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The *Caulobacter crescentus* GTPase $CgtA_C$ is required for progression through the cell cycle and for maintaining 50S ribosomal subunit levels

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Summary

The Obg subfamily of bacterial GTP-binding proteins are biochemically distinct from Ras-like proteins raising the possibility that they are not controlled by conventional guanine nucleotide exchange factors (GEFs) and/or guanine nucleotide activating proteins (GAPs). To test this hypothesis, we generated mutations in the *Caulobacter crescentus obg* gene (*$cgtA_C$*) which, in Ras-like proteins, would result in either activating or dominant negative phenotypes. In *C. crescentus*, a P168V mutant is not activating *in vivo*, although *in vitro*, the P168V protein showed a modest reduction in the affinity for GDP. Neither the S173N nor N280Y mutations resulted in a dominant negative phenotype. Furthermore, the S173N was significantly impaired for GTP binding, consistent with a critical role of this residue in GTP binding. In general, conserved amino acids in the GTP-binding pocket were, however, important for function. To examine the *in vivo* consequences of depleting $CgtA_C$, we generated a temperature-sensitive mutant, G80E. At the permissive temperature, G80E cells grow slowly and have reduced levels of 50S ribosomal subunits, indicating that $CgtA_C$ is important for 50S assembly and/or stability. Surprisingly, at the non-permissive temperature, G80E cells rapidly lose viability and yet do not display an additional ribosome defect. Thus, the essential nature of the *$cgtA_C$* gene does not appear to result from its ribosome function. G80E cells arrest as predivisional cells and stalkless cells. Flow cytometry on synchronized cells reveals a G1-S arrest. Therefore, $CgtA_C$ is necessary for DNA replication and progression through the cell cycle.

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Introduction

In recent years, it has become apparent that in addition to the well-characterized GTPases involved in specific steps in translation, such as IF2, EF-Tu, EF-G and RF3, there are a number of additional widely conserved GTP-binding proteins that play other roles in ribosome function (Leipe *et al*., 2002; Caldon and March, 2003). Among this latter class is the Obg family of GTPases, named after the *Bacillus subtilis* protein (named because of its coexpression with *spoOB*; Trach and Hoch, 1989). The Obg proteins are found in all genomes sequenced thus far. The bacterial Obg proteins are highly conserved throughout the N-terminal OBG-fold (Buglino *et al*., 2002) and within the signature GTP-binding domain. The C-terminal domains of these proteins, however, can vary in both length and sequence, which may result in functional differences between distantly related bacteria. In eukaryotes, the mitochondrial Obg proteins are similar to those found in bacteria. Eukaryotes also possess a nucleolar Obg protein, Nog1p, that is critical for biogenesis of the 60S ribosomal subunit (Jensen *et al*., 2003; Kallstrom *et al*., 2003) and two related cytoplasmic proteins in *Saccharomyces cerevisiae* (called Rbg1 and Rbg2) that are associated with translating ribosomes (P. Wout and J.R. Maddock, unpubl.). Archaea possess two Obg proteins of unknown function that are related to Nog1p and Rbg1p.

The bacterial Obg GTPases are ribosome associated. The *Caulobacter crescentus* $CgtA_C$ and *Escherichia coli* $CgtA_E$ (also called Obg_E and YhbZ) proteins are primarily associated with the 50S ribosomal subunit (Lin *et al*., 2004; Wout *et al*., 2004). Furthermore, the *B. subtilis* Obg protein co-fractionates with ribosomes and binds to the large ribosomal subunit L13 (Scott *et al*., 2000). Moreover, the *B. subtilis* Obg protein interacts with a number of proteins (Scott and Haldenwang, 1999) involved in controlling σ^B activity, all of which are also found associated with ribosomes (Scott *et al*., 2000). Recently, we have shown that $CgtA_E$ interacts with SpoT (Wout *et al*., 2004) and that SpoT is also ribosome associated (P. Wout and J.R. Maddock, unpubl.). Thus, it may be that all Obg proteins are ribosome-associated as are their interacting partners.

Accumulating evidence raises the possibility that in addition to associating with large ribosomal subunits, the bacterial Obg proteins, like the eukaryotic Nog1p protein, play a role in the assembly and/or stability of the large ribosomal subunit. In *E. coli*, overexpression of $CgtA_E$ suppresses the ribosome assembly defect of a Δ*rrmJ* mutant (Tan *et al*., 2002). RrmJ is a methyltransferase that modifies the 23S rRNA (Caldas *et al*., 2000) rather late in the maturation process of the 50S subunit. Suppression of Δ*rrmJ* by $CgtA_E$ is consistent with a role in ribosome assembly. Moreover, in *C. crescentus*, long-term depletion of $CgtA_C$ results in a diminution of translating ribosomes (Lin *et al*., 2004), consistent with a role in late assembly of the 50S.

In addition to an interaction with ribosomal subunits, all other known interacting partners of Obg proteins are involved in stress response. In *B. subtilis*, Obg interacts with RsbW, RsbT and RsbX, three proteins involved in the activation of σ^B, presumably via the activation of RsbT (Scott and Haldenwang, 1999). Furthermore, the *E. coli* $CgtA_E$ protein interacts with the stringent response protein SpoT (Wout *et al*., 2004). Perhaps non-coincidentally, the *B. subtilis* Obg protein co-crystallized with the SpoT/RelA product, ppGpp (Buglino *et al*., 2002). Interestingly, SpoT has a TGS motif. The TGS motif is predicted to be involved in binding to RNA and is found in a limited number of proteins, including threonyl tRNA synthetases and the cytosolic eukaryotic Obg proteins (Wolf *et al*., 1999). We have recently shown that in *S. cerevisiae*, Rbg1p is associated, on polysomes, with the stress response protein Gcn1p, through its coupling protein Gir2p (P. Wout and J.R. Maddock, unpubl.). Thus, in both prokaryotes, and eukaryotes, the Obg proteins physically interact with stress response proteins, raising the possibility that they are also universally involved in this cellular process.

Obg proteins bind guanine nucleotides with modest affinity, exchange guanine nucleotides rapidly but have intrinsic hydrolysis rates comparable to Ras-like GTPases (Welsh *et al*., 1994; Lin *et al*., 1999; Buglino *et al*., 2002; Wout *et al*., 2004). Here we investigate the *in vivo* and *in vitro* consequences of altering conserved residues in the GTP-binding pocket of $CgtA_C$. These studies reveal both similarities and differences between $CgtA_C$ and Ras-like proteins. Moreover, to gain insight into the function of $CgtA_C$ in *C. crescentus*, we generated and characterized a temperature-sensitive allele. These studies provide evidence that $CgtA_C$ is involved in two critical cellular functions, progression through the cell cycle and in 50S biogenesis.

Results

The binding of guanine nucleotides is critical for $CgtA_C$ function

The guanine nucleotide binding pocket of Ras-like proteins has been well characterized both structurally and genetically. The structures of the *B. subtilis* (Buglino *et al*., 2002) and *Thermus thermophilus* (Kukimoto-Niino *et al*., 2004) Obg proteins reveal that conserved residues also play critical roles in co-ordinating the guanine nucleotide in these essential Obg GTPases. To examine the consequences of altering these conserved amino acid residues in terms of $CgtA_C$ function, we individually changed amino acids predicted to play critical roles in guanine nucleotide binding and GTP hydrolysis, typically to alanine (Fig. 1). We also made the double mutant *$cgtA_C$D213AG216A*, the triple mutant *$cgtA_C$G171AK172AS173A* and a combined mutant, *$cgtA_C$G171AK172AS173A D213AG216A*, hereafter called DxxG, GKS and $CgtA_C$5A respectively. Single mutants will be referred to by their amino acid change (i.e. P168V). Each mutant allele was cloned into a plasmid that

