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The *Caulobacter crescentus* $CgtA_C$ Protein Cosediments with the Free 50S Ribosomal Subunit

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The Obg family of GTPases is widely conserved and predicted to play an as-yet-unknown role in translation. Recent reports provide circumstantial evidence that both eukaryotic and prokaryotic Obg proteins are associated with the large ribosomal subunit. Here we provide direct evidence that the *Caulobacter crescentus* $CgtA_C$ protein is associated with the free large (50S) ribosomal subunit but not with 70S monosomes or with translating ribosomes. In contrast to the *Bacillus subtilis* and *Escherichia coli* proteins, $CgtA_C$ does not fractionate in a large complex by gel filtration, indicating a moderately weak association with the 50S subunit. Moreover, binding of $CgtA_C$ to the 50S particle is sensitive to salt concentration and buffer composition but not guanine nucleotide occupancy of $CgtA_C$. Assays of epitope-tagged wild-type and mutant variants of $CgtA_C$ indicate that the C terminus of $CgtA_C$ is critical for 50S association. Interestingly, the addition of a C-terminal epitope tag also affected the ability of various $cgtA_C$ alleles to function in vivo. Depletion of $CgtA_C$ led to perturbations in the polysome profile, raising the possibility that $CgtA_C$ is involved in ribosome assembly or stability.

GTP-binding proteins play roles in diverse cellular processes ranging from signal transduction to vesicle fusion. While much attention has been focused on the role of the eukaryotic Ras-like GTPases, less attention has been paid to a large number of GTPase subfamilies that are conserved among all living organisms sequenced to date (4, 5, 27, 33). On the basis of the evolutionary relationships and emerging experimental data, it has been proposed that these relatively widely conserved GTP-binding proteins act as translation factors (27).

The Obg subfamily is a distinct group of monomeric GTP-binding proteins that share a conserved GTP-binding domain. *Bacteria* encode one Obg protein and *Archaea* typically encode two related Obg proteins, whereas eukaryotes usually encode four Obg proteins, including three distinct protein types. The bacterial and the eukaryotic mitochondrial Obg proteins are likely to be homologous, as sequences flanking the GTP-binding domain are also conserved. The biochemical features of the Obg proteins are distinct from those of the well-characterized Ras-like proteins. For example, all bacterial Obg proteins examined thus far bind guanine nucleotides with modest (in the micromolar range) affinity (28, 47, 52). More strikingly, the *Caulobacter crescentus* and the *Escherichia coli* Obg proteins, $CgtA_C$ and $CgtA_E$, respectively ($CgtA_E$ is also called Obg_E or YhbZ), have rapid GDP and GTP exchange rate constants but relatively slow GTP hydrolysis rates (28). To date, we and others have favored models for Obg function that involve Obg acting as a sensor of intracellular GTP/GDP pools (28, 39). We further proposed that in vivo, rapid exchange of guanine nucleotides might be inhibited and that GTP hydrolysis may play a role in regulating the activation of the Obg proteins (30). The inhibition of guanine nucleotide exchange could be accomplished by the association of Obg in a complex.

Accumulating evidence suggests that the Obg proteins are ribosome associated. The *Bacillus subtilis* Obg protein fractionates in a large cytoplasmic complex by gel filtration, coelutes with ribosomal proteins, and interacts specifically with the 50S ribosome subunit protein L13 (42). Similarly, the *E. coli* $CgtA_E$ protein also fractionates with a large RNA-containing complex by gel filtration and interacts with L13 (K. Pu and J. R. Maddock, unpublished data). $CgtA_E$ has been shown genetically to interact with RrmJ (FtsJ) (47), a methyltransferase that modifies the 23S rRNA (3). Overexpression of $CgtA_E$ suppresses both the growth defect and the polysome profile defect of the Δ*rrmJ* mutant, suggesting that $CgtA_E$ plays an active role in ribosome assembly or stability (47).

In this report we demonstrate that the *C. crescentus* $CgtA_C$ protein is associated with the 50S ribosomal subunit but not with the 70S monosomes or with polyribosomes, indicating that $CgtA_C$ is not associated with translating ribosomes. The observed association is dependent on both the salt concentration and the magnesium counter ion used in the assay. Moreover, the C-terminal acid domain of $CgtA_C$, which is important for function, is also critical for 50S association, and the addition of a C-terminal epitope tag affects both function and ribosome association. Finally, the long-term effect of $CgtA_C$ depletion is a reduction in 70S monosomes and polyribosomes.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. *E. coli* was grown at 37°C in Luria-Bertani broth or on Luria-Bertani agar containing antibiotics as required (see below). *C. crescentus* strains were derived from CB15N and were grown in PYE medium at 30°C. All DNA manipulation was performed in *E. coli* strain DH5α. Plasmids were introduced into *C. crescentus* by conjugal transfer via the *E. coli* strain S17 (12). Antibiotics were used at the following concentrations: for *C. crescentus*, oxytetracycline (Tet) (concentration, 1 μg ml^{-1}), naladixic acid (20 μg ml^{-1}), ampicillin (Amp) (10 μg ml^{-1}), or kanamycin (Kan) (5 μg ml^{-1}); and for *E. coli*,

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
E. coli		
DH5a	Δ(*lac*)*U169* ϕ80 Δ(*lacZ*)M15 *hsdR17 endA1 gyrA96 recA1 relA1 supE44 thi-1*	17
S17-1	F^- *recA*; RP4-2 Tc^r::Mu Tm^r::Tn7	44
C. crescentus		
CB15N	Synchronizable derivative of wild type (also known as NA1000)	13
JM1108	*Pxyl*::*cgtA* integrated at the *cgtA* locus	30
Plasmids		
pMR20	Mini-RK2 cloning vector; Tet^r	45
pCR2.1-TOPO	PCR cloning vector; Kan^r Amp^r	Invitrogen
pT7blue-3	Blunt cloning vector; Kan^r Amp^r	Novagen
pET28a(+)	Expression vector for N-terminal His-tagged fusions; Kan^r	Novagen
pFAGa-3HA-TRP	Source of 3HA tag	31
pJM625	Full-length *cgtA* in pET28a	28
pJM746	Full-length *cgtAT192A* in pET28a	30
pJM747	Full-length *cgtAT193A* in pET28a	30
pJM1402	1.06-kb *Nco*I-*Bam*HI PCR fragment containing $cgtA^{1-347}$ in pT7blue-3	This study
pJM1759	2.25-kb *Pst*I-*Hin*dIII *cgtA* in pMR20; *Nco*I site at inititating codon	30
pJM2374	2.3-kb *Nco*I-*Hin*dIII $cgtA^{1-347}$ in PCR2.1-TOPO	This study
pJM2383	$cgtA^{1-347}$-*3HA* in pMR20	This study
pJM2384	$cgtAT192A^{1-347}$-*3HA* in pMR20	This study
pJM2385	$cgtAT193A^{1-347}$-*3HA* in pMR20	This study
pJM2395	1.09-kb *Nco*I-*Pac*I PCR fragment containing *cgtA* in PCR2.1-TOPO	This study
pJM2403	*cgtA-3HA* in pMR20	This study
pJM2404	*cgtAT192A-3HA* in pMR20	This study
pJM2405	*cgtAT193A-3HA* in pMR20	This study
	$cgtA^{1-347}$ in pMR20	This study

