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Alanine scan mutagenesis of the switch I domain of the *Caulobacter crescentus* CgtA protein reveals critical amino acids required for *in vivo* function

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Summary

The *Caulobacter crescentus* CgtA protein is a member of the Obg/GTP1 subfamily of monomeric GTP-binding proteins. *In vitro*, CgtA displays moderate affinity for both GDP and GTP and displays rapid exchange rate constants for either nucleotide, indicating that the guanine nucleotide-binding and exchange properties of CgtA are different from those of the well-characterized Ras-like GTP-binding proteins. The Obg/GTP1 proteins share sequence similarity along the putative effector-binding domain. In this study, we examined the functional consequences of altering amino acid residues within this conserved domain, and identified that T193 was critical for CgtA function. The *in vitro* binding, exchange and GTP hydrolysis of the T192A, T193A and T192AT193A mutant proteins was examined using fluorescent guanine nucleotide analogues (mant-GDP and mant-GTP). Substitution of either T192 and/or T193 for alanine modestly reduced binding to GDP and significantly reduced the binding affinity for GTP. Furthermore, the T193A mutant protein was more severely impaired for binding GTP than the T192A mutant. The T193A mutation appeared to account solely for the impaired GTP binding of the T192AT193A double mutation. This is the first report that demonstrates that a confirmed defect in guanine nucleotide binding and GTP hydrolysis of an Obg-like protein results in the lack of function *in vivo*.

Introduction

The Obg/GTP1 GTP-binding proteins are essential proteins found ubiquitously in prokaryotic and eukaryotic life forms. Despite the widespread conservation of these proteins, their cellular role remains unknown. We are investigating the role of the *Caulobacter crescentus* member of the Obg/GTP1 family, CgtA. We have previously demonstrated that CgtA is essential for cell viability and is present in low levels throughout the cell cycle (Maddock *et al*., 1997). Furthermore, we have shown that the CgtA displays unique guanine nucleotide binding and exchange parameters (Lin *et al*., 1999). Whereas the well-studied eukaryotic Ras-like proteins bind guanine nucleotides with high affinity, CgtA binds guanine nucleotides with moderate affinity. In addition, CgtA has rapid GDP and GTP exchange rate constants. These biochemical features are consistent with a model in which the intracellular guanine nucleotide pools govern the guanine nucleotide occupancy of CgtA.

In line with this model, the onset of differentiation in sporulating bacteria appears to be determined, in part, by the balance of Obg protein and GTP levels. In *Streptomyces* spp., overproduction of Obg does not affect vegetative growth but does prevent the development of aerial mycelium (Okamoto *et al*., 1997). The inhibition of GMP synthetase results in the restoration of aerial mycelium production in *Streptomyces* spp. strains overproducing Obg (Okamoto and Ochi, 1998). In addition, specific *obg* mutant alleles display dominant effects on sporulation (Okamoto and Ochi, 1998). In *Bacillus subtilis,* the Obg protein appears to be involved in communicating signals to the SpoOA sporulation pathway by an unknown mechanism (Vidwans *et al*., 1995).

Recent evidence points to a role for these proteins in ribosome function. In *B. subtilis*, the Obg protein exists as a large cytoplasmic complex, coelutes with ribosomal subunits and specifically interacts with the ribosomal protein L13 (Scott *et al*., 2000). These observations suggest that the Obg proteins may play a role in ribosome assembly or in monitoring ribosome assembly state. Thus, the challenge ahead is to delineate the relationship between ribosome function, the guanine nucleotide-bound state of the Obg protein and the interactions between Obg and other proteins.

