that increase with higher (p)ppGpp concentrations, whereas 

\[ V_{\text{max}} \] values remain constant (Table S3).

To determine the degree of inhibition, we extracted the inhibitory constants (\( K_i \)) for both alarmones from the v/S characteristic (compare with Fig. S7) using the equation 

\[ v = \frac{V_{\text{max}} S}{K_i S + S} \] yielding 

\[ K_i \] values of 

\[ 77 \pm 6 \text{ and } 337 \pm 50 \mu M \] for ppGpp and 

\[ 372 \pm 23 \text{ and } 829 \pm 115 \mu M \] for pppGpp in absence or presence of 50S ribosomal subunits, respectively (Fig. 6C and Table S4). It is noteworthy that in presence of the 50S ribosomal subunits, RbgA activity is 2- and 5-fold less inhibited by pppGpp and ppGpp, respectively, as evidenced by the shift in their 

\[ K_i \] values. Taken together, our results provide evidence that ppGpp and pppGpp act as competitive inhibitors of RbgA GTPase activity in the absence and presence of the 50S ribosomal subunits.

**Discussion**

**Mechanism of RbgA GTPase inhibition by (p)ppGpp**

In this study, we revealed how the stringent response alarmones (p)ppGpp suppresses the GTPase activity of the evolutionary widely conserved and essential large ribosomal subunit biogenesis cgGTPase RbgA. Our crystal structures of S. aureus RbgA suggest that ribosome-free RbgA might exist in a conformation that is incompatible with hydrolysis of GTP, characterized by the displaced G2 and G3 motifs (switch I + II) that leads to a deficiency of the active site coordination of Mg\(^{2+}\) and K\(^+\). However, our structural dynamics analysis by HDX-MS showed that RbgA undergoes structural rearrangements in solution depending on the identity of the associated nucleotide, which illustrates that the identical configuration observed in the crystal structures might not necessarily be the same in solution. We could show that the dynamics observed for GMPPNP-
Figure 6. Alarmones competitively inhibit the GTPase activity of B. subtilis RbgA. A, B. subtilis RbgA was incubated with GTP, ppGpp, or pppGpp in the absence (gray bars) or presence (black bars) of 50S subunits, and its hydrolytic activity was determined by HPLC. Error bars indicate standard deviations derived from three individual measurements. B, Lineweaver–Burk plots of B. subtilis RbgA GTPase activity without or with purified 50S subunits in the presence of increasing concentrations of ppGpp (left panels) or pppGpp (right panels). The GTP concentration and initial velocity are given in mmol min⁻¹ and mmol RbgA⁻¹ min⁻¹, respectively. C, the initial velocities of GTPase activity of B. subtilis RbgA in presence of 1 mm GTP and increasing amounts of ppGpp (red line, squares) or pppGpp (green line, circles). Solid and dashed lines indicate the presence or absence of 50S subunits, respectively. Error bars represent standard deviations, derived from triplicates. The inhibitory constants (Kᵢ) are shown on the right side.

Our data support the idea that (p)pGpp likely sequesters RbgA-containing 45S and 50S particles because of GTPase suppression, which is substantiated by the observation that ppGpp increases the affinity of RbgA for mature 50S subunits (19). This, in turn, might withdraw mature 50S subunits from the formation of translationally active 70S ribosomes and consequently shuts down not only ribosome maturation but also protein production to economize cellular resources during starvation (6, 19). Interestingly, we demonstrated in our enzyme kinetic analysis that ppGpp and pppGpp become less potent inhibitors in the presence of the 50S ribosomal subunits. A plausible reason for this behavior might be that the rRNA contact–mediated rearrangement of the G domain and RNA-
(p)ppGpp-mediated ribosomal large subunit arrest by RbgA

binding domain in a GTPase active conformation competes with the association of (p)ppGpp by clashing via the G2 motif with the δ- and ε-phosphate moieties. Taken together, our crystal structures, structural dynamics, and enzyme kinetic analyses of RbgA suggest that the alarmones (p)ppGpp prohibit formation of the GTPase active configuration by sterically precluding association of the G2 motif via the δ- and ε-phosphate moieties in a GTP-competitive manner.