tetracycline (12 μg ml^{-1}), Kan (30 μg ml^{-1}), or Amp (100 μg ml^{-1}). The ability of $cgtA_C$ alleles to function in *C. crescentus* strain JM1108 was assayed on PYE agar plates containing 0.2% xylose (PYE+Xyl) or 0.2% glucose (PYE+Glu), Tet, and Kan. No antibiotics were added in the final liquid culture for polyribosome preparation.

Construction of epitope-tagged $CgtA_C$-3HA. Relevant plasmids used in this study are listed in Table 1. C-terminal deletion $cgtA_C^{1-347}$ was generated by PCR amplification using primers *cgtA-Nco*I (5′-GGACCCCATGGAATTCTTGGACCA) and *cgtA*-347delta (5′-TCTAGAGGATCCCCTCGTCGACGTGATCCT), whereas $cgtA_C^{1-334}$ was generated using primers *cgtA-Nco*I and *cgtA*-334delta (5′-TCTAGAGGATCCCGAGATCACCGCGACGGAT). Through a series of subclones, a C-terminal *Bam*HI-*Hin*dIII influenza virus hemagglutinin tag (3HA tag) from pFA6a-3HA-TRP1 (31) was added to create the $cgtA^{1-347}$-*3HA* fusion construct (pJM2383). 3HA-tagged mutant alleles (T192A and T193A) were generated by substitution of the *Nco*I-*Xho*I fragments from the appropriate mutant allele clones into pJM2383 (30). The $cgtA^{1-347}$ and the $cgtA^{1-347}$-*3HA* fusions were placed under the control of the *cgtA* promoter by replacement of the *Nco*I-*Hin*dIII fragment from pJM1759 (a modified *C. crescentus* pMR20 plasmid that contains the *cgtA* promoter and *cgtA* with an engineered *Nco*1 site at the initiating codon) with $cgtA^{1-347}$ or $cgtA^{1-347}$-*3HA*. The full-length *cgtA-3HA* construct was generated by PCR amplification of $cgtA_C$ through the use of CgtA-*Nco*I and C-PacIXbaI-CgtA (5′ GCTCTAGATCTTAATTAACGGCGTCCAGCC), tagging with 3HA, and expression from the *cgtA* promoter in a manner analogous to that used for the $cgtA^{1-347}$ and $cgtA^{1-347}$-*3HA* fusion constructs. The final constructs of all 3HA-tagged *cgtA* alleles were verified by DNA sequencing of the *cgtA* gene, junction regions of the *PcgtA* promoter, and the 3HA tag.

Affinity purification of $CgtA_C$ antibody. Polyclonal rabbit anti-$CgtA_C$ antibody was affinity purified from crude antiserum with Affi-Gel 15 (Bio-Rad) agarose beads coupled with purified $CgtA_C$ protein (28). All steps were performed at 4°C unless otherwise specified. Approximately 25 mg of purified $CgtA_C$ protein was incubated with 1 ml of Affi-Gel 15 beads in a final volume of 1.5 ml of HEPES buffer (100 mM, pH 8) for 30 min. The coupling efficiency was at least 95%, as determined by a Bradford assay (Bio-Rad) (catalog no. 500-000). The remaining active sites on the Affi-Gel were subsequently blocked with 10 mM ethanolamine-HCl (1 M stock, pH 8) for 1 h. The $CgtA_C$-coupled Affi-Gel ($CgtA_C$-Affi-Gel) was equilibrated with phosphate-buffered saline (100 mM NaCl, 80 mM Na_2HPO_4, 20 mM NaH_2PO_4, pH 7.5) containing 0.05% Tween 20 (PBST) and 0.2% sodium azide.

For immunoaffinity purification of anti-$CgtA_C$ antibody, approximately 0.3 ml of $CgtA_C$-Affi-Gel beads was combined with 0.5 ml of crude antiserum and brought to a final volume of 1 ml with PBST and 10% bovine serum albumin (BSA). The mixture was incubated overnight with gentle shaking. The beads were incubated on ice for 5 min, pelleted in a 1.75-ml microcentrifuge tube (with a small cut 2 to 4 mm from the bottom) at 2,000 × *g* for 3 min, and then washed five times with 1 ml of PBST. Anti-$CgtA_C$ was eluted from beads three times with 0.1 ml of glycine-HCl (50 mM [pH 2.5] in 0.1% Tween 20), once with 0.1 ml of BSA (10% in PBST), and once with 0.1 ml of Tris-HCl (1.5 M, pH 7.5) by centrifugation at 2,000 × *g* for 3 min. Eluted samples were combined and stored at −80°C in 10-μl aliquots. The specificity and titer of the purified anti-$CgtA_C$ were tested by immunoblot analysis.

Immunoblot analysis. Proteins were separated by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS–12% PAGE) and electroblotted to polyvinylidene difluoride membranes (NEN Life Science Products) with a Hoeffer semidry transfer apparatus as recommended by the manufacturer. The membranes were blocked with 10% skim milk in PBST, probed with either a 1:2,000 dilution of affinity-purified polyclonal rabbit anti-CgtA or a 1:1,000 dilution of monoclonal mouse anti-HA (ascites fluid, clone 16B12; BAbCO), and washed with PBST; bound antibody was detected with a 1:20,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit (Pierce) or rabbit anti-mouse (Sigma) antibody, respectively, and visualized by fluorography using ECL (Amersham Pharmacia Biotech) as recommended by the manufacturer.