We are interested in identifying critical amino acids that may play a role in effector binding of CgtA, with the ultimate goal of identifying proteins that bind specifically to this domain. Monomeric GTP-binding proteins communicate with their regulators and downstream effectors via amino acids within the effector domain (switch I and switch II; Bourne *et al*., 1991; Goldberg, 1999; Mott *et al*.,

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1999). Crystallographic analysis of the human H-Ras p21 protein indicates that both switch I (residues 30–37) and switch II (residues 60–76) undergo a major conformational change after the conversion between the GTP- and GDP-bound forms (Milburn *et al.*, 1990; Schlichting *et al.*, 1990). The members of the Obg/GTP1 family share considerable conservation along the putative switch I and switch II domains. This conservation implies that these regions are also involved in protein–protein interactions. Within the switch I domain of all monomeric GTP-binding proteins is a conserved threonine that participates in the co-ordination of the GTP and Mg ion (Milburn *et al.*, 1990; Pai *et al.*, 1990). In the Obg/GTP1 proteins, two threonines (T192 and T193) are potential candidates for this critical residue.

In this study, we investigated the cellular consequences of specific amino acid substitutions on CgtA function. We have introduced single amino acid substitutions in the switch I and switch II domains, and examined these mutant alleles for their ability to function *in vivo*. We show here that substitution of T193, but not T192, led to a protein incapable of functioning *in vivo*. To confirm that T193 was involved in GTP-binding, we examined the *in vitro* guanine nucleotide binding, exchange and GTP hydrolysis of the single and double threonine substitutions.

Results

Depletion of CgtA using the repressible Pxyl promoter

The activity of P_{xyl} promoter is strictly dependent on xylose. P_{xyl} is fully induced in PYE+Xyl and repressed when xylose is removed from the growth medium (PYE+Glu) (Meisenzahl *et al.*, 1997). We placed *cgtA* expression exclusively under the control of the P_{xyl} promoter by integrating a C-terminally truncated P_{xyl}*::cgtA* fusion at the *cgtA* locus to create JM1108. The integration resulted in a partial duplication of *cgtA* with a non-functional copy behind the *cgtA* promoter and the intact *cgtA* gene controlled by the xylose promoter.

CgtA levels in JM1108 were depleted by a carbon shift from PYE+Xyl to PYE+Glu. The CgtA protein levels remained relatively constant for at least 2 h in PYE+Glu and then decreased to low, but detectable, levels for at least 12 h after the carbon shift (Fig. 1A). Concomitant with the reduction in CgtA protein levels, mid-log JM1108 cells shifted to PYE+Glu showed a decrease in growth after 4 h (Fig. 1B) as well as a reduction in cell viability (to $\sim 1 \times 10^8$ ml^{-1}). No loss in viability was observed for cells grown continuously in PYE+xylose (Fig. 1C). A low level of cell growth was observable on PYE + glucose plates (Fig. 3). However, complementation of P_{xyl}*::cgtA* with a plasmid harbouring a wild-type *cgtA* gene resulted in robust growth on either PYE+Xyl or PYE+Glu plates