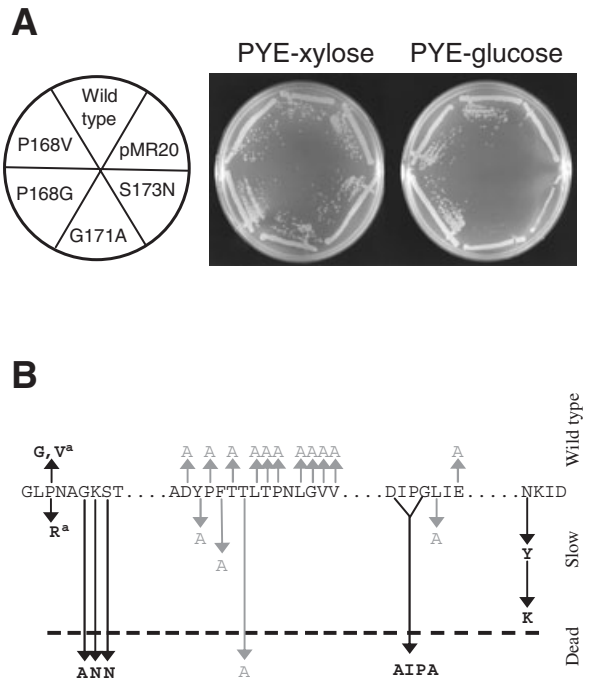

Fig. 1. Mutants affecting $CgtA_C$ function. The ability of each mutant *$cgtA_C$* allele to support growth was monitored in a strain (JM1108) in which the chromosomal copy of *$cgtA_C$* (P_{xyl}::*$cgtA_C$*) is repressed on PYE-glucose plates.
A. Cells expressing plasmid-borne wild-type *$cgtA_C$*, no *$cgtA_C$* (pMR20), or representative *$cgtA_C$* mutants, as identified in the cartoon on the left, after growth at 30°C on PYE-xylose or PYE-glucose plates.
B. Summary of the growth of the $CgtA_C$ mutants. The relevant amino acids in the GTP-binding domain are shown. Specific amino acid replacements and their ability to support growth when expressed in JM1108 on PYE-glucose plates compared with cells harbouring wild-type *$cgtA_C$* are indicated by the arrows and their positions. Mutants in the GTP-binding domains generated in this study are shown in bold type, whereas those in the Switch I and Switch II domains reported previously (Lin *et al*., 1999) are in grey. [a] indicates these mutants grew more slowly at 15°C than control cells.

replicates in *C. crescentus* as previously described (Lin *et al*., 2001). Wild-type cells expressing the mutant *cgtA*$_C$ alleles, wild-type *cgtA*$_C$, or containing a control vector grew equally well at 30°C and 37°C, indicating that none of the episomal *cgtA*$_C$ alleles conferred a dominant negative effect on colony growth (data not shown).

To address whether the *cgtA*$_C$ mutant alleles could support *C. crescentus* growth as the only expressed copy of *cgtA*$_C$ in the cells, we transformed JM1108, a strain in which the chromosomal copy of *cgtA*$_C$ is controlled by the xylose promoter (Lin *et al*., 2001), with each mutant plasmid. CgtA$_C$ levels in JM1108 are depleted by a shift of carbon source from xylose to glucose (Lin *et al*., 2001). JM1108 cells expressing an episomal wild-type *cgtA*$_C$ gene grow well on PYE-glucose whereas cells without *cgtA*$_C$ on the helper plasmid (pMR20) do not (Fig. 1A). Each mutant allele was tested for its ability to function *in vivo* by assessing the ability to complement the chromosomal P_{xyl}::*cgtA*$_C$ on PYE-glucose plates (summarized in Fig. 1B). None of the multiple mutants (DxxG, GKS or CgtA$_C$5A) grew, indicating that binding to guanine nucleotides is critical for CgtA$_C$ function (data not shown). Several of the single mutants, such as G171A, K172N and S173N, also failed to support growth. Furthermore, three alleles (P168R, N280Y, N280K) complemented poorly, although to different degrees (as judged by colony size) whereas P168G and P168V grew as well as cells expressing wild-type *cgtA*$_C$ (Fig. 1A). Similar results were seen when these strains were grown in PYE-glucose at either 37°C or 15°C with the exception of the P168V- and P168R-containing cells (data not shown). In these cases, growth on PYE-glucose at 15°C was impaired, indicating a mild cold sensitivity. Immunoblot analysis of all mutant strains revealed that the levels of CgtA$_C$ were comparable to that of strains harbouring wild-type CgtA$_C$ even after growth in PYE-glucose media for 24 h (data not shown). The inability of strains expressing the G171A, K172N and S173N alleles to support growth confirms the critical role of these amino acids in CgtA$_C$ function. Furthermore, the requirement for these residues is consistent with the conclusions of structural studies: the guanine nucleotide binding pocket of the Obg proteins is structurally similar to that of other Ras-like GTPases (Buglino *et al*., 2002; Kukimoto-Niino *et al*., 2004).

Guanine nucleotide binding, exchange and GTP hydrolysis of the P168V protein

The P168V mutant is of interest because this mutation does not appear to affect CgtA$_C$ function *in vivo*, whereas the equivalent mutation in Obg from *Streptomyces coelicolor* was reported to be essential (Okamoto and Ochi, 1998). In order to investigate whether this mutation affected the kinetic properties of the protein, P168V was expressed in *E. coli* cells and purified as described previously (Lin *et al*., 1999; 2001). To assess the ability of the

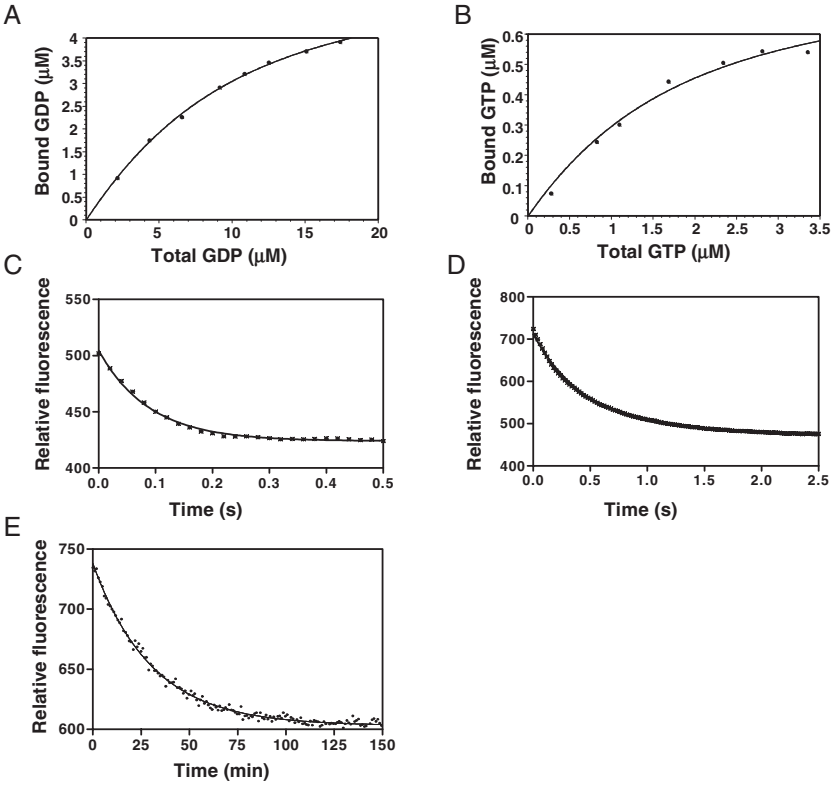

Fig. 2. Guanine nucleotide kinetics of P168V.
A and B. Equilibrium binding of P168V to (A) GDP and (B) GTP. P168V (4.9 μM) was incubated with increasing concentrations of either [8-^{3}H]-GDP or [^{32}P]-GTP in buffer containing 12 mM $MgCl_2$. Aliquots were removed and bound nucleotide was quantified. Results from a representative experiment are shown.
C and D. Guanine nucleotide exchange by P168V bound to (C) mGDP and (D) mGTP. P168V (5.1 μM) was pre-bound to 2 μM mGDP or mGTP in buffer containing 12 mM $MgCl_2$. This solution was rapidly mixed with excess GDP (150 μM) in a stopped-flow fluorometer, and the decrease in fluorescence intensity was monitored over time. Data were fitted to a single exponential decay curve. Curves shown represent the average of 10 data sets.
E. Hydrolysis of mGTP by P168V. P168V (3.7 μM) was pre-bound to mGTP (0.3 μM) in a buffer containing 12 mM $MgCl_2$. The reduction in fluorescence intensity resulting from hydrolysis of mGTP to mGDP in the protein–mGTP complex was recorded over time. Data were fitted to a single exponential decay equation; the results shown are from a representative experiment.