Preparation of *C. crescentus* cell lysates. *C. crescentus* cell lysates were prepared according to Ohta et al. (38) and Flessel et al. (15) with the following modifications. Briefly, 500 ml of PYE cultures of *C. crescentus* cells was grown at 30°C to an optical density at 600 nm (OD_{600}) of 0.6 to 0.8. Chloramphenicol was added to a final concentration of 100 μg/ml 3 min prior to harvesting. Cells were immediately chilled by pouring over an equal volume of crushed ice and harvested by centrifugation (10,000 × *g*, 15 min). The cell pellet was washed with 20 ml of ice-cold TE (100 mM Tris-HCl [pH 7.5], 1 mM EDTA) and resuspended in 5 ml of SETS buffer (100 mM NaCl, 1 mM EDTA, 100 mM Tris-HCl [pH 7.5], 17% sucrose) containing 100 mg of lysozyme (Sigma)/ml and 100 μg of phenylmethylsulfonyl fluoride (10 mg/ml of stock in ethanol)/ml. All subsequent operations were performed at 4°C. After 30 min of lysozyme treatment, $MgCl_2$ (1 M

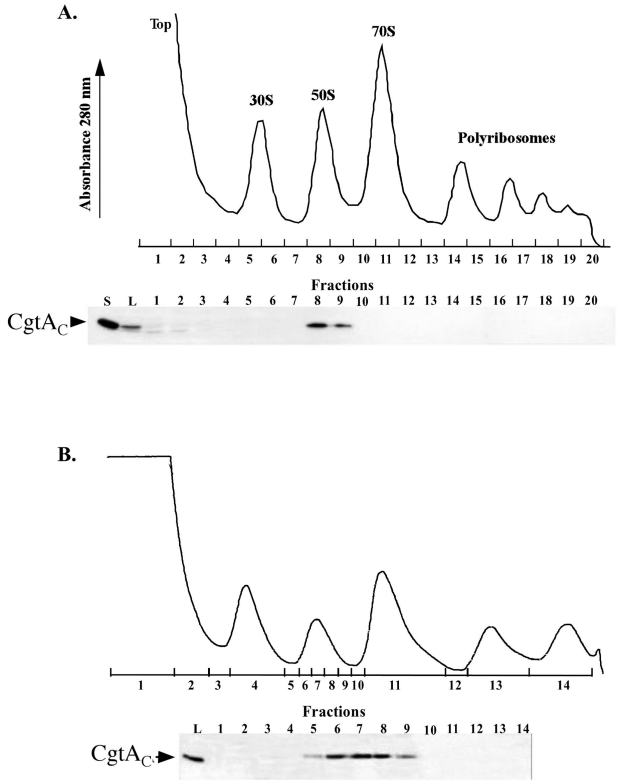

FIG. 1. CgtA$_C$ cofractionates with the 50S ribosomal subunit by sucrose density centrifugation. CB15N cells were grown to logarithmic phase in PYE medium and harvested, and cell lysates were prepared and sedimented through a 15 to 45% sucrose gradient at 210,000 × *g* for 3 h (A) or a 20 to 40% sucrose gradient at 84,000 × *g* for 12 h (B). UV profiles were monitored at 280 nm. The positions of the 30S, 50S, and 70S monosome and polyribosome peaks relative to those of the fractions collected are indicated. Immunoblots of relevant fractions (10 μl of each 1-ml fraction) separated by SDS–12% PAGE and the levels of CgtA$_C$ detected with anti-CgtA$_C$ antibodies are shown below each polyribosome trace. S, 10 ng of purified CgtA$_C$; L, 1 μl of cell lysate (OD_{260} of 0.1).

stock) was added to restore the magnesium concentration to 20 mM unless otherwise indicated. Spheroplasts were monitored under a phase-contrast microscope and centrifuged for 20 min at 10,000 × *g*. The pellet was resuspended in 2 ml of TM buffer (10 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$) containing 100 μg of chloramphenicol/ml, 100 μg of phenylmethylsulfonyl fluoride/ml, and Complete EDTA-free protease inhibitor cocktail (Roche Molecular Biochemicals) (1 tablet/50 ml). The spheroplasts were disrupted by the addition of sodium deoxycholate (10% stock) and Brij 58 (10% stock) to achieve a final concentration of 1% each. The crude lysate was frozen in liquid nitrogen and slowly thawed in an ice-water bath. After centrifugation at 20,000 × *g* for 20 min, the clarified lysate was stored at −80°C in 0.5-ml aliquots. UV absorbance of the cell lysate was determined in a 1-ml quartz cuvette.

Polyribosome fractionation. Cell lysates (200 μl) at an OD_{260} of approximately 20 were subjected to ultracentrifugation in 10 ml of 15 to 45% sucrose (ARCOS) RNase- and DNase-free gradients for 3 h at 41,000 rpm (210,000 × *g*) and 0°C in a Beckman SW41Ti rotor. Higher-resolution separation was performed using 20 to 40% sucrose gradients at a lower speed (84,000 × *g*) (Fig. 1B). Sucrose solutions contained 100 mM NH_4Cl in TM buffer, and the gradients were prepared (10). The resulting polyribosomes were fractionated by a Brandel gradient fractionator (model BR-186) (2-mm-path-length flow cell) connected to a syringe pump (model SYR-101). The syringe was filled with 50% sucrose in distilled water, and the pump flow rate was set to 0.75 ml/min. The UV absorbance (280 nm) of the sample was monitored and recorded by an ISCO UA-5 detector. A total of 10 μl from each fraction was analyzed by SDS-PAGE and immunoblotting. In experiments examining the effects of excess guanine nucleotides on CgtA-ribosome association, the cell lysates were preincubated with 10 mM GDP or GTP and sedimented through a sucrose gradient in standard buffer with additional 250 μM GDP or GTP, respectively. A parallel blank control (loaded with 200 μl of 10 mM GDP on top of the sucrose gradient containing 250 μM GDP) was used, and the resulting UV absorbance profile confirmed that the distribution of guanine nucleotide in the sucrose gradient after ultracentrifugation was unaltered (data not shown).

Gel filtration chromatography. *C. crescentus* cell lysates (0.5 ml) were loaded onto a 100-ml (1.5 by 70 cm) Toyopearl HW-55S column (TosoHaas) eluted (0.4 ml/min) with TMA buffer (10 mM TrisHCl [pH 7.5], 10 mM $MgCl_2$, 100 mM NH_4Cl) at 4°C. Fractions (1 ml) were collected from elution volumes of between 20 and 100 ml. When indicated, the cell lysates were preincubated with 10 mM GDP or GTP for 30 min on ice prior to loading and subsequently eluted with TMA buffer containing 1 mM GTP or 1 mM GDP, respectively. Samples (20 μl) from alternate fractions were subjected to SDS-PAGE followed by immunoblot analysis. The column was calibrated with a 0.5-ml mixture of thyroglobulin (Sigma) (669 kDa, 0.25 mg), β-amylase (Sigma) (200 kDa, 0.25 mg), BSA (Sigma) (66 kDa, 0.25 mg), and bovine carbonic anhydrase (Sigma) (29 kDa, 0.25 mg) under the same elution conditions.