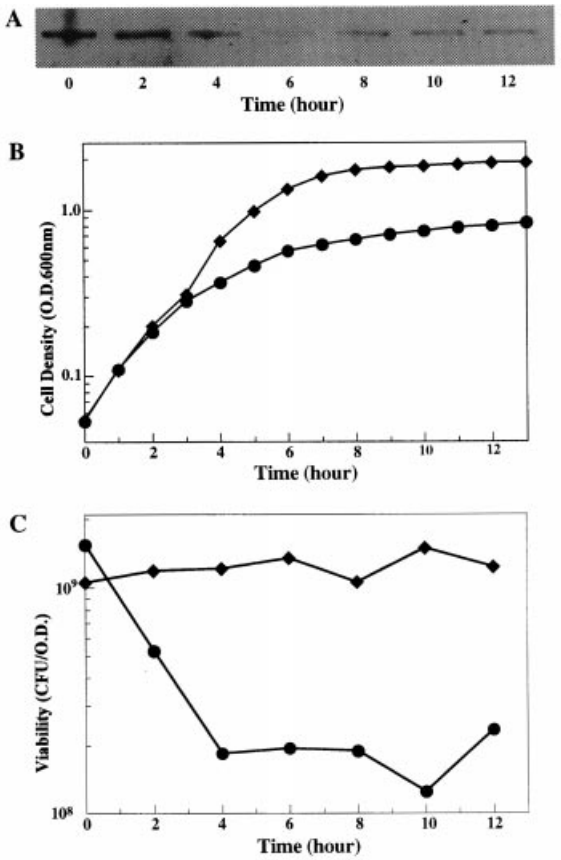

Fig. 1. Depletion of CgtA.
A. Immunoblot analysis of JM1108 cells [a strain in which the chromosomal copy of *cgtA* (P_{xyl}*::cgtA*) is repressed on PYE glucose] shifted from PYE+Xyl (time 0) to PYE+Glu for the indicated times reveals a reduction of CgtA protein.
B. Growth rates of JM1108 cells grown in PYE+Xyl (◆) or PYE+Glu (●).C. Viability of JM1108 cultures grown in PYE+Xyl (◆) or PYE+Glu (●).

(Fig. 3). Taken together, these experiments demonstrated that repression of P_{xyl}*::cgtA* resulted in a reduction, but not elimination, of CgtA protein levels, which resulted in impaired cell growth. More importantly, the slow growth of JM1108 in glucose could be exploited to assay for CgtA function by complementation.

Alanine scan mutagenesis of the CgtA effector domains reveal residues critical for CgtA function

The switch I and switch II domains of Ras-like GTP-binding proteins are involved in the interactions between the GTPase and its cellular target and regulatory proteins. Numerous mutations in these domains that affect the interaction between the GTP-binding protein and the regulatory proteins or the effector have been isolated (Adari *et al.*, 1988; Moodie *et al.*, 1995; Yoder-Hill *et al.*, 1995). As a first step in determining the importance of these potential CgtA domains in protein interaction and

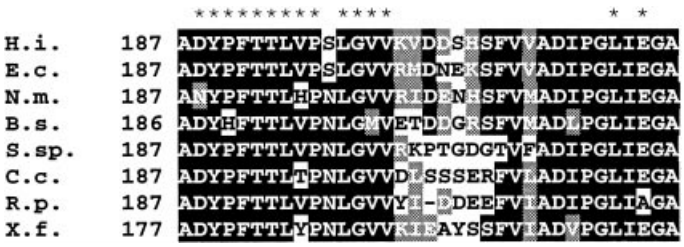

Fig. 2. Alignment of the switch I and switch II domains. Shown are the amino acids in the conserved switch I and switch II domains of several members of the Obg/GTP1 subfamily of GTP-binding proteins. Sequences in black boxes are identical whereas grey boxes indicate conserved amino acids. *Haemophilus influenzae* (H.i.), *Escherichia coli* (E.c.), *Neisseria meningitidis* (N.m.), *Bacillus subtilis* (B.s.). *Synechocystis* sp. (S.sp.), *Caulobacter crescentus* (C.c.), *Rickettsia prowazekii* (R.p.), and *Xylella fastidiosa* (X.f.). The amino acid number of the first residue shown is listed. Amino acids altered in this study are marked with an asterisk.

guanine nucleotide binding, we individually changed 15 of the conserved amino acids in the switch I and switch II domains to alanine (indicated in Fig. 2 by an asterisk). We also made the double mutant T192AT193A and the triple mutant F191AT192AT193A.

Wild-type cells expressing from plasmids, *cgtA* mutant alleles, wild-type *cgtA* or containing a control vector grew equally well at 30°C and 37°C, indicating that none of the episomal *cgtA* alleles conferred a dominant negative effect on colony growth (data not shown). Similarly, JM1108 cells containing plasmid-borne copies of wild type, mutant *cgtA* alleles or a control vector formed similar size colonies at either temperature on PYE+Xyl plates (Fig. 3 and data not shown). These results demonstrate that none of the episomal *cgtA* alleles displays a dominant negative phenotype.