Implications for final 50S ribosomal subunit maturation

Final maturation of the large subunit is to some extent conserved between prokaryotic and eukaryotic ribosomes and incorporation of uL16 coincides with Lsg1/RbgA GTPase activation and release (12, 17). However, the process appears uniquely more intricate in eukaryotes than in prokaryotes. In brief, delivery and incorporation of uL16 has been shown in S. cerevisiae to require the dedicated chaperone Sqt1 that shields uL16’s N-terminal domain before incorporation at the central protuberance close to the P site (26). Incorporation of uL16 into mature large subunits is to be performed with the release of Sqt1, the activation and dissociation of the GTPase Lsg1, and the release of large subunit export adapter Nmd3 (15–17, 26). However, it is not precisely understood whether the GTPase activity of Lsg1 is required to assemble uL16 or whether assembly of uL16 leads to activation of Lsg1 and Nmd3 release to signal for subunit maturation. A similar but less complex scenario has been observed in the prokaryotic 50S maturation. RbgA-depleted cells enrich uL16 deficient pre-45S particles, and the presence of uL16 is required for stimulation of the GTPase activity and release of RbgA (12). Cryo-EM of pre-45S particles from RbgA-depleted cells revealed that four 45S subunit regions have a particularly high degree of conformational flexibility: the central protuberance; helix 38 (A-site finger); helices H89–93 of the peptidyl-transferase center (PTC); and helices H67–71, which are required for ribosomal intersubunit contacts (11, 12). Because the binding interface of Lsg1 is located in the same region of the large subunit and the ribosome maturation function is conserved between the homologues, we speculate that the RbgA-binding interface on the 23S rRNA also encompasses helices 67–71. It is noteworthy that the distance between the putative binding site of RbgA and the incorporation site of uL16 between helix 38 and 89 of the 23S rRNA are ~40 Å apart. Hence, RbgA might not be activated by a direct contact with uL16. It seems more conceivable that incorporation of uL16 induces structural rearrangement in the 23S rRNA, which propagates toward H68–71 of the large subunit. The mature 50S arrangement of H69 and H71 might eventually signal for large ribosomal subunit completion to allow for GTPase activation by proper positioning of the G motifs (G1–G3) and subsequent GTPase release.

Experimental procedures

Cloning of expression constructs

S. aureus rbgA was amplified by PCR from S. aureus USA300 genomic DNA using a forward primer that contained a Ncol restriction site and the coding sequence for a hexahistidine tag and a reverse primer, which contained a BamHI restriction site. The B. subtilis rbgA homologue was amplified from B. subtilis 168 genomic DNA using a forward primer that contained a Ncol restriction site and a reverse primer, which contained a BamHI restriction site after a 4xGS linker and a hexahistidine tag coding sequence. The fragments were digested with Ncol and BamHI and cloned into pET24d (Novagen).

Production and purification of RbgA

Constructs were transformed in E. coli BL21(DE3) (Novagen) for overexpression. Cells were grown in 2 liters of lysogeny broth medium, supplemented with 25 g of lactose and kanamycin (50 mg/liter). The cells were incubated at 30 °C overnight under rigorous shaking (180 rpm). The cells were harvested by centrifugation (3,500 x g, 20 min, 4 °C) and resuspended in 20 ml of buffer A (20 mM HEPES-Na, pH 8.0, 250 mM NaCl, 20 mM KCl, 20 mM MgCl2, 40 mM imidazole) before lysis in a M-110L Microfluidizer (Microfluidics). The lysate was cleared at 47,850 x g for 20 min at 4 °C, and the supernatant was applied onto two 1-ml HisTrap FF columns (GE Healthcare) for nickel–nitrilotriacetic acid affinity chromatography. After a wash step with 15 column volumes of buffer A, proteins were eluted with three column volumes of buffer B (20 mM HEPES-Na, pH 8.0, 250 mM NaCl, 20 mM KCl, 20 mM MgCl2, 500 mM imidazole).
Proteins were concentrated to 1 ml and further purified by size-exclusion chromatography (SEC). SaRbgA was purified using a HiLoad 26/600 Superdex 75 column (GE Healthcare) equilibrated in buffer C (20 mM HEPES-Na, pH 7.5, 200 mM NaCl). The main peak fractions were concentrated to 1.5 ml and dia-lyzed overnight at 4 °C against 200 ml of buffer C containing 10 g of HCl-activated charcoal and 1 mM EDTA to remove Mg\(^{2+}\) and co-purified nucleotides. RbgA was subsequently subjected to a second SEC step using a HiLoad 26/600 Superdex 75 column (GE Healthcare) equilibrated in buffer D (20 mM HEPES-Na, pH 7.5, 20 mM KCl, 20 mM MgCl\(_2\), 200 mM NaCl). BsRbgA was purified using a HiLoad 16/600 Superdex 200 column (GE Healthcare) equilibrated in buffer E (0.2 M potassium sulfate, 20% PEG 3350); and 1 mM of RbgA was subsequently subjected to anion-exchange chromatography using a ResourceQ 6-ml column (GE Healthcare) at a flow rate of 6 ml/min and pppGpp eluted with a gradient of LiCl. pppGpp-containing fractions were pooled, and lithium chloride was added to a final concentration of 1 M. After addition of four volumes of ethanol, the suspension was incubated at −20 °C for 20 min and centrifuged (5000 × g, 20 min, 4 °C). The resulting pellet was washed with ethanol, dried, and stored at −20 °C.