RESULTS

CgtA$_C$ cosediments with free 50S ribosomal subunits. In *B. subtilis*, Obg fractionates as a large cytoplasmic complex containing ribosomal proteins and interacts with the 50S ribosomal subunit protein L13, according to the results of an affinity blot assay (42). The *E. coli* CgtA$_E$ protein also interacts with L13 and purifies in a large RNA-containing complex (Pu and Maddock, unpublished) but does not associate with the large ribosomal subunit after sucrose density centrifugation (25). To examine the relationship between the ribosome and *C. crescentus* CgtA$_C$ protein, we separated cell lysates by ultracentrifugation through a sucrose gradient (10, 15, 38), monitored the polyribosome profile by UV absorbance, and detected the migration of CgtA$_C$ by immunoblot analysis (Fig. 1). As expected, the majority of the cellular proteins and small molecules were in the top of the gradient, followed by the peaks for the 30S and 50S subunits and the 70S ribosomes and polyribosomes. CgtA$_C$ was located in fractions corresponding to the peak of free 50S ribosomal subunits (Fig. 1A). To examine whether CgtA$_C$ cosedimented with ribosomal intermediate particles adjacent to the 50S peak (32, 40), the profile of CgtA$_C$ was examined after sucrose density centrifugation under conditions that result in a greater separation of the ribosomal subunits. Under these conditions, CgtA$_C$ also coeluted with the mature 50S subunit, although low levels of CgtA$_C$ were found in fractions preceding the 50S peak, perhaps due to the dissociation of CgtA$_C$ from the 50S subunits during centrifugation or due to an interaction with the pre-50S subunits (Fig. 1B).

Salt dependence of the association of CgtA$_C$ with 50S ribosomal subunits. Ammonium chloride (NH_4Cl) has been widely employed as a salt in buffers used for polyribosome profiles, although specific assay conditions differ widely among different reports (36). To test the effects of NH_4Cl concentration on CgtA$_C$-50S subunit association, lysates of *C. crescentus* were sedimented through sucrose gradients containing 10 mM Tris-HCl (pH 7.5) supplemented with 10 mM $MgCl_2$ and concentrations of NH_4Cl ranging from 0 to 200 mM. No difference in gradient profile was observed regardless of the concentration of NH_4Cl present (Fig. 2A). Moreover, when separated in the absence of NH_4Cl, CgtA$_C$ was still found predominantly in 50S fraction peaks with a trace amount of CgtA$_C$ at the top of the

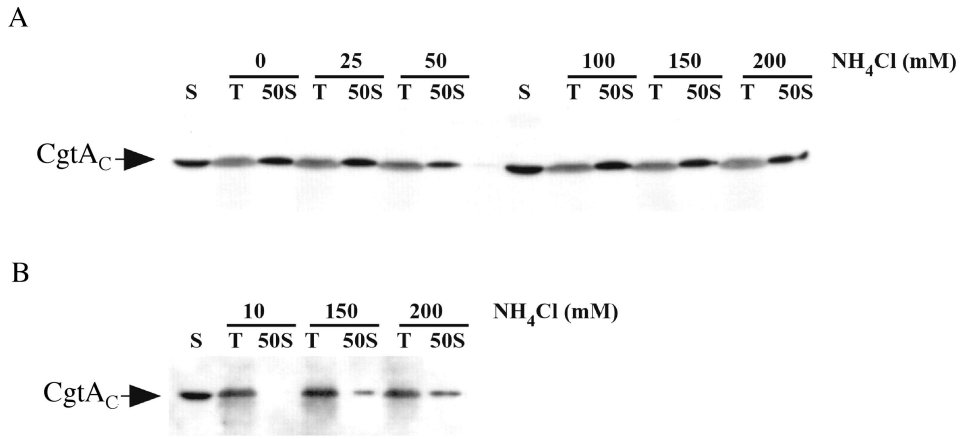

FIG. 2. Salt dependence of the association of $CgtA_C$ with free 50S ribosomal subunits. CB15N cell lysates (200 μl, OD_{260} of 20) were sedimented through 15 to 45% sucrose gradients containing 0 to 200 mM NH_4Cl (as indicated) in 10-mM Tris-HCl (pH 7.5) buffer supplemented with either 10 mM $MgCl_2$ (A) or 10 mM $MgSO_4$ (B). Polyribosome profiles similar to that shown in Fig. 1 were obtained, and the fractions corresponding to the top (T) of the gradients and the 50S peaks (50S) were separated by SDS–12% PAGE. Each lane was loaded with 10 μl of a 1-ml fraction, and the levels of $CgtA_C$ were detected by immunoblotting using anti-$CgtA_C$ antibodies. S, 2 ng of purified $CgtA_C$.

gradient (Fig. 2A) and in fractions prior the 50S peak (data not shown). Thus, $CgtA_C$ bound to free 50S ribosomal subunits independently of the NH_4Cl concentrations used.

Information regarding the source of the Mg^{2+} ion present in sucrose gradient buffers also differs among different reports. To address whether the source of the Mg^{2+} ion was important in the binding of $CgtA_C$ to the 50S subunit, we examined the consequences of the use of various levels of NH_4Cl in 10 mM Tris-HCl (pH 7.5) supplemented with 10 mM $MgSO_4$. Under these conditions, we observed an NH_4Cl concentration-dependent 50S association of $CgtA_C$ with the 50S peak. In buffers containing low levels of NH_4Cl, $CgtA_C$ did not bind to the 50S subunit and was exclusively found at the top of the gradient (Fig. 2B). Some $CgtA_C$-50S cofractionation was observed in buffers containing higher NH_4Cl concentrations; even in the presence of 200 mM NH_4Cl, however, a significant amount of $CgtA_C$ was in the top of the gradient (Fig. 2B). Thus, the combination of the Mg^{2+} counter ion used and NH_4Cl concentration is critical for the observed association of $CgtA_C$ with the 50S ribosomal subunit.