Each mutant allele was tested for its ability to function *in vivo* by assessing the ability to complement the chromosomal P_{xyl}::*cgtA* on PYE+Glu plates. JM1108 cells expressing an episomal *cgtA* gene grew on PYE+Glu, whereas cells without *cgtA* on the helper plasmid did not (Fig. 3). Most of the *cgtA* effector domain mutants supported growth on PYE+Glu and therefore had little or no effect on CgtA function (Fig. 3 and data not shown). In contrast, the JM1108 cells expressing the T193A, T192AT193A or F191AT192AT193A allele failed to complement. Those harbouring the F191A allele formed smaller colonies on PYE+Glu at 30°C (Fig. 3) and displayed a more severe growth defect at 37°C (data not shown). The lack of complementation by T192A or T192AT193A was not caused by a defect in protein level, as CgtA protein was similar to that of JM1108 cells harbouring wild-type *cgtA* (data not shown). These results demonstrate that amino acid T193 is critical for CgtA function.

We performed a plasmid shuffle technique in order to address whether plasmid-borne *cgtA* alleles would complement a chromosomal *cgtA* null allele. To do this, we first generated a strain in which the chromosomal *cgtA* allele was disrupted and wild-type *cgtA* was carried on an episomal plasmid with the *sacB* gene (JM674). We transformed JM674 with each of the mutant *cgtA* alleles (on replicating plasmids) and then selected for loss of the plasmid harbouring the wild-type *cgtA* gene by plating the cells on sucrose. We successfully recovered complemented

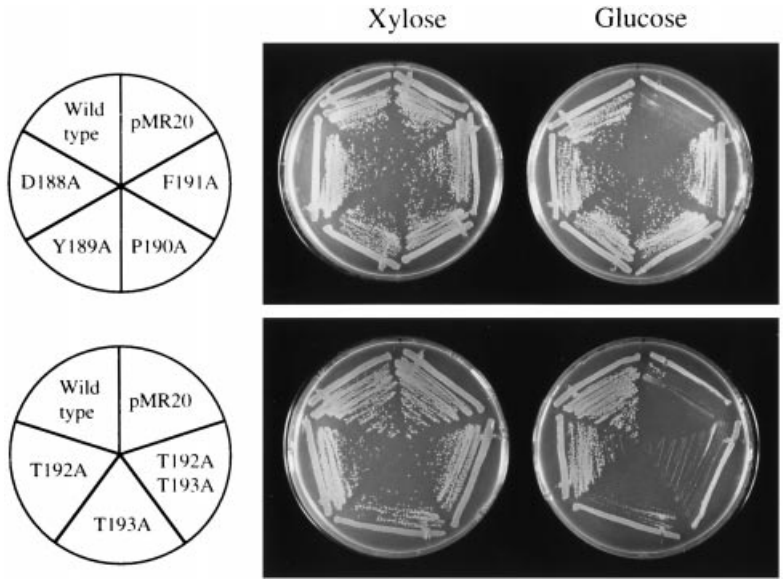

Fig. 3. Complementation analysis of the effector domain mutants. The ability of each mutant *cgtA* allele to support growth was monitored in a strain in which the chromosomal copy of *cgtA* (P_{xyl}::*cgtA*) is repressed on PYE glucose plates (JM1108). A cartoon of the alleles (harboured on vector pMR20) assayed on each plate is indicated on the left. Photographs of colonies after 2–3 days at 30°C on PYE xylose or PYE glucose plates are presented as indicated.

strains (those in which the only copy of *cgtA* contains a mutation) for all alleles examined except T193A, T192AT193A, F191AT192AT193A and F191A (L217A and E219A were not tested). The inability to obtain complemented strains with the T193A and T192AT193A alleles confirmed that these alleles do not support *C. crescentus* growth. Growth of all viable mutants was examined at 23, 30 and 37°C and found to be similar to *cgtA::spec* complemented with wild-type *cgtA* (data not shown).

The T192A and T193A mutant proteins are impaired for binding to GTP but not GDP

All monomeric GTP-binding proteins possess a conserved threonine in the switch I region that probably plays a role in co-ordination of the Mg ion and thus plays a role in the binding of GTP. In the Obg/GTP1 subfamily of GTP-binding proteins, both T192 and T193 are absolutely conserved. Given the *in vivo* requirement for T193, we performed an in depth analysis of the guanine nucleotide exchange and GTP hydrolysis parameters of the T192A, T193A and T192AT193A mutant proteins.