Crystallization

Purified S. aureus RbgA was concentrated to 20 mg/ml. Nucleotides (GDP, GMPPNP, ppGpp, or pppGpp) were added at a final concentration of 5 mM, and RbgA was subsequently subjected to crystallization by sitting-drop vapor diffusion at 20 °C. Block shaped crystals grew within 2 days in drops containing 1 μl of RbgA-GDP and 1 μl of crystallization buffer (0.2 M lithium sulfate, 0.1 M MES, pH 6.0, 35% (v/v) 2-methyl-2,4-pentanediol (MPD)); 1 μl of RbgA-GMPPNP and 1 μl of crystallization buffer (0.2 M potassium fluoride, 0.1 M MES, pH 6.0, 20% PEG 3350); 1 μl of RbgA-pppGpp and 1 μl of crystallization buffer (0.2 M potassium sulfate, 20% PEG 3350); and 1 μl of RbgA-ppppGpp and 1 μl of crystallization buffer (0.2 M potassium fluoride, 0.1 M MES, pH 6.0, 20% PEG 3350). The crystals were transferred into crystallization buffer containing 20% (v/v) glycerol as cryo-protectant, subsequently flash-frozen, and stored in liquid nitrogen. No cryo-protectant was added to the crystals of RbgA-GDP because of the presence of 35% MPD in the crystallization buffer.

Data collection and structure determination

Diffraction data were collected at Beamlines ID-30B, ID29, and ID23-1 of the European Synchrotron Radiation Facility (Grenoble, France) (28). Data were processed with the XDS program package for data reduction (29), and merging and scaling was performed using the AIMLESS program as implemented in the CCP4 package (30). The RbgA-GDP data set was solved by molecular replacement using the crystal structure of B. subtilis RbgA (PDB code 1PUJ) via the CCP4 implemented program Phaser (31). Coot (32) in combination with Refmac5 (CCP4 package) and phenix.refine (PHENIX package (33)) was used for iterative model building and refinement. The GMP-PNP, ppGpp, and pppGpp RbgA state data sets were subsequently solved by molecular replacement using the GDP cleared S. aureus RbgA crystal structure (this study) via the CCP4 implemented program Phaser and refined using the phenix.refine software. The figures were prepared in PyMOL.

Affinity measurements using microscale thermophoresis

MST was performed on a Monolith NT.115 (NanoTemper Technologies GmbH, Munich, Germany) at 21 °C (red LED power was set to 70% and IR laser power to 25%) (34). RbgA (50 μM) was labeled with the dye NT 647 according to the supplier’s protocol (NanoTemper Technologies). 200 mM RbgA was titrated with GTP, GDP, ppGpp, or pppGpp starting from a concentration of 0.5 mM in buffer C (20 mM HEPES-Na, pH 7.5, 200 mM NaCl). To each measurement Tween 20 (Sigma) was added to a final concentration of 0.05 mM. At least nine independent MST experiments were recorded at 680 nm and processed by NanoTemper analysis 1.2.009. For fitting of the experimental data and K_d determination, Origin8G was used.

Hydrogen–deuterium exchange MS (HDX-MS)

Prior HDX-MS SaRbgA (40 μM) was incubated without any nucleotide or in presence of 5 mM GDP, GMPPNP, ppGpp, or pppGpp. Preparation of samples for HDX-MS analysis was aided by a two-arm robotic autosampler (LEAP Technologies). Hydrogen–deuterium exchange was started by 10-fold dilution of SaRbgA in D_2O-containing SEC buffer followed by incubation at 25 °C for 10/30/95/1000/10,000 s. The reaction was stopped by mixing the HDX reaction with an equal volume of ice-cold quench buffer (400 mM KH_2PO_4/H_3PO_4, 2 M guanidine HCl, pH 2.2) and subsequently injected into an ACQUITY UPLC M-class system with HDX technology (Waters) (35). Undeuterated samples of SaRbgA were prepared similar by 10-fold dilution in H_2O-containing SEC buffer followed by quench and injection into the LC-MS system. SaRbgA was digested online using immobilized porcine pepsin at 12 °C at 100 μl/min flow rate of water + 0.1% (v/v) formic acid, and the resulting peptides were trapped on a C18 column kept at 0.5 °C. After 3 min, the C18 trap column was placed in line with an ACQUITY UPLC BEH C18 1.7-μm 1 × 100-mm column (Waters), and the peptides were eluted at 0.5 °C using a gradient of water +0.1% (v/v) formic acid (A) and acetonitrile +0.1% (v/v) formic acid (B) at 30 μl/min flow rate as follows: 0–7 min/95–65% A, 7–8 min/65–15% A, 8–10 min/15% A, 10–11 min/5% A, 11–16 min/95% A. Mass spectra were acquired on a G2-Si high-definition MS (HDMS) (Waters) mass spectrometer in HDMS or enhanced HDMS positive ion mode for deuterated and undeuterated samples, respectively (36, 37). Continuous lock mass correction was performed using [Glu1]-(p)ppGpp-mediated ribosomal large subunit arrest by RbgA
Fibrinopeptide B standard (Waters). Three replicates were measured for each incubation time. To reduce peptide carryover, the pepsin column was washed three times with 80 μl of 4% (v/v) acetonitrile and 0.5 m guanidine hydrochloride during each LC run, and an additional blank run was performed between each sample. Peptide identification and determination of deuterium uptake was carried out as described previously (38–40) aided by the PLGS and DynamX 3.0 software (Waters).