Inclusion of excess GTP or GDP does not affect the cosedimentation of $CgtA_C$ with the 50S subunit. As a guanine nucleotide protein, $CgtA_C$ undergoes conformational changes among the GTP-bound, GDP-bound, and apo states (30). It is therefore possible that the guanine nucleotide occupancy of $CgtA_C$ could affect its association with free 50S subunits. To examine whether this is the case, *C. crescentus* cell lysates preincubated with either 10 mM GDP or GTP were sedimented through 15 to 45% sucrose gradients in standard buffers containing 250 μM GDP or GTP, respectively. Because in vitro $CgtA_C$ binds guanine nucleotides with moderate affinity (~1 μM) and displays a rapid guanine nucleotide exchange rate (28), we predicted that under these conditions the majority of the $CgtA_C$ protein would be nucleotide bound. As observed in the absence of guanine nucleotides (Fig. 2), the majority of $CgtA_C$ cofractionated with the 50S ribosomal subunit irrespective of the presence of either GDP or GTP (Fig. 3). Therefore, it appears that $CgtA_C$ binds to the free 50S ribosomal subunit independently of its nucleotide occupancy state.

$CgtA_C$ is not associated with the 50S particle by gel filtration. The bacterial 50S ribosomal subunit consists of an ~1,000-kDa 23S rRNA molecule, an ~40-kDa 5S rRNA molecule, and over 31 ribosomal proteins with an average molecular mass of 15 kDa each (34). Thus, the overall size of the 50S subunit complex exceeds 1.4 MDa. To examine whether the $CgtA_C$ protein binds tightly to the 50S particle, we examined the elution of $CgtA_C$ from *C. crescentus* cell lysates separated by gel filtration on a 100-ml Toyopearl HW-55S gel filtration column (fractionation range of 1 to 10^3 kDa for globular proteins) (Fig. 4). The majority of $CgtA_C$ eluted in fractions corresponding to 30 to 200 kDa, peaking at approximately 50 kDa (Fig. 4), indicating that a significant amount of $CgtA_C$ fractionates as monomers or small complexes. To assay whether the guanine nucleotide binding state of $CgtA_C$ affected its chromatographic migration, cell lysates were preincubated with 10 mM GDP or GTP and eluted in buffer containing 1 mM GDP or 1 mM GTP, respectively. Under these conditions,

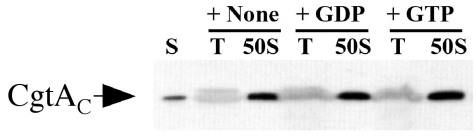

FIG. 3. The addition of excess guanine nucleotides does not affect the association of $CgtA_C$ and free 50S ribosomal subunits. CB15N cell lysates (200 μl, OD_{260} of 20) were preincubated on ice without (+None) or with 10 mM GDP (+GDP) or 10 mM GTP (+GTP) for 30 min prior to sedimentation through a 15 to 45% sucrose gradient supplemented with no nucleotide, 250 μM GDP, or 250 μM GTP, respectively. Polyribosome profiles similar to that shown in Fig. 1 were obtained, and the fractions corresponding to the top (T) of the gradients and the 50S peaks (50S) were separated by SDS–12% PAGE. Each lane was loaded with 10 μl of a 1-ml fraction, and the levels of $CgtA_C$ were detected by immunoblotting using anti-$CgtA_C$ antibodies. S, 2 ng of purified $CgtA_C$.

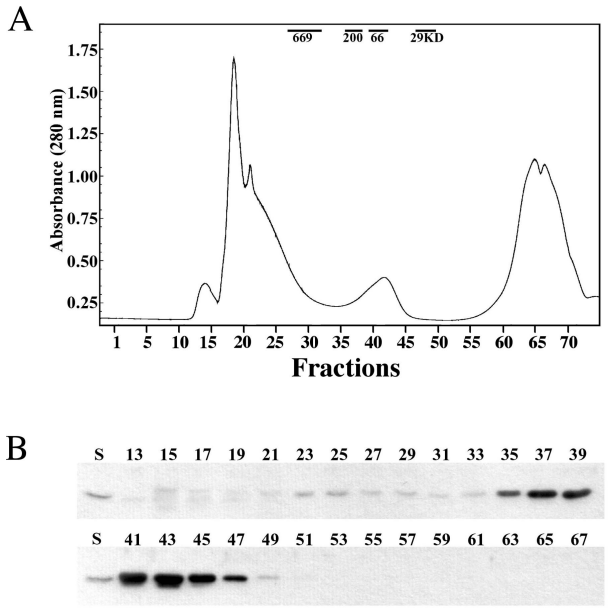

FIG. 4. Gel filtration chromatography of a *C. crescentus* CB15N cell lysate reveals that the majority of CgtA$_C$ elutes as a monomer or in a small complex. CB15N cell lysates (0.5 ml) were fractionated through a 100-ml (1.5 by 70 cm) Toyopearl HW-55S column (Toso-Haas) at a flow rate of 0.4 ml/min in TMA buffer (10 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 100 mM NH_4Cl). (A) The elution of samples was monitored by UV absorbance at 280 nm, and 1-ml fractions were collected using elution volumes between 20 and 100 ml. The elution positions of control proteins are indicated. (B) The indicated fractions (10 μl each) were separated by SDS–12% PAGE. The levels of CgtA$_C$ were detected by immunoblotting using anti-CgtA$_C$ antibodies. S, 2 ng of CgtA.

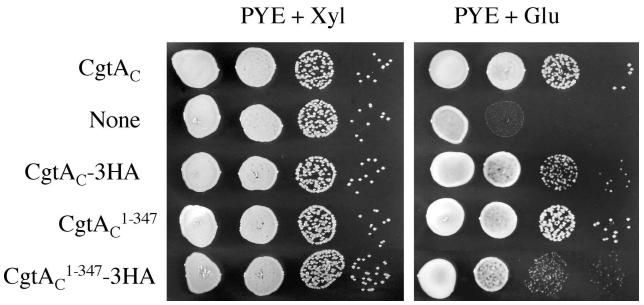

FIG. 5. Modification of the CgtA$_C$ C terminus affects function. Serial dilutions of cultured JM1108 cells containing pMR20 plasmids expressing *cgtA$_C$*, no protein (None), *cgtA$_C$*-3HA, *cgtA$_C^{1-347}$*, or *cgtA$_C^{1-347}$*-3HA were spotted onto PYE+Xyl and PYE+Glu plates. The plates were incubated at 30°C for 2 days.

no change in CgtA$_C$ elution was detected (data not shown). Thus, we conclude that, regardless of its nucleotide occupancy, CgtA$_C$ does not bind tightly to the 50S ribosomal subunit.