To determine whether these mutant proteins were capable of binding GTP, we used a UV cross-linking approach. Protein (10 μM) was incubated with 0.05 μM [γ-^{32}P]GTP, exposed to UV irradiation and the proteins separated using SDS–PAGE. Wild-type CgtA bound GTP tightly. In contrast, T192A, T193A or T192AT193A bound [γ-^{32}P]GTP weakly and showed a 24-, 33- and 29-fold reduction, respectively, in radioactivity associated with the CgtA protein (data not shown).

To explore binding to guanine nucleotides further, we determined the equilibrium binding constants of the mutant proteins for GDP and GTP in the presence of 12 mM Mg^{2+} (Fig. 4, Table 1). At this magnesium ion concentration, wild-type CgtA binds either GDP or 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 mutant proteins resulted in a typical hyperbolic plot (Fig. 4 and data not shown). However, compared with wild-type CgtA, the T192A mutant displayed a 2.5-fold and sevenfold lower affinity for GDP and GTP respectively (apparent K_D of 1.3 ± 0.1 and 7.4 ± 0.5 respectively; Table 1). The T193A mutant bound GDP with fivefold lower affinity than wild-type CgtA, and GTP binding was reduced 22-fold (apparent K_D of 2.6 ± 0.2 and 24 ± 3 respectively; Table 1). The guanine nucleotide-binding equilibrium constants for the T192AT193A mutant resembled those of the T193A mutant (apparent K_D of 2.7 ± 0.4 and 25 ± 3, for GDP and GTP respectively). Therefore, substitution of either T192 and/or T193 for alanine modestly reduced binding to GDP and significantly reduced the binding affinity for GTP. Furthermore, the T193A mutant protein was more severely impaired for binding GTP than the T192A mutant and the T193A mutation appeared to account solely for the effects of the T192AT193A double mutation.

The T192 and T193 mutant proteins display enhanced dissociation rate constants for mant-GTP but not mant-GDP

Mant-nucleotides are extremely useful analogues for guanine nucleotide-binding studies. Upon binding of the mant-nucleotide to protein, the fluorescence intensity of the mant moiety changes, almost invariably increasing relative to the intensity of the free mant-nucleotide. Moreover, there is typically a difference in the intensity between bound mant-GTP and bound mant-GDP, whereas the intensity of the free states of these nucleotides is equivalent. These differences allow for a differentiation between both the free nucleotides and the protein-bound forms. We have previously used mant-nucleotides to determine the rate constants of guanine nucleotide exchange and GTP hydrolysis of wild-type CgtA (Lin *et al.*, 1999). In this study, we examined the excitation spectra of the mutant CgtA–mant-nucleotide complexes. Upon binding of mant-GDP or mant-GTP to the mutant CgtA proteins, an increase in fluorescence was observed. However, unlike wild-type CgtA, in which the CgtA–mGDP and CgtA–mGTP forms differ (1.3- and 1.5-fold increase over baseline respectively; Lin *et al.*, 1999), CgtA(T192A)–mant-GTP and CgtA (T192A)–mant-GDP displayed overlapping spectra with a 1.5-fold increase in fluorescence (Fig. 5A). Similar overlapping spectra were observed for

Table 1. Nucleotide binding, exchange rate constants and mGTP hydrolysis of CgtA in 12 mM Mg^{2+}.

CgtA protein	Equilibrium binding K_D (μM)		Nucleotide exchange k_d (s^{-1})		mGTP hydrolysis $T_{1/2}$ (min)
	GDP	GTP	mGDP	mGTP	
Wild-type[a]	0.52 ± 0.03	1.1 ± 0.1	1.43 ± 0.04	1.28 ± 0.02	23 ± 2
T192A	1.3 ± 0.1	7.4 ± 0.5	1.11 ± 0.01	7.1 ± 0.4	29 ± 4
T193A	2.6 ± 0.2	24 ± 3	1.17 ± 0.02	8.9 ± 0.2	170 ± 30
T192AT193A	2.7 ± 0.4	25 ± 3	1.07 ± 0.01	8.8 ± 0.5	200 ± 10

a. Wild-type data obtained from Lin, *et al.*, 1999.