Table 1. Nucleotide binding, exchange rate constants and mGTP hydrolysis of $CgtA_C$.

	Equilibrium binding K_D (μM)		Nucleotide exchange k_d (s^{-1})		
$CgtA_C$ protein	GDP	GTP	mGDP	mGTP	mGTP hydrolysis $t_{1/2}$ (min)
Wild type[a]	0.52 ± 0.03	1.1 ± 0.1	1.43 ± 0.04	1.28 ± 0.02	23 ± 2
P168V	6.3 ± 1.1	1.4 ± 0.3	11.3 ± 0.3	2.1 ± 0.02	19.3 ± 0.4
S173N	19.4	ND	21.7 ± 0.9	ND	ND

a. Wild-type data reproduced from Lin *et al.* (1999).
ND, no data.

P168V to bind guanine nucleotides, the equilibrium binding constants for GDP and GTP were determined in the presence of 12 mM Mg^{2+} (Fig. 2; Table 1). At this magnesium ion concentration, wild-type $CgtA_C$ binds both GDP and GTP with moderate affinity (0.52 ± 0.03 μM and 1.1 ± 0.1 μM respectively) (Lin *et al.*, 1999). The equilibrium binding profiles obtained for the P168V mutant protein were typical hyperbolic plots (Fig. 2A and B); the P168V bound GTP with an affinity similar to that of wild-type $CgtA_C$ (1.4 ± 0.3 μM), whereas binding to GDP was reduced 12-fold (6.3 ± 1.1 μM; Table 1). Thus, a moderate reduction in the affinity for GDP does not affect $CgtA_C$ function.

Guanine nucleotide exchange and hydrolysis rates were assayed as described previously, using fluorescent *N*-methyl-3′-*O*-anthranoyl (mant) GTP and GDP analogues, mant-GTP and mant-GDP (Lin *et al.*, 1999; 2001; Sullivan *et al.*, 2000). Because the fluorescence of the mant moiety increases in a hydrophobic environment, the binding of protein to the mant-nucleotide can be measured as a function of the increase in fluorescence intensity (monitored with an excitation wavelength of 361 nm and an emission wavelength of 446 nm) of the mant group in the presence versus the absence of protein. As seen with $CgtA_C$ protein (Lin *et al.*, 1999; 2001; Lin and Maddock, 2001), binding of mant-GDP or mant-GTP to the P168V mutant protein resulted in an 1.2- and 1.6-fold increase in mant-nucleotide fluorescence respectively (data not shown). To measure dissociation constant for the mant-nucleotide, an excess of non-fluorescent GDP or GTP competitor was rapidly added to a prebound P168V–mant-GNP complex. The decrease in fluorescence intensity was measured over time and the data were fitted to a single-phase exponential decay equation (Fig. 2C and D). In the presence of 12 mM Mg^{2+}, wild-type $CgtA_C$ displays a rapid exchange of both GDP and GTP (1.43 ± 0.04 and 1.28 ± 0.02 s^{-1}; (Lin *et al.*, 1999). The P168V protein had a dissociation rate for mant-GTP similar to that of wild-type protein (2.1 ± 0.02 s^{-1}); the rate of dissociation of mant-GDP, however, was eightfold faster (11.3 ± 0.3 s^{-1}) (Table 1). Thus, neither a modest reduction in GDP affinity nor a modest increase in GDP exchange is detrimental to $CgtA_C$ function.

In Ras, the G12V mutant protein is significantly impaired for GTP hydrolysis (Gibbs *et al.*, 1988). Hydrolysis of GTP is also important for $CgtA_C$ function; we have previously reported that a primary effect of the loss-of-function Switch I mutation, T173A, is on GTP hydrolysis rate. To determine whether GTP hydrolysis was affected in the P168V mutant protein (analogous to G12V in Ras), we measured GTPase activity by monitoring the decrease in fluorescence that occurs as mant-GTP is hydrolysed to mant-GDP (Fig. 2E). The half-time of hydrolysis with P168V was 19.3 ± 0.4 min, which is comparable to that of wild-type $CgtA_C$ (Table 1). Therefore, the P168V mutation in *cgtA*$_C$, unlike the analogous mutation in *ras*, does not affect GTP hydrolysis.

The S173N mutant protein is significantly impaired for GTP binding and modestly impaired for GDP binding and exchange

In Ras-like GTPases, the stabilization of GTP requires a Mg^{2+} ion, which is oriented by the S17 residue. This coordinating serine is absolutely critical for GTP binding and affinity (John *et al.*, 1993). To determine whether this was also the case for the analogous $CgtA_C$ residue, we examined the biochemical properties of the S173N mutant protein. Binding of both GDP and GTP were severely reduced with the S173N as compared with the wild-type protein. The affinity for GDP was reduced ≈40-fold (Table 1), and the binding to GTP was insufficient to be detected in our assay. The exchange of mant-GDP was 15 times more rapid for the S173N mutant than for wild-type $CgtA_C$ (Table 1), consistent with the lower affinity for GDP. Because we were unable to monitor binding of S173N to mant-GTP, we could determine neither the exchange constant nor the hydrolysis rate.

The growth and viability of G80E mutant cells is impaired at the non-permissive temperature

A temperature-sensitive *B. subtilis obg* mutant (Kok *et al.*, 1994) and an analogous *E. coli cgtA*$_E$ mutant (Kobayashi *et al.*, 2001) have been previously isolated. The original *B. subtilis* mutant *obg* allele had two nucleotide changes

that resulted in G79E and D84N substitutions, both changes in the extended OBG fold (Buglino *et al*., 2002). Intragenic second-site suppressors mapped to amino acid 79, either restoring it to a glycine or altering it to an alanine, strongly suggesting that the G79E substitution alone was sufficient to confer temperature sensitivity. To investigate this in *C. crescentus* therefore we generated an analogous mutation in *cgtA*$_C$: *cgtA*$_C$*G80E.* Using a plasmid shuffle technique (Lin *et al*., 2001), we constructed a strain (JM1240) in which the only copy of *cgtA*$_C$ was a plasmid-borne *cgtA*$_C$*G80E* mutant allele.

At the permissive temperature of 23°C, cells expressing G80E grow more slowly than control cells harbouring a wild-type *cgtA*$_C$ gene (JM929) (4.5 h and 2.5 h doubling, respectively; Fig. 3A). The growth rate of cells expressing wild-type *cgtA*$_C$ increased after a shift to the non-permissive temperature of 37°C (2.2 h doubling), as is typical for *C. crescentus* at this higher temperature. Initially, the growth of G80E cells also increased at 37°C (3 h doubling). After approximately two generations, however, growth rapidly ceases (Fig. 3A). Furthermore, a dramatic reduction in viability of G80E cells occurs after the shift to the non-permissive temperature (Fig. 3B and C). No significant loss in cell viability was observed in the control cells.

It is possible to explain the loss in growth and viability at the non-permissive temperature by either the absence of CgtA$_C$ protein or the presence of non-functional CgtA$_C$. Immunoblot analysis of CgtA$_C$ levels from cells grown at the permissive and non-permissive temperature, however, revealed that there was no significant reduction in CgtA$_C$ protein after 8 h at 37°C (Fig. 3D), a time when viability had dropped 1000-fold. Therefore, the reduction in growth and the loss of viability appears to result from the loss of function of G80E at higher temperature.