C-terminally epitope-tagged CgtA$_C$ variants are impaired for in vivo function. CgtA$_C$ is 354-amino-acid tripartite protein possessing an N-terminal glycine-rich domain called the Obg-fold (amino acids 1 to 159) (2), a GTP-binding domain (amino acids 160 to 314), and a C-terminal acidic domain (EEEIDDDEDHVDE; amino acids 335 to 347). We have previously shown that the N-terminal Obg-fold is critical for CgtA$_C$ function (29). To address whether the charged C terminus of CgtA$_C$ is also important for function, we made C-terminal truncation constructs and examined their ability to support growth as the sole expressed source of CgtA$_C$. To do this, we took advantage of a *C. crescentus* strain (JM1108) in which the chromosomal *cgtA$_C$* is under the control of the Pxyl promoter such that expression of chromosomal *cgtA$_C$* is repressed by a change of carbon source from xylose (Xyl) to glucose (Glu). JM1108 cells containing a plasmid-borne *cgtA$_C$* allele grow in PYE+Glu (30) (Fig. 5). Episomal expression of *cgtA$_C$* lacking the terminal 7 amino acids (*cgtA$_C^{1-347}$*) also supports growth (Fig. 5), whereas CgtA$_C$ lacking the acidic domain (*cgtA$_C^{1-334}$*) does not (data not shown). Thus, modest C-terminal deletions are not deleterious to CgtA$_C$ function, whereas removal of the acidic domain results in a nonfunctional protein.

To examine whether CgtA$_C^{1-347}$ associates with the 50S ribosomal subunit, we epitope-tagged full-length CgtA$_C$ and *cgtA$_C^{1-347}$* so that they could be specifically identified in cell lysates expressing untagged chromosomal *cgtA$_C$*. The full-length protein, CgtA$_C$-3HA, supports growth of JM1108 on PYE+Glu, albeit at a slightly reduced rate compared to untagged CgtA$_C$ (Fig. 5). Likewise, the *cgtA$_C^{1-347}$-3HA* allele supports growth but at a lower rate than that with its untagged counterpart (Fig. 5). Moreover, whereas the growth rates of cells expressing CgtA$_C$ or CgtA$_C^{1-347}$ were indistinguishable, cells expressing the *cgtA$_C^{1-347}$-3HA* allele grew more slowly than those with the *cgtA$_C$-3HA* allele. Thus, it appears that the C-terminal addition of the 3HA tag results in a partial loss of CgtA$_C$ function and that the combination of deletion of the seven C-terminal amino acids and addition of the 3HA tag further perturbs CgtA$_C$, underlining the importance of the C-terminal acidic domain to CgtA$_C$ function. To test whether the observed differences in in vivo function were caused by a variation in protein expression, JM1108 cells harboring 3HA-tagged *cgtA$_C$* alleles were grown in PYE+Xyl and analyzed by immunoblotting using monoclonal anti-HA and/or affinity-purified anti-CgtA$_C$ antibodies. All 3HA-tagged proteins (including the T192A and T193A variants described below) containing full-size CgtA$_C$ or C-terminally truncated CgtA$_C^{1-347}$ were expressed at similar levels, confirming that the growth differences among strains growing in glucose were not due to changes in steady-state protein levels (data not shown).

There are two conserved threonine residues within the GTP-binding domain of CgtA$_C$. The first, T192, is critical for CgtA$_C$ function, whereas the adjacent threonine, T193, is not essential but exhibits a minor defect in binding GDP and GTP (30). As expected, plasmid-encoded CgtA$_C$T192A-3HA does not support growth of JM1108 on PYE+Glu (Fig. 6). Interestingly, whereas plasmid-encoded CgtA$_C$T193A supports growth of JM1108 in PYE+Glu (30), CgtA$_C$T193A-3HA does not (Fig. 6). We propose that the slight defect in guanine nucleotide binding caused by the T193A mutation and the reduction in function caused by the addition of the C-terminal 3HA tag are negatively synergetic.

To examine whether the tagged CgtA$_C$ variants were associated with the 50S ribosome subunit in the context of wild-type CgtA$_C$, polyribosome profiles of JM1108 expressing the tagged CgtA$_C$ were examined by immunoblot analysis. In

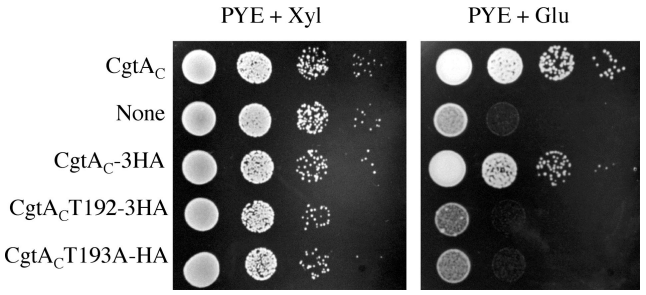

FIG. 6. Growth of tagged CgtA$_C$ GTP-binding domain mutants. Serial dilutions of cultured JM1108 cells containing pMR20 plasmids expressing *cgtA$_C$*, no protein (None), *cgtA$_C$*-3HA, *cgtA$_C$T192A*-3HA, or *cgtA$_C$T193A*-3HA were spotted onto PYE+Xyl and PYE+Glu plates. The plates were incubated at 30°C for 2 days.

JM1108 cells expressing plasmid-encoded CgtA$_C$-3HA, the chromosomally encoded CgtA$_C$ was found in the 50S fractions (Fig. 7B) whereas the CgtA$_C$-3HA was in the top of the gradient (Fig. 7A). Not surprisingly, the plasmid-encoded 3HA-tagged mutant CgtA$_C$ proteins CgtA$_C^{1-347}$-3HA, CgtA$_C$T192-3HA, and CgtA$_C$T193A-3HA were also found in the top of the gradient in their respective cell lysates (Fig. 7). Thus, the 3HA-tagged proteins either were unable to bind the 50S subunits, were displaced during the sucrose density centrifugation, or could not compete with wild-type CgtA in binding with 50S ribosomal subunits.

Depletion of CgtA$_C$ in *C. crescentus* resulted in a modest reduction of 70S ribosomes and polyribosomes. To further investigate the relationship between ribosomes and the function of CgtA$_C$, the effects of depleting CgtA$_C$ on polyribosome profiles were examined. Wild-type cells (CB15N) and cells containing *cgtA* under the control of the *Pxyl* promoter (JM1108) were grown to mid-exponential phase in PYE+Xyl, washed with PYE, resuspended at low density in either PYE+Xyl or PYE+Glu medium, and grown at 30°C for 6 h, a time when the vast majority of CgtA$_C$ protein is depleted, cell viability decreases, and cell growth is slowed (30). Lysates were subjected to sedimentation through a sucrose gradient. In CB15N cells, a shift from PYE+Xyl to PYE+Glu had no effect on the resulting polyribosome profile (Fig. 8). JM1108 cells grown in PYE+Xyl had profiles similar to that of CB15N (Fig. 8). In contrast, JM1108 cells grown in PYE+Glu contained reduced levels of 70S monosomes and polyribosomes whereas the levels of free 30S and 50S ribosomal subunits were not affected (Fig. 8). These data demonstrate that a long-term consequence of CgtA$_C$ depletion (either direct or indirect) is a reduction in translating ribosomes. A minor 60S peak is observed in all of our preparations but is particularly visible in JM1108 grown in glucose, due to the reduction of the 70S peak. A similarly sized peak has been reported previously (50), and is predicted to be due to alternate 30S-50S couples.