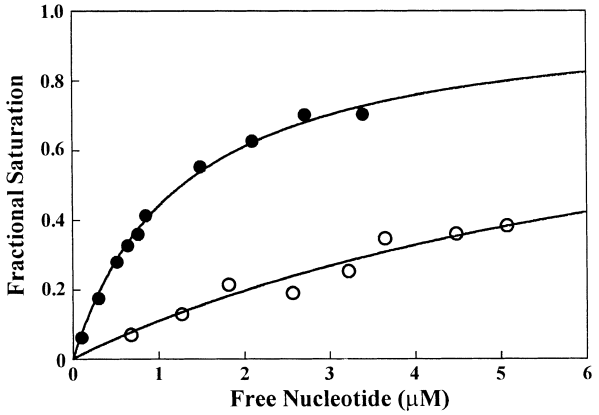

Fig. 4. Equilibrium binding of CgtA (T192A) to guanine nucleotides plotted as fractional saturation. CgtA (T192A) was incubated at 30°C with increasing [8–^{3}H]GDP (●) or [γ-^{32}P]GTP (○) in 12 mM Mg^{2+}. Aliquots were removed and free nucleotide was quantified using scintillation counting as described in *Experimental procedures*. Shown are the hyperbolic binding curves. The corresponding equilibrium binding constant, K_D, for GDP and GTP was 1.3 ± 0.1 μM and 7.4 ± 0.5 μM respectively.

T193A and T192AT193A mutant proteins (data not shown). We confirmed that these spectra were obtained at saturating concentrations of protein by determining the saturation profiles for binding of CgtA(T192A) to mant-GTP and mant-GDP (Fig. 5B). Maximum fluorescence of 1 μM mant-GDP occurs after the addition of 10 μM CgtA whereas maximum fluorescence of 1 μM mant-GTP occurs after the addition of 20 μM CgtA. Therefore, under the conditions used to obtain the excitation spectra (1 μM mant-nucleotide, 25 μM protein), virtually all of the mant-nucleotides were bound to CgtA protein. Furthermore, the different saturation profiles observed demonstrate that CgtA binds to mant-GDP with higher affinity than mant-GTP.

We used the fluorescence changes that occur upon the release of mant-nucleotides from CgtA to quantify the guanine nucleotide dissociation rate constants, k_d, of the mutant CgtA proteins (Table 1). Wild-type CgtA 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 T192A, T193A and T192AT193A CgtA proteins had dissociation rates for mant-GDP similar to that of wild-type protein (1.11 ± 0.01, 1.17 ±0.02, and 1.07 ± 0.01 s^{-1} respectively).

In contrast, the discussion rates of mant-GTP were 5.5- to 7-fold faster (7.1 ± 0.4, 8.9 ± 0.2 and 8.8 ± 0.5 s^{-1} respectively).

Mant-GTP hydrolysis is impaired in the T193A and T192AT193A mutant proteins

To measure the single-turnover hydrolysis rate constant of wild-type CgtA, we monitored the decrease in fluorescence that accompanied the conversion of bound mant-GTP to mant-GDP (Lin *et al.*, 1999). However, because the fluorescence intensities of mant-GTP and mant-GDP bound to the mutant proteins were identical, we could not employ this approach to monitor the GTPase activity of the CgtA mutants. However, we observed a biphasic nucleotide exchange curve, using mant-GTP prebound to the mutant proteins (Fig. 6), that was the result of the combined contributions of mant-GTP and low levels of mant-GDP contaminating the mant-GTP stock. The fast phase (85% of the signal) was attributed to the dissociation of mant-GTP and the slower phase was attributed to the dissociation of mant-GDP. To confirm that the slower phase was indeed caused by mant-GDP as a product of hydrolysis, we examined the relative contribution of each component upon prolonged incubation of mant-GTP with the mutant CgtA proteins. Over