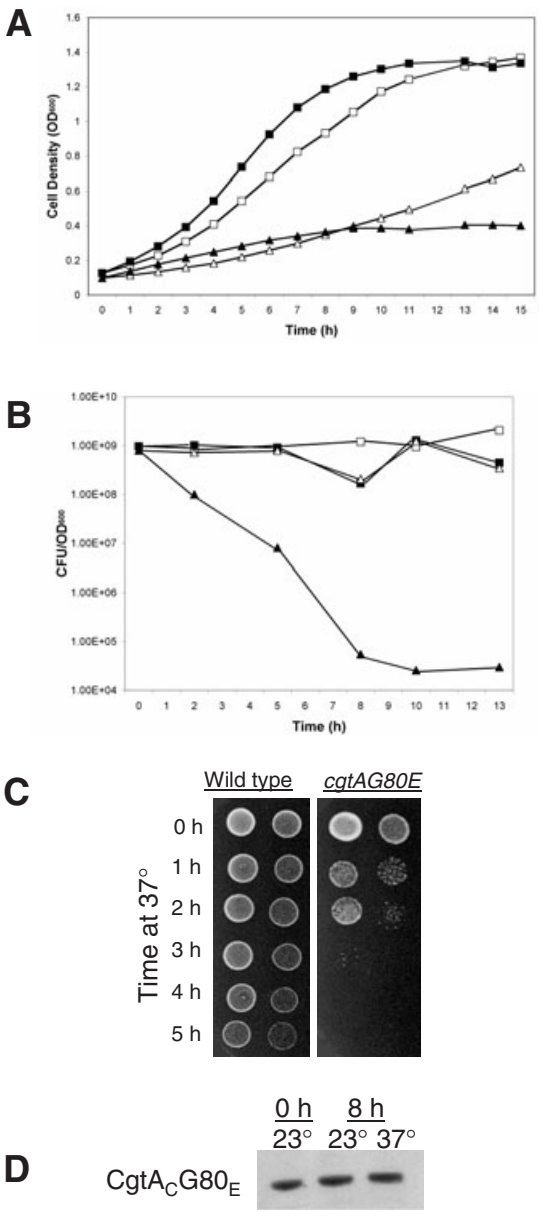

Fig. 3. Growth and viability of G80E mutant cells.
A. Representative growth curves of cells expressing either CgtA$_C$ (JM929, squares) or G80E (JM1240, triangles) at either 23°C (open) or 37°C (filled).
B. Corresponding viability of cells from (A).
C. Colony formation (two serial dilutions) of cells incubated at 37°C for the indicated time and plated on PYE-Tet-Spec at 23°C.
D. Immunoblot showing G80E protein levels before and after a shift from 30°C to 23°C or 37°C for 8 h.

CgtA$_C$ *affects the levels of 50S ribosomal subunits*

We have previously shown that CgtA$_C$ is associated with the 50S ribosomal subunit, and that long-term depletion of CgtA$_C$ results in a reduced level of 70S monosomes and polyribosomes (Lin *et al*., 2004). With the goal of determining the more immediate effect(s) of a loss of CgtA$_C$ function on ribosomal synthesis or stability, we examined the polysome profiles of cells harbouring either a wild-type *cgtA*$_C$ allele or the *cgtA*$_C$*G80E* mutant allele (JM929 and JM1240 cells respectively). Cells carrying the wild-type *cgtA*$_C$ gene display a normal ribosome profile at all temperatures (Fig. 4A). Cells expressing G80E, in contrast, exhibit a reduction in the 50S and 70S peaks relative to the 30S peak at all temperatures examined (Fig. 4B). As G80E mutants are slow growing at lower temperatures, it was not surprising to detect a polysome defect at the permissive temperature; however, we were somewhat surprised that we did not observe an enhanced defect at the non-permissive temperature. Two critical conclusions can be drawn from these results. First, the G80E mutant, even at the permissive temperature, is defective in 50S subunit synthesis or stability. This defect may be a direct consequence of a defective CgtA$_C$ protein, or due to downstream defects in this mutant strain. Second, G80E

mutant cells are not inviable at 37°C by a further perturbation in ribosome biogenesis and/or stability. Thus, the essential requirement for $CgtA_C$ probably results from a function independent of ribosome assembly.

G80E mutants display a cell cycle arrest

Caulobacter crescentus is particularly amenable to cell cycle studies. After cell division, predivisional cells give rise to two distinct cell types: the motile swarmer cell and the sessile stalked cell (Fig. 5A). The stalked cell immediately begins to elongate and initiates a new round of DNA synthesis. The swarmer cell, however, first differentiates into a stalked cell before the onset of DNA replication (Fig. 5A). These cell cycle changes can be observed both morphologically, as the cell types are visually distinct (Fig. 5B), and by DNA content using flow cytometry (see below). To determine whether $CgtA_C$ played a role in cell cycle progression, we determined the terminal phenotype of a population of G80E cells by electron microscopy of whole cells mounted on formvar grids. A subtle phenotype is apparent with G80E cells grown at 23°C (Fig. 5C). The G80E swarmer cells were slightly shorter than those of wild type, although most swarmer cells were flagellated and motile as observed by light microscopy (data not shown). The stalked cells were similar in size and shape to those of control cells, although the stalks themselves occasionally (<15%) had misformed ends (flat or pointed). Predivisional cells had similar stalk defects. In addition, late predivisional cells had extended septation sites and occasional cells were observed in which the two cell halves appeared to be rotated 180° relative to one another. One hour after being shifted to 37°C, few swarmer cells were detected in the population (Fig. 5C). Stalks were significantly shorter than wild type, misformed, or missing from the stalk cells and the predivisional cells. Predivisional cells were abundant and the elongated septal zone seen in cells grown at 23°C was more apparent. After 3 h at 37°C, similar phenotypes were observed and the stalk defects were more pronounced (Fig. 5C). After 5 h at the non-permissive temperature, elongation of some predivisional cells was detected (Fig. 5C). Thus, it appears that the G80E cells arrest at two points in the cell cycle: as stalkless cells and as late predivisional cells.

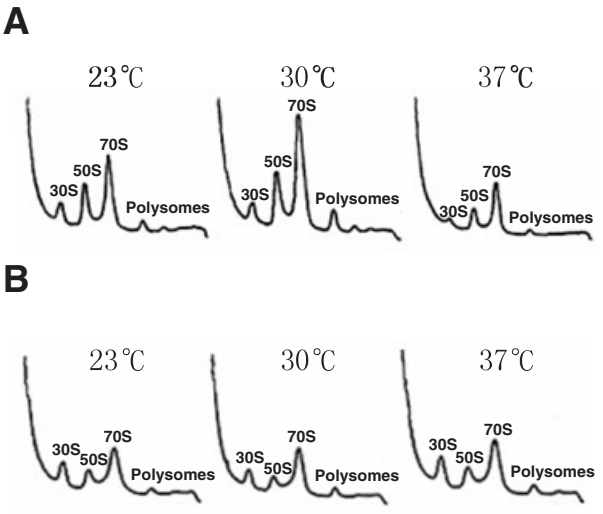

Fig. 4. Polysome profiles of cells expressing $CgtA_C$ or G80E. JM929 (A) and JM1240 (B) cells were grown at 23°C and shifted to either 30°C or 37°C or allowed to continue growth at 23°C for 2 h, as indicated. Polysome profiles from the cell lysates were monitored by UV absorbance as the sample was withdrawn from the top (left) to bottom (right) of the gradient. The 30S and 50S ribosomal subunits, the 70S monosome and the polysome peaks are indicated.

To examine more carefully the terminal phenotype of the G80E mutant, we quantified the DNA content of the cells by flow cytometry (Fig. 5D and E). In control cells expressing $cgtA_C$ episomally (JM929), at 23°C, the DNA profiling revealed that the population consisted of swarmer cells (G1 cells with 1N DNA content), stalked cells (S cells with a replicating chromosome) and predivisional cells (G2 cells with 2N DNA content). A shift of these cells to 37°C for 1 or 3 h had little effect on the distribution of the DNA profile. After 5 h at 37°C, however, a small population of cells with three chromosomes appears, consistent with the observation of elongated cells at this temperature. In contrast, the initial profile of the G80E-expressing cells shows an increase in the number of predivisional cells relative to the control strain. Furthermore, after a shift to 37°C for only 1 h, a remarkable alteration in the DNA content of the population occurs: the majority of the cells (> 80%) contain two chromosomes. At later times, some 1N cells appear, as do cells with 3N chromosomes. Thus, the G80E mutant displays a partial cell division arrest as initially significant numbers of predivisional cells accumulate. After extended incubation at the non-permissive temperature, some of the predivisional cells divide, resulting in 1N cells, or replicate one chromosome, resulting in 3N cells. It is likely that the elongated cells visualized by electron microscopy are these 3N cells.