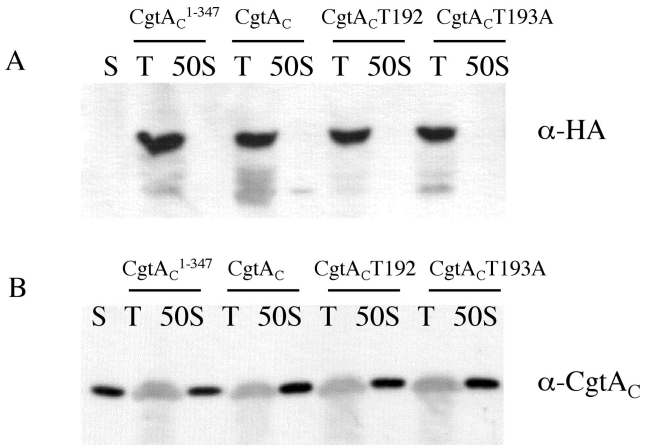

FIG. 7. The C-terminally 3HA-tagged CgtA$_C$ proteins failed to cosediment with free 50S ribosomal subunits in the cell lysates. *C. crescentus* JM1108 cells expressing *cgtA$_C^{1-347}$-3HA* (second and third lanes), *cgtA$_C$-3HA* (fourth and fifth lanes), *cgtA$_C^{T192A}$-3HA* (sixth and seventh lanes), and *cgtA$_C^{T193A}$-3HA* (eighth and ninth lanes) episomally from pMR20 were grown to logarithmic phase in PYE+Xyl medium and harvested. Cell lysates were independently sedimented through a 15 to 45% sucrose gradient prepared in 10 mM Tris-HCl (pH 7.5)–10 mM $MgCl_2$–100 mM NH_4Cl. A total of 10 μl of each 1-ml fraction from the top of the gradient (T) and the free 50S ribosomal subunit peak (50S) was separated by SDS–12% PAGE. The results of immunoblotting with duplicate gels prepared using monoclonal anti-HA antibody (A) and anti-CgtA$_C$ (B) antibodies are shown. S, 2 ng of purified CgtA$_C$.

DISCUSSION

Ribosome assembly occurs through the coordinated assembly of specific proteins on the nascent rRNA coupled with temporal rRNA processing and modification. In eukaryotes, there are approximately 200 proteins required for the biogenesis and export of the ribosomal subunits (see reference 49 for a review). Putative remodeling proteins such as helicases, AAA ATPases, and GTPases are included in these ribosomal assembly factors. In *E. coli*, active small and large ribosomal subunits can be spontaneously assembled in vitro without the need for ribosome assembly factors (8, 20). This in vitro assembly, however, requires elevated temperatures, high Mg^{2+} levels, and extended incubation times, indicating that in vivo, additional factors are necessary for optimal ribosomal assembly.

GTP-binding proteins associated with ribosomal precursor particles are likely candidates for such assembly factors. Interestingly, the eukaryotic Obg protein, Nog1p, copurifies with a wide range of pre-60S intermediates (1, 14, 18, 37) and is critical for 60S assembly (22, 24). Therefore, it is possible that the Obg proteins in archaea and bacteria also play a role in large ribosome biogenesis. In this study, we demonstrated that CgtA$_C$ is associated with the free 50S ribosomal subunits but not with the mature 70S monosomes or with polyribosomes. Since CgtA$_C$ is not a protein associated with translating ribosomes but is exclusively associated with the free mature or pre-50S ribosomal subunits, it is a strong candidate for a bacterial ribosomal assembly factor.

The conditional dependence on NH_4Cl concentration for CgtA$_C$ binding to the 50S subunit is of interest. In the presence of 10 mM $MgCl_2$, CgtA$_C$ cosediments with the 50S subunit at similar levels over a wide range of NH_4Cl concentrations. In buffers containing 10 mM $MgSO_4$, however, the majority of CgtA$_C$ remained in top of the gradient and the amount of CgtA detected in the fractions of the 50S subunit increased in correspondence to the addition of excess NH_4Cl. Recently, it was reported that the *E. coli* CgtA$_E$ protein was not detected in

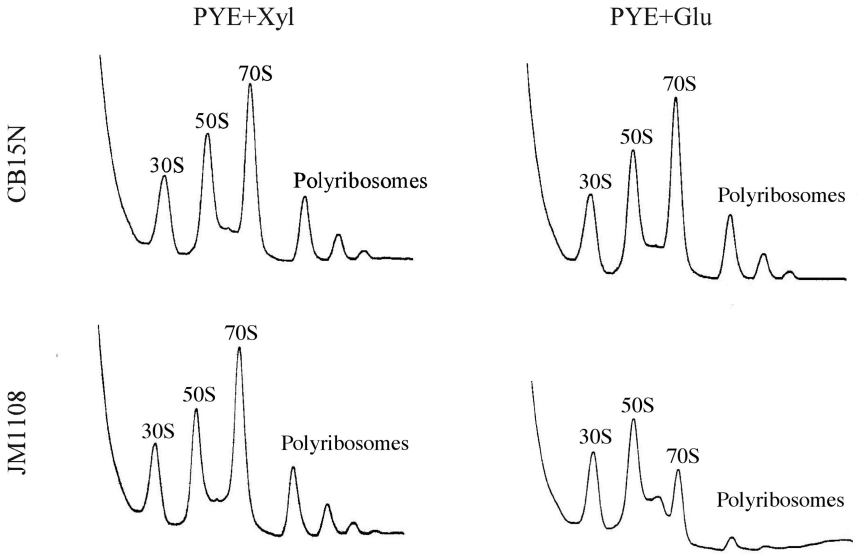

FIG. 8. Long-term depletion of CgtA$_C$ reduces the levels of 70S ribosomes and polyribosomes. Wild-type *C. crescentus* cells (CB15N) and cells containing *cgtA* under the control of the *Pxyl* promoter (JM1108) were grown to mid-exponential phase in PYE+Xyl, washed with PYE, resuspended at low density in either PYE+Xyl or PYE+Glu medium, and grown at 30°C for 6 h. Cell lysates were sedimented through 15 to 45% sucrose gradients, and the resulting UV absorbance profiles were recorded.

ribosomal fractions when the cell lysates were prepared and sedimented in buffers containing 100 mM $(NH_4)_2Ac$ and 15 mM MgAc (25). It is possible that divalent anions such as SO_4^{2-} or Ac^{2-} might inhibit CgtA$_C$ binding to 50S subunits.