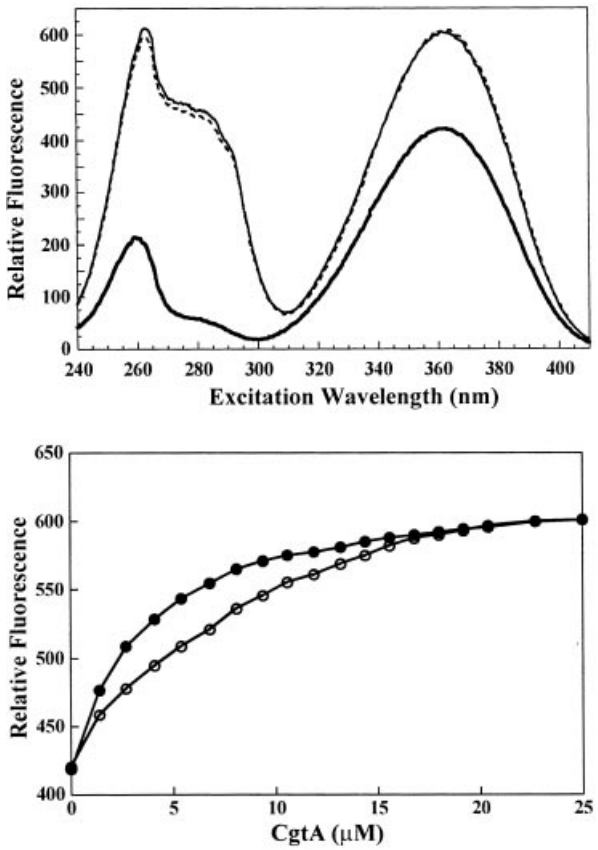

Fig. 5. Increase in mant fluorescence upon interaction with CgtA(T192A) protein.
A. Excitation spectra of CgtA(T192A)-bound and free mant-nucleotides. The relative excitation spectra of 1 μM mant-GDP (thick line), and 1 μM mant-GDP or mant-GTP incubated with 25 mM CgtA(T192A) (thin and dashed lines respectively) is shown. The spectra of free mant-GTP overlays that of free mant-GDP (data not shown).
B. Saturation profile for CgtA(T192A) to mant-nucleotides. 1 μM mant-GDP (●) or mant-GTP (○) was incubated with increasing amounts of CgtA(T192A) protein in the presence of 12 mM Mg^{2+}, as indicated. Shown is the increase in fluorescence of the mant-nucleotides upon binding to CgtA.

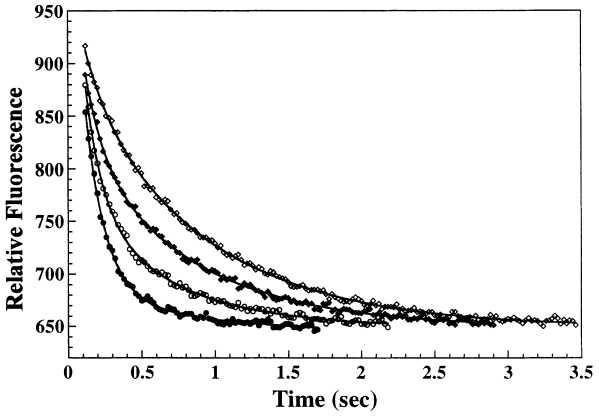

Fig. 6. Dissociation kinetics of mant-GTP from CgtA(T192A). Shown are the curve fitted dissociation curves of CgtA(T192A)●mant-GTP at 0 min (●), 10 min (○), 30 min (◆), and 70 min (◇). Data were collected after rapid mixing of CgtA(T192A)●mant-GTP complexes incubated at 30°C for the indicated times with excess GDP in a stopped-flow fluorometer. All curves fit with two additive exponential decay phases, the relative contribution of each is reported in Table 2.

time, the contribution of the fast exponential decreased and the contribution of the slow component increased (Fig. 6, Table 2) as mant-GTP was hydrolysed to mant-GDP. We took advantage of the change in relative contribution of each dissociation phase to obtain the half-life ($T_{1/2}$) of hydrolysis (Table 1).