The morphology of the G80E mutants suggested that they were arrested as stalkless cells and predivisional cells. The DNA content analysis of the population of G80E cells provides evidence for a partial predivisional arrest. To determine whether the stalkless cells also possess a DNA replication defect, we performed cell synchrony experiments.

Shown in Fig. 6A is a cell cycle flow cytometry analysis of synchronized cells expressing $cgtA_C$ episomally (JM929). Isolated swarmer cells were incubated in PYE medium at 23°C and at the indicated times the DNA content of the cells was assessed. Under these condi-

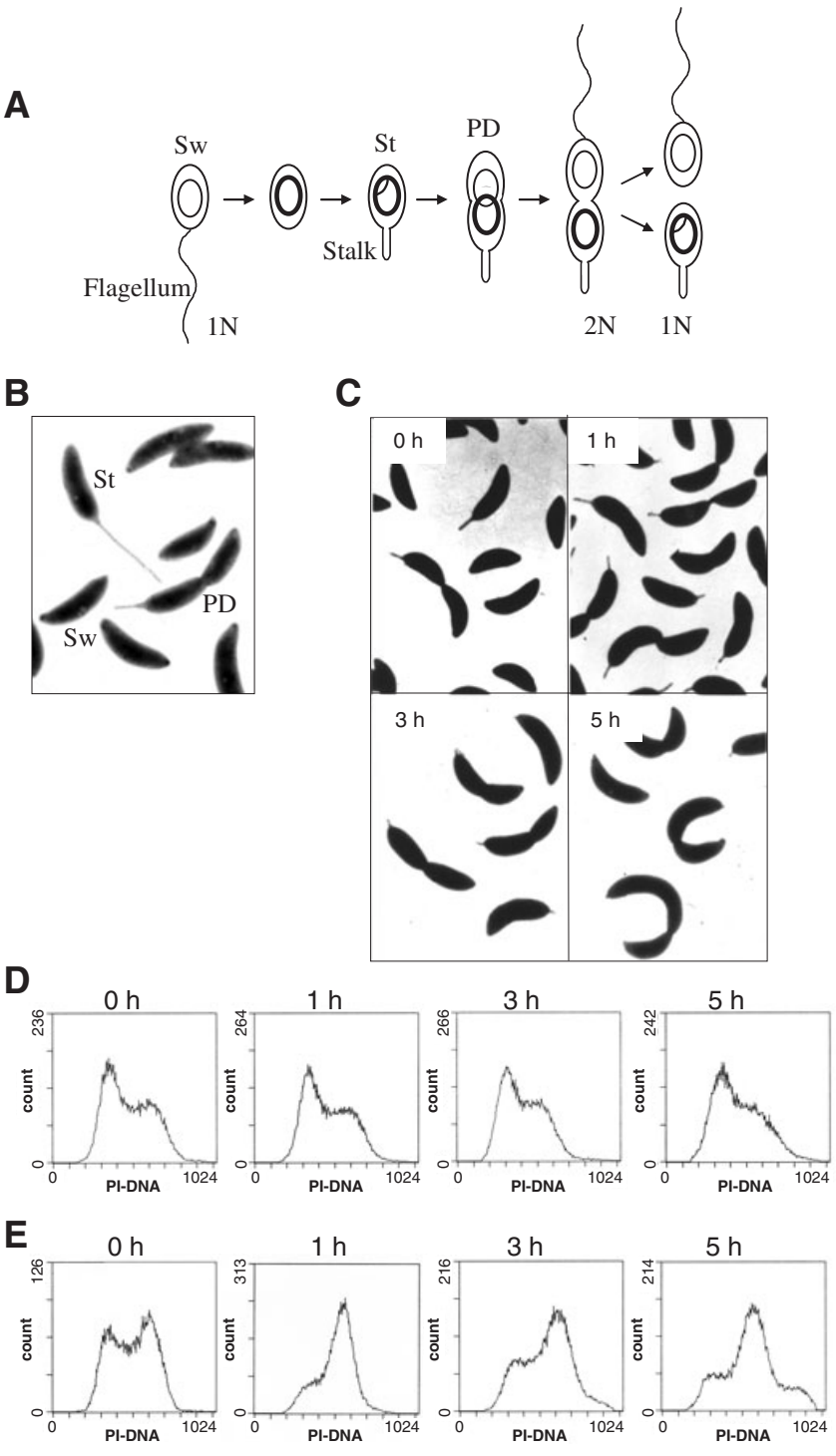

Fig. 5. Cellular morphology and DNA content of cells harbouring G80E at the non-permissive temperature.
A. Cartoon of the *C. crescentus* cell cycle. The circular chromosome is depicted by the internal oval. The swarmer (Sw) cell possesses a single polar flagellum and one chromosome (1N). The transition to a stalk (St) cell is accompanied by the initiation of DNA replication and the generation of the stalk. Predivisional cells (PD) divide to produce one swarmer and one stalk cell.
B and C. Electron micrographs of cells expressing (B) CgtA$_C$ (JM929) grown at 23°C or (C) G80E (JM1240) shifted to 37°C for the indicated period of time. Representative Sw, St and PD cells are labelled.
D and E. Flow cytometry of cell populations expressing either (D) CgtA$_C$ (JM929) or (E) G80E (JM1240) shifted to 37°C for the indicated period of time. The *y*-axis is the cell count and the *x*-axis is the DNA content as measured by propidium iodide fluorescence. A minimum of 50 000 cells were counted per experiment.

tions, JM929 has a 2.5 h doubling time (Fig. 3A). Immediately upon synchronization, the vast majority of the cells have 1N DNA content, consistent with the initial population consisting primarily of swarmer cells (Fig. 6A). After the cells differentiate into stalked cells (after ≈60 min), DNA replication ensues, as indicated by a partial shift in the DNA content. By 120 min post synchronization, the vast majority of the cells are in the midst of replicating their chromosome or are predivisional cells. After 150 min, some predivisional cells have divided, resulting in the reappearance of a population of 1N cells (swarmer and stalked cells).

G80E swarmer cells were also isolated and allowed to grow at 23°C. Under these conditions, the G80E cells