The total intracellular ionic concentration in bacterial cells is estimated to be in the range of 150 to 550 mM, depending on the osmolarity of the medium (7, 21, 46); therefore, the buffers containing 100 mM NH_4Cl used in this study should represent an ionic strength close to that of the intracellular ionic environment. In sucrose gradient sedimentation, the CgtA$_C$-50S ribosomal subunit complex withstands NH_4Cl concentrations ranging from 0 to 200 mM, suggesting that CgtA$_C$ binds free 50S ribosomal subunits in vivo. In gel filtration experiments, however, CgtA$_C$ from cell lysates did not elute with the 50S ribosomal particle in buffer containing 100 mM NH_4Cl. This paradox could be explained by the equilibrium between the CgtA$_C$-50S subunit complexes and free CgtA$_C$ monomers. In single-phase solutions such as cell lysates and sucrose gradients, CgtA$_C$ would have access to the 50S subunits and the binding equilibrium would favor the CgtA$_C$-50S complexes. In a multiphase environments such as gel filtration, however, dissociated 50S subunits and CgtA$_C$ protein would be separated into two mobile phases. The large 50S complex would elute in the void volumes, whereas the free CgtA$_C$ would migrate slowly through the porous resin. Interestingly, the *B. subtilis* Obg and *E. coli* CgtA$_E$ proteins have been shown to fractionate in a large cytoplasmic complex by gel filtration (42) (Pu and Maddock, unpublished). Perhaps the use of low-salt buffers in these studies increased the stability of the protein-50S complex. Alternatively, there may be species-specific differences in the affinity of the Obg proteins with the 50S ribosomal subunits.

The addition of excess GDP or GTP to the cell lysates had no detectable effects on the association of CgtA$_C$ with 50S ribosomal particles. CgtA$_C$ has a moderate affinity for both GTP and GDP and rapidly exchanges its bound nucleotide in vitro (28). Therefore, in vivo, unless exchange is inhibited in the CgtA$_C$-50S complex the occupancy state of CgtA$_C$ should reflect that of the nucleotide pools. The results in this study suggest that the state of guanine nucleotide occupancy of CgtA$_C$ does not affect its binding or dissociating free 50S ribosomal subunits. It will be of interest to examine the association of CgtA$_C$ with 50S ribosomal subunits in cells at the stationary phase, when the intracellular levels of GDP should increase relative to that of GTP.

The details of CgtA$_C$ association with the 50S ribosomal subunit are unknown. Since the *B. subtilis* and *E. coli* Obg proteins associate with L13 (42) (Pu and Maddock, unpublished), it is likely that interaction with L13 mediates at least part of this interaction. In addition, CgtA$_E$ interacts with SpoT in a yeast two-hybrid screen (Pu and Maddock, unpublished); therefore, contacts with SpoT may also be critical for ribosome association. In this study, we uncovered a requirement for the C-terminal seven amino acids for both optimal CgtA$_C$ function and for association with the 50S ribosomal particles. Interestingly, strains expressing full-length or slightly truncated *cgtA$_C$* alleles epitope tagged with 3HA grew more slowly than the cells expressing their untagged counterparts, indicating that the addition of the tag was also detrimental for protein function. Moreover, none of the CgtA$_C$-3HA proteins associated with the 50S ribosomal particle when coexpressed with wild-type CgtA$_C$. Thus, either deletion of C-terminal sequences or the addition of a C-terminal tag affects CgtA$_C$ function and/or ribosome association.

CgtA$_C$ may play a role in ribosome assembly. The majority of CgtA$_C$ associates with a 50S particle but not with the 70S monoribosomes or with polyribosomes. The 50S peak is composed of newly synthesized 50S ribosomal subunits, recycled 50S ribosomal subunits (i.e., subunits dissociated from runoff mature ribosomes after transcription), and late pre-50S ribo-

somal precursors (23, 32, 35). The long-term effect of $CgtA_C$ depletion was a reduction in the level of mature 70S ribosomes and polyribosomes, whereas the levels of both free 30S and 50S subunits were relatively unchanged. It is possible that $CgtA_C$ acts at a late step in 50S subunit maturation. Such a role would be consistent with the ability of the *E. coli* $CgtA_E$ protein to act as a high-copy-number suppressor of both the growth and polysome defect of an rRNA methyltransferase mutant, Δ*rrmJ* (47).

The bacterial Obg proteins clearly play a cellular role beyond that of a translation factor. Obg proteins display essential functions in a wide variety of distinct cellular processes, such as cell growth and differentiation (39, 48, 51), DNA replication (26), chromosome segregation, and cell division (9, 11, 25, 43). In addition, the *B. subtilis* Obg protein is necessary for activation of the general stress response transcription factor, σ^B (41). Under conditions of environmental stress (i.e., heat, ethanol, salt, or acid treatment) or during energy depletion (i.e., decrease of cellular ATP concentration), σ^B is activated through the coordinate activities of the Rsb proteins and Obg. Obg in turn, interacts directly with RsbT, RsbW, and RsbX (42). A widespread role for Obg function in stress response, however, cannot be mediated through σ^B, as most bacteria do not have a σ^B-regulated stress response pathway. In *E. coli*, for example, this activity is primarily regulated by σ^S, a sigma factor that regulates many cellular responses that accompany entry into stationary phase, nutrient starvation (stringent condition), or shift to high osmolarity or low pH (for a review, see reference 19). The activation of σ^S is controlled, in part, by the alarmone (p)ppGpp (16). (p)ppGpp is synthesized by the ribosome-associated synthase, RelA. When cells enter into the stationary phase or are starved for one or more amino acids, RelA is activated and SpoT [a (p)ppGpp synthetase-hydrolase] is inactivated, resulting in increasing levels of (p)ppGpp (see reference 6 for a review). Intriguingly, the *E. coli* $CgtA_E$ protein interacts directly with SpoT (Pu and Maddock, unpublished), raising the possibility that $CgtA_E$ is also involved in stress response. One intriguing possibility is that the Obg proteins are involved in coordinating ribosome assembly and stress response. Studies are in progress to directly test this possibility.

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