50S ribosome preparation for RbgA GTPase assay

*B. subtilis* 3610 cells were inoculated from an overnight culture in 200 ml of lysogeny broth medium and grown to an A600 of 0.5 at 37 °C. The cells were rapidly cooled down on ice and harvested at 4 °C for 20 min at 4000 x g. The cells were resuspended in ribosome buffer 1 (25 mM HEPES pH 7.5, 15 mM Mg(OAc)2, 100 mM KOAc, 6 mM β-mercaptoethanol, 0.025% DDM) and lysed by the use of a microfluidizer (Microfluidics). Cell debris was removed at 60,000 x g for 30 min and the supernatant loaded on a 17.5% (w/v) sucrose cushion. Ribosomes were harvested at ultracentrifugation at 200,000 x g for 2 h at 4 °C. The ribosomal pellet was resuspended in 400 μl of ribosome buffer 1 by stirring on ice for 20 min. The ribosomes were transferred to a fresh reaction tube, and undissolved material was removed by centrifugation (8000 x g, 5 min, 4 °C). 100 μl of resuspended ribosomes were loaded on top of a 10–60% (w/v) sucrose gradient. Gradients were prepared with a Gradient station (Biocomp) using the 10–50% long protocol. Gradient ultracentrifugation was performed within Ultra-Clear thin-wall tubes (14 ml, 14*95 mm) in an SW40 Ti rotor at 200,000 x g for 3 h at 4 °C. The gradient was analyzed with the gradient station coupled to a Biocrop Triax UV cell and 50S subunits manually fractionated. Fractions were pooled and concentrated with an Amicon Ultra centrifugal filter (100,000 Da molecular mass cutoff) to an A260 nm of 10.

Analysis of GTPase activity of BsRbgA

In end-point measurements, 5 μM BsRbgA were incubated together with 1 mM of nucleotides (GTP, ppGpp, pppGpp, or combinations thereof) at 37 °C in a buffer containing 50 mM HEPES-K, pH 7.4, 100 mM KCl, 50 mM KOAc, 12.5 mM Mg(OAc)2, 5 mM MgCl2, and 1 mM DTT for 100 min. Where indicated, 50S were employed in a concentration of 5 μM. The reactions were quenched by addition of two volume parts chloroform, followed by vigorous mixing for 5 s, heating at 95 °C for 15 s, and flash-freezing in liquid nitrogen. While thawing, samples were centrifuged (17,300 x g, 30 min, 4 °C), and the aqueous phase analyzed by HPLC on an Agilent 1260 Series system (Agilent Technologies) equipped with a C18 column (EC 250/4.6 Nucleodur HTec 3 μm; Macherey–Nagel). The nucleotides were eluted with a buffer containing 50 mM KH2PO4, 50 mM K2HPO4, 10 mM tetrapotassiumammonium bromide, and 25% (v/v) acetonitrile and detected at 253 nm in agreement with standards.

Enzyme kinetics of GTP hydrolysis by BsRbgA was determined by incubating 5 μM enzyme together with varying concentrations of GTP (i.e. 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, and 1 mM) at 37 °C. Where indicated, 50S ribosomes were present in a final concentration of 5 μM. Alarmones ppGpp and pppGpp were employed in concentrations of 25, 100, 250, or 1000 μM. Samples were taken after 25, 50, 75, and 100 min in the presence of 50S or after 75, 150, 225, or 325 min in the absence of 50S and subsequently quenched and analyzed as described above.

The initial velocities of GTP hydrolysis were obtained from the slope of the linear regression of the amount of GTP quantified at different incubation times. The so-obtained initial velocities were plotted against the concentration of GTP (v/S) or in the double-reciprocal plot (Lineweaver–Burk). Values of K99 and Vmax ± standard deviation were obtained from the v/S plot using the equation 

\[ v = \frac{V_{max}}{K_m + S} \]

Inhibitory constants were obtained from the v/S characteristic fitted with the equation

\[ v = \frac{V_{max} S}{K_m + [1 + I/K_i] + S} \]

Analysis of all kinetic data were carried out using GraphPad Prism version 6.04 for Windows (GraphPad Software, San Diego, CA).

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