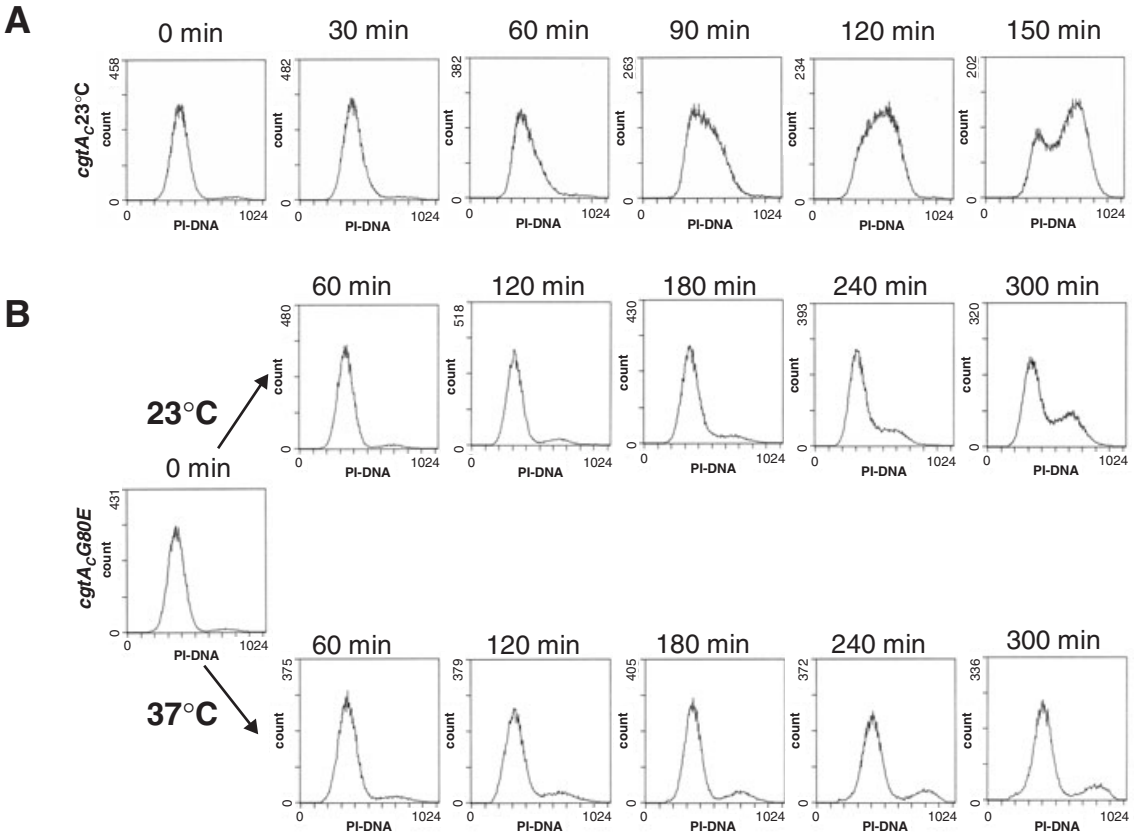

Fig. 6. Cell cycle analysis of the DNA content of cells expressing $CgtA_C$ or G80E. Cells were grown at 23°C and swarmer cells isolated as described in *Experimental procedures*.
A. Flow cytometry of synchronized JM929 cells (*cgtA*$_C$) grown in PYE at 23°C for 150 min.
B. Flow cytometry of synchronized JM1240 cells (*cgtA*$_C$*G80E*) grown at 23°C (top) or shifted to 37°C (bottom) for 300 min.
DNA measurements were taken at the indicated times. The *y*-axis is the cell count and the *x*-axis is the DNA content as measured by propidium iodide fluorescence. A minimum of 50 000 cells were counted per experiment.

doubled in 4.5 h (Fig. 3A). Consistent with the slow growth and slight morphological abnormalities observed in the G80E mutant at the permissive temperature (Figs 3 and 4), a defect in cell cycle progression is also apparent. The DNA content profile of G80E cells is strikingly different from that of control cells in that, even allowing for the increased doubling time, the majority of cells appear to remain for extended times as 1N cells with only limited number initiating and completing DNA replication (Fig. 6B). When G80E swarmer cells were shifted to the non-permissive temperature, the G1 block was even more dramatic (Fig. 6B). A small number of G2 cells accumulate after extended times at 37°C; however, the number of cells in S phase is small, as evidenced by the dip in the curve between the G1 and G2 cell peaks. These data indicate that cells lacking $CgtA_C$ activity do not effectively replicate their DNA. The minor population of G80E cells that do progress through the cell cycle may result from leaky G80E function or perhaps is attributable to cells that were already beyond the $CgtA_C$ checkpoint at the beginning of the synchrony. Regardless, these data reveal that $CgtA_C$ is also required for cell cycle progression from G1 to S.

Discussion

In this study, our first goal was to investigate the cellular consequences of amino acid substitutions in putative GTP-binding domains on $CgtA_C$ function. These studies were, in large part, motivated by the concern that mutations in these biochemically distinct bacterial GTPases may not render phenotypes analogous with those found in the eukaryotic Ras-like proteins. We, and others, have previously demonstrated that the Obg proteins bind guanine nucleotides specifically, but with modest affinity (Welsh *et al.*, 1994; Lin *et al.*, 1999; Wout *et al.*, 2004). Furthermore, guanine nucleotides are rapidly exchanged (Lin *et al.*, 1999; Wout *et al.*, 2004), raising the possibility that the Obg proteins are not controlled by guanine nucleotide exchange factors (GEFs) and/or guanine nucleotide activating proteins (GAPs). These biochemical features are also found in other bacterial GTPases predicted to

play roles in ribosome function such as the *E. coli* Era (Sullivan *et al*., 2000), Der (Hwang and Inouye, 2001), YjeQ (Daigle *et al*., 2002) and YihA (Lehoux *et al*., 2003) proteins. Therefore, we were particularly concerned with the widespread assumption that the generation of alleles that in Ras-like proteins are 'activating' (such as RasG12V) or 'dominant negative' (such as RasS17N) would produce similar phenotypes in these bacterial GTPases. We show here that these analogous mutations in *cgtA$_C$* are distinctly different from their Ras counterparts.

One of the most widespread mutations generated in Ras-like proteins is the activating mutation G12V. The G12V mutant protein is significantly impaired for both intrinsic and GAP-stimulated hydrolysis (Gibbs *et al*., 1988), and therefore, is constitutively active. We report here that this is not the case for the analogous CgtA$_C$ mutant, P168V. In all Obg proteins, the RasG12 position is a proline, although we show here that a *C. crescentus* P168G mutant is fully functional (Fig. 1). Unlike the G12V activating mutant in *ras*, expression of P168V in a wild-type *C. crescentus* background does not result in a dominant negative phenotype. Moreover, cells expressing only P168V or P168R are also viable and show no obvious growth defects at 30°C, indicating that these alleles are functional *in vivo*. We do, however, observe a modest cold-sensitive phenotype for both of these mutant alleles. Biochemically, GTP binding, exchange and most notably GTP hydrolysis are not impaired in the P168V protein (Fig. 2; Table 1). There is, however, a modest defect in both the affinity and guanine nucleotide exchange of GDP, perhaps contributing to the cold sensitivity.

Our biochemical analysis of the *C. crescentus* P168V mutant protein sheds light on a previous study in *S. coelicolor* (Okamoto and Ochi, 1998). Starvation in *S. coelicolor* results in the formation of aerial mycelium and ultimately the formation of spores. Overexpression of wild-type Obg in *S. coelicolor* results in the suppression of aerial mycelium (Okamoto and Ochi, 1998). It has been proposed that this suppression results from the presence of the excess Obg bound to GTP under conditions when the Obg would normally be bound to GDP (Okamoto and Ochi, 1998). Interestingly, overexpression of the *S. coelicolor* P168V mutant protein led to a further suppression of aerial mycelium (Okamoto and Ochi, 1998). Based on the phenotype of the G12V *ras* mutant, the authors proposed that the *S. coelicolor* P168V mutant protein would be bound predominantly to GTP because of an inability to be regulated by its cognate GAP (Okamoto and Ochi, 1998). Given the biochemical features of *C. crescentus* P168V mutant protein, we propose an alternate explanation of these data: that *in vivo*, the *S. coelicolor* P168V mutant protein is more tightly bound to GTP, but due to a lower affinity for GDP and not because of a defect in hydrolysis. Thus, although the details of the mechanism differ, our data support the model for how GTP occupancy could control development in *S. coelicolor* (Okamoto and Ochi, 1998).

In general, *ras* mutants that result in proteins with impaired binding to GTP, such as S17N, N116Y or D119N, behave as dominant negative alleles (Farnsworth and Feig, 1991; Cool *et al*., 1999). RasS17N, for example, displays a decreased affinity to GTP and an increased affinity for its exchange factor, effectively sequestering the GEF and inhibiting the activation of the wild-type Ras (Farnsworth and Feig, 1991). This is clearly not the case for the Obg proteins as neither the *S. coelicolor* S173N (Okamoto and Ochi, 1998) or the *C. crescentus* S173N and N280Y mutant proteins display a dominant negative phenotype under conditions assayed. As expected, based on the conserved guanine nucleotide pockets of all GTPases, the *C. crescentus* S173N protein is, however, severely impaired for nucleotide binding (Table 1). The affinity for GDP was reduced >35-fold and the exchange rate constant increased 15-fold. We could not detect any binding to GTP.

Despite the modest affinity for nucleotides, guanine nucleotide occupancy is a critical requirement for the function of Obg proteins. We show here that mutations in conserved amino acids of CgtA$_C$ predicted to be critical for the binding of guanine nucleotides, such as DxxG, GKS or CgtA$_C$5A, S173N, G171A or K172N were unable to function *in vivo*. In *S. coelicolor*, G171A is probably non-functional as well as chromosomal insertions were not obtained using a two-step integration/excision approach (Okamoto and Ochi, 1998). These investigators were also unable to obtain an *obgP168V* mutant by this method. Given that the *cgtA$_C$P168V* is fully functional and the GTP-binding domains of all Obg proteins are quite similar, it may be that this allele was not obtained in *S. coelicolor* because of unequal recombination frequencies in the DNA flanking the *obg* gene. Regardless, it is clear that despite the modest affinity for guanine nucleotides by the Obg proteins, guanine nucleotide binding is an essential feature of their function.

The second goal of our study was to provide evidence for a function for CgtA$_C$. Because of evolutionary relationships between GTPases, it was proposed that the Obg proteins are involved in some aspect of translation (Leipe *et al*., 2002). Based on recent publications and work presented here, it is likely that the majority of Obg proteins are involved in assembly of the large ribosomal subunit. Such a role is clearly well documented for the nucleolar *S. cerevisiae* Nog1p protein which functions specifically in the maturation of the 60S subunit (Jensen *et al*., 2003; Kallstrom *et al*., 2003). Similarly, we have recently shown the *S. cerevisiae* mitochondrial Obg protein, Mtg2p is associated with the large ribosomal subunit and required

for mitochondrial translation (K. Datta and J.R. Maddock, unpubl.). Interestingly, the two cytoplasmic *S. cerevisiae* Obg proteins, Rbg1p and Rbg2p, do not appear to play a role in assembly, but rather, are associated with the polysomes and interact with the stress response pathway (P. Wout and J.R. Maddock, unpubl.). In bacteria, accumulating evidence points to a role in 50S assembly. In *E. coli*, overexpression of $CgtA_E$ suppresses the assembly defect of rRNA methyltransferase mutant (Tan *et al*., 2002). We have recently shown that both the *E. coli* $CgtA_E$ (Wout *et al*., 2004) and *C. crescentus* $CgtA_C$ proteins (Lin *et al*., 2004) interact with the 50S ribosomal subunit. This is probably the case for the *B. subtilis* Obg protein as it binds to the large subunit protein L13 and co-fractionates with ribosomal proteins (Scott *et al*., 2000).

We have previously shown that long-term depletion of $CgtA_C$ resulted in a polysome defect (Lin *et al*., 2004). In order to determine the immediate consequences of impairing $CgtA_C$ function, we created and examined the temperature-sensitive *cgtA*$_C$*G80E* allele. Strains expressing this mutation grew slowly at the permissive temperature and died rapidly at the non-permissive temperature (Fig. 3). Polysome profiles revealed a defect in the accumulation of 50S subunits, even at the permissive temperature (Fig. 4), consistent with a role for $CgtA_C$ in ribosome assembly and/or stability. Similar slow growth and a reduction in 50S subunit are commonly seen in proteins involved in ribosome assembly in *E. coli* (Marvaldi *et al*., 1979; Dabbs, 1982; Herold *et al*., 1986; Franceschi and Nierhaus, 1990; Wower *et al*., 1998). Thus, it appears that $CgtA_C$ plays a role in the assembly and/or stability of the large ribosomal subunit.

Interestingly, at the non-permissive temperature, we do not see a further polysome profile perturbation in cells expressing G80E indicating that the essential function of $CgtA_C$ is distinct from its role in ribosome biogenesis. At the non-permissive temperature, G80E cells arrest as stalkless cells and as late predivisional cells (Fig. 5), both times in the cell cycle before the onset of DNA replication (Degnen and Newton, 1972; Marczynski *et al*., 1995). Flow cytometry analysis confirms that these cells are impaired for DNA replication (Figs 5 and 6). This DNA replication arrest is particularly interesting given that in *E. coli*, $CgtA_E$ is associated with both the 50S subunit and with the stringent response protein, SpoT (Wout *et al*., 2004). In *E. coli*, the levels of the alarmone (p)ppGpp are controlled by the activities of the (p)ppGpp synthetase RelA (Alfoldi *et al*., 1962) and the (p)ppGpp synthetase/hydrolase SpoT (Hernandez and Bremer, 1991). Both RelA and SpoT are associated with (Ramagopal and Davis, 1974; Gentry and Cashel, 1995; P. Wout and J.R. Maddock, unpubl.) and controlled by (Haseltine and Bock, 1973; Richter, 1980) ribosomes. It has been previously noted that in *E. coli* (p)ppGpp is an effector that couples DNA replication with growth rate control through expression of the initiator protein, DnaA (Chiaramello and Zyskind, 1990).

An interesting possibility is that $CgtA_E$ is involved in the control of SpoT function and therefore indirectly involved in control of DNA replication. Three lines of evidence support such a model. First, in *cgtA*$_E$(ts) mutants at the non-permissive temperature, replication of λ replicons is reduced as is the level of DnaA (Ulanowska *et al*., 2003) suggesting that $CgtA_E$ plays a role in expression of *dnaA*. Second, *cgtA*$_E$(ts) mutants also show a DNA partitioning defect (Kobayashi *et al*., 2001). At the time, the authors proposed a direct role of $CgtA_E$ in chromosome partitioning (Kobayashi *et al*., 2001). An alternate possibility, however, is that this phenotype results from the lower expression of the *dnaA* operon. Coexpressed with *dnaA* is *gyrB* (B subunit of DNA gyrase) and *gyrB* mutants form filamentous cells and fail to segregate chromosomes (Orr *et al*., 1979), a phenotype very similar to that reported for *cgtA*$_E$(ts) (Kobayashi *et al*., 2001). Finally, in this report, we show that in *C. crescentus*, G80E mutant cells arrest before the onset of DNA replication. In *C. crescentus*, accumulation of (p)ppGpp results in the inability of swarmer cells to differentiate into stalked cells (Chiaverotti *et al*., 1981). One intriguing possibility is that $CgtA_C$ also interacts with Rel_C, the only predicted (p)ppGpp synthetase/hydrolase in *C. crescentus*. If true, perhaps the cell cycle arrest we observe in the G80E mutant is a result of altered (p)ppGpp levels. We are currently examining these possibilities.

Experimental procedures

Bacterial strains and culture conditions

Caulobacter crescentus strains were derived from NA1000 (a synchronizable but otherwise wild-type strain) and were typically grown at 30°C in PYE medium (Poindexter, 1964) or PYE with 2% glucose or xylose, as indicated. JM1108 is a strain with *cgtA*$_C$ expression controlled exclusively by the P_{xyl} promoter (Lin *et al*., 2001). JM929 possesses a chromosomal null allele of *cgtA*$_C$ (Maddock *et al*., 1997) and wild-type *cgtA*$_C$ on pRM20 (Stephens *et al*., 1997). Other mutant strains are described below.

Antibiotics were used at the following concentrations: 1 μg ml^{-1} oxytetracycline (Tet), 25 μg ml^{-1} spectinomycin (Spec), 20 μg ml^{-1} naladixic acid (Nal) or 5 μg ml^{-1} kanamycin (Kan), when appropriate. Plasmids were introduced into *C. crescentus* from the *E. coli* strain S17 by conjugal transfer (Ely, 1979). *E. coli* cells were grown in Luria broth (LB; 10 g of tryptone, 5 g of yeast extract, 10 g of NaCl per litre) or LB agar at 37°C containing antibiotics at the following concentrations: 12 μg ml^{-1} tetracycline, 100 μg ml^{-1} spectinomycin, 30 μg ml^{-1} kanamycin or 100 μg ml^{-1} ampicillin (Amp).

Generation of mutations and mutant strains

Various *cgtA*$_C$ mutations were generated using the oligonu-

Table 2. Oligonucleotides used to generate *cgtA$_C$* mutations.

Name	Amino acid altered	Sequence[a]
P168V	168	5′-CTGGTGGGCCTG**GT**CAACGCCGGCAAG-3′
P168G	168	5′-CTGGTGGGCCTG**GG**CAACGCCGGCAAG-3′
P168R	168	5′-CTGGTGGGCCTG**CG**CAACGCCGGCAAG-3′
G171A	171	5′-CTGCCCAACGCCG**C**CAAGTCGACCTTC-3′
K172N	172	5′-CCCAACGCCGGC**A**ACTCGACCTTCCTG-3′
S173N	173	5′-AACGCCGGCAAG**AA**CACCTTCCTGGCG-3′
GKS	171, 172, 173	5′-CTGCCCAACGCCG**C**C**GC**G**GC**GACCTTCCTGGCG-3′
DxxG	213, 216	5′-TTCGTGCTGGCCG**C**CATTCCC**G**C**C**CTGATCGAAGGC-3′
N280K	280	5′-ATCCTGGCGCTGAA**G**AAGATCGACGCC-3′
N280Y	280	5′-ATCCTGGCGCTG**T**ACAAGATCGACGCC-3′
REK	25, 26, 27	5′-GTGTCGTTCCGC**GC**CG**C**G**GC**GTACATCGAGTA-3′
G80E	80	5′-AGCGCGGCATG**AG**GCGGCCGGCGAAGA-3′

a. The nucleotides that differ from wild type are in bold and the altered codons are underlined.

cleotides listed in Table 2 and Altered Sites II mutagenesis as described previously (Lin *et al*., 2001). After confirming the mutations by DNA sequencing, the *cgtA$_C$* alleles were cloned into pMR20 (Stephens *et al*., 1997), as described (Lin *et al*., 2001). Mutant *cgtA$_C$* alleles on pMR20 were introduced into JM1108 and maintained on PYE Kan Tet + 0.1% xylose plates.

The G80E temperature-sensitive mutant (JM1240) was isolated as follows: pJS22 (*cgtA$_C$G80E*) was introduced into JM557, a strain containing *δcgtA$_C$::Ω* integrated at the *cgtA$_C$* locus (Maddock *et al*., 1997), by conjugation. Loss of the integrated plasmid was selected for as described (Maddock *et al*., 1997), and surviving cells were screened for Kan^S temperature-sensitive cells to identify JM1240.

Growth and viability measurements

Cultures of JM1240 and JM929 were grown on PYE Tet Spec Nal at 23°C to an OD_{600} of ≈0.1. The cultures were split and either returned to 23°C or shifted to the non-permissive temperature (37°C). Cell growth was monitored by measuring the OD_{600}. Cell viability was measured by plating appropriate dilutions onto PYE plates and observing growth at 23°C. These experiments were performed in duplicate on parallel cultures. Similar results were seen in all cases. The complementation of *cgtA$_C$* mutants in JM1108 was assayed by cell growth on PYE + 0.1% xylose and PYE + 0.1% glucose (Tet Spec) at 18°C, 23°C, 30°C and 37°C.

Electron microscopy

Mid-log phase cells grown in PYE at room temperature or at 37°C for the indicated times were allowed to settle for ≈20 min on formvar-coated grids (prepared by placing grids on the film produced after dropping 0.5% formvar on ddH_2O). After one wash in distilled water, cells were stained with 1% uranyl acetate and washed extensively in water. Microscopy was performed on a Philips CM10 at 60 kV and images were recorded on Kodak 4489 film.

Flow cytometry

Cells were grown to mid-log phase at room temperature in PYE with the appropriate antibiotics. For the temperature shift experiments, the 23°C culture was split and grown at 23°C and 37°C. Cell synchronies were performed as described (Evinger and Agabian, 1977) with the following modifications: single colonies of JM1240 or JM929 cells were inoculated into 5 ml of PYE Tet Nal and grown to an OD_{600} of 0.8. Cells were pelleted and synchronized in microcentrifuge tubes. For flow cytometry, cells were pelleted, washed in staining buffer (50 mM NaCitrate, pH 7.2, 10 mM Tris-HCl, pH 7.2, 1 mM EDTA), resuspended in 70% ethanol in staining buffer and incubated at –20°C overnight. The cells were then washed in staining buffer and resuspended in the same buffer with the addition of 2 μg ml^{-1} RNase and 20 μg ml^{-1} propidium iodide. Flow cytometry was performed at the Cell Sorting Facility at the University of Michigan. A minimum of 50 000 cells were counted for each time point.

Polysome profiles

Caulobacter crescentus cells were grown in PYE at 23°C to an OD_{600} of 0.3 and cells were shifted to either 30°C or 37°C or were allowed to continue growing at 23°C for 2 h. *C. crescentus* cell lysates were prepared as described (Lin *et al*., 2004). Approximately 20 OD_{260} of cell lysate was subjected to sucrose density centrifugation in a 10 ml 15–45% sucrose gradient for 3 h at 41 000 r.p.m. at 0°C as described (Lin *et al*., 2004). The resulting polysomes were fractionated by a Brandel gradient fractionator connected to a syringe pump and the UV absorbance (260 nm) was monitored.

Overexpression and purification of CgtA$_C$

Escherichia coli Bl21(DE3) pLys cells expressing CgtA$_C$ were grown in 1 l of LB to an OD_{600} of 0.6 and induced with 1 mM IPTG for 2–4 h. Cells were harvested (6000 *g*, 10 min 4°C), resuspended in CORE buffer (50 mM Tris-HCl, pH 8.0, 10% glycerol, 1 mM DTT) supplemented with 1 mM PMSF, lysed by two passages through a French pressure cell, and the lysate was clarified by centrifugation at 28 000 *g* for 30 min at 4°C. The supernatant was passed through a 0.45 μm filter and applied to a 20 ml Cibacron Blue column. The column was washed with 300 ml of CORE buffer and the protein was eluted at a flow rate of 1 ml min^{-1} with a 240 ml linear gradient

of CORE buffer containing 0–0.6 M KCl. The fractions were analysed by 15% SDS-PAGE, and appropriate fractions were pooled, diluted twofold with CORE buffer and loaded on a 50 ml DEAE column. The column was washed with 100 ml of CORE buffer and protein eluted at a flow rate of 1 ml min^{-1} with a 250 ml linear gradient of 0–0.5 M KCl in CORE buffer. The fractions were analysed by 15% SDS-PAGE, and appropriate fractions were concentrated using a Centiprep 10 concentrator (Amicon), loaded onto a 100 ml gel filtration column (source), and eluted with CORE buffer containing 150 mM KCl. Fractions were collected at a flow rate of 0.5 ml min^{-1} and analysed by 15% SDS-PAGE. Concentrated fractions were pooled, dialysed against CORE buffer and stored at –80°C before use. The concentration of the protein was determined by the Bradford method (Bradford, 1976).

Equilibrium binding assays

Each binding reaction contained 4.9 μM purified P168V or S173N protein in binding buffer (50 mM Tris-HCl pH 8.0, 10% glycerol, 50 mM KCl, 2 mM DTT, 10 μM ATP, 1 mM EDTA) with 12 mM $MgCl_2$. [α-^{32}P]-GTP was varied over a range of 0.25–5 μM. [8-^{3}H]-GDP was varied over a range of 2–18 μM for reactions containing P168V, whereas for reactions containing S173N, [8-^{3}H]-GDP was varied over a range of 10–40 μM. Aliquots (60 μl) were taken directly for scintillation counting to measure the total nucleotide concentration, whereas the remainder of the reaction was transferred to an Amicon spin column. After a centrifugation at 16 000 *g* for 10 min, 60 μl of the filtrate was withdrawn for scintillation counting to determine the free nucleotide concentration. The bound nucleotide concentration was calculated by subtracting the free from the total nucleotide concentration. Equilibrium binding constants (K_D) were calculated from a binding curve (bound versus total nucleotide) by fitting to a one-site binding hyperbola equation using GraphPad Prism software. Reactions for P168V were performed in triplicate; S173N binding was estimated from a single reaction.

Measurement of dissociation constants

To measure the dissociation constant for the mant-nucleotide, 5.1 μM purified protein was pre-bound to 1 μM mant-nucleotide in binding buffer containing 12 mM $MgCl_2$ at 30°C. To initiate dissociation of the mant-nucleotide, 150 μM non-fluorescent GDP or GTP competitor was rapidly added to the pre-bound complex using a stop flow fluorometer. The decrease in peak fluorescence (excitation 361 nm, slit width 15 nm; emission 446 nm, slit width 15 nm) was measured. The data were fitted to a single-phase exponential decay equation using the GraphPad Prism software. The dissociation rate constant, k_d for mGTP and mGDP, was determined by averaging the k_d from a minimum of 10 trials.

GTP hydrolysis assays

To determine the rate of GTP hydrolysis by P168V, 4 μM protein was pre-bound to 0.3 μM mGTP in binding buffer containing 12 mM $MgCl_2$ at 30°C. The GTPase activity was monitored by decrease in P168V-mant fluorescence over a period of 3 h. The peak fluorescence was recorded at 1 min intervals. The data were fitted to single exponential decay (first order) equation using GraphPad Prism software, and the rate constant, the half-life ($t_{1/2}$) of a single turnover mant-GTP hydrolysis, was determined by averaging three independent trials.

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