These studies showed that the $T_{1/2}$ of hydrolysis for the T193A and T192AT193A mutant proteins was severely compromised, increasing to 170 ± 30 and 200 ± 10 min respectively. The T192A mutant had a hydrolysis rate similar to wild-type CgtA (29 ± 4 and 23 ± 2 min respectively). Taken together with the equilibrium binding and dissociation data, these results indicate that T193 plays a critical role in both binding to and hydrolysis of GTP.

Limited proteolysis defined a structural change in the P-loop-switch I domains upon binding of CgtA to guanine nucleotides

We used limited proteolysis to identify a conformational change that occurs in CgtA upon binding to guanine nucleotides. As shown in Fig. 7, limited proteolysis of CgtA by trypsin or chymotrypsin results in the production of a number of fragments. Apo–CgtA differed from CgtA–GDP and CgtA–GTP complexes in that one or two bands (trypsin and chymotrypsin respectively) present in the unbound CgtA sample were absent when CgtA was bound to nucleotides (Fig. 7). These fragments were subjected to N-terminal sequencing. The 18.8 kDa chymotrypsin fragment was shown to begin at the N-terminus of CgtA, whereas the 19.2 kDa chymotrypsin fragment began at L176 within the predicted guanine nucleotide-binding domain of CgtA. The sizes of these products are consistent with one cleavage event, resulting in these two peptides. Similarly, limited proteolysis with trypsin also revealed an ~18 kDa peptide present in the unbound CgtA samples but not in the CgtA–GDP or CgtA–GTP complexes (Fig. 7B). This trypsin fragment started at the N-terminus of CgtA and is probably a result of a specific cleavage between the P-loop and switch I as well (either S173 or I186). Thus, binding of CgtA to either guanine nucleotide results in a protection of a trypsin and chymotrypsin cleavage, and provides evidence that CgtA undergoes a conformational shift upon binding of guanine nucleotides.

We next examined the sensitivity to limited proteolysis of the T192A and T193A mutant proteins. The pattern of limited proteolysis for wild-type CgtA, T192A and T193A is identical, demonstrating that the tertiary structure of these proteins is roughly equivalent. Furthermore, the mutant proteins apparently undergo a similar conformational change upon binding to guanine nucleotides because the same pattern of trypsin and chymotrypsin protection is observed in these mutants as was seen in the wild-type protein (Fig. 7). Thus, any differences in guanine nucleotide binding, exchange or hydrolysis observed in this study was not the result of gross structural differences in these mutant proteins. In contrast, the T192AT193A double mutant showed a slightly increased sensitivity to proteolysis, indicating that the conformation of at least part of this mutant protein sample is conformationally different from wild-type protein. Furthermore, the addition of guanine nucleotides to the T192AT193A mutant protein resulted in only a partial protection of the L176 chymotrypsin cleavage and no reduction of the ~18 kDa trypsin fragment.

Table 2. Relative amplitudes of the two components of mGTP biphasic kinetics of T192A protein.

Incubation time (min)	Amplitude[a] (relative fluorescence)	
	Fast phase	Slow phase
0	297 ± 25	52 ± 20
5	232 ± 7	89 ± 9
10	205 ± 7	111 ± 7
20	159 ± 14	143 ± 5
30	140 ± 10	180 ± 11
50	90 ± 5	219 ± 4
70	57 ± 20	252 ± 8

a. Data are an average of three experiments at 30°C

Neither T192A nor T193A is the site of autophosphorylation

In *B. subtilis*, Obg can be autophosphorylated by the γ-phosphate from the bound GTP on a His residue (Welsh *et al.*, 1994). Era, a different bacterial GTPase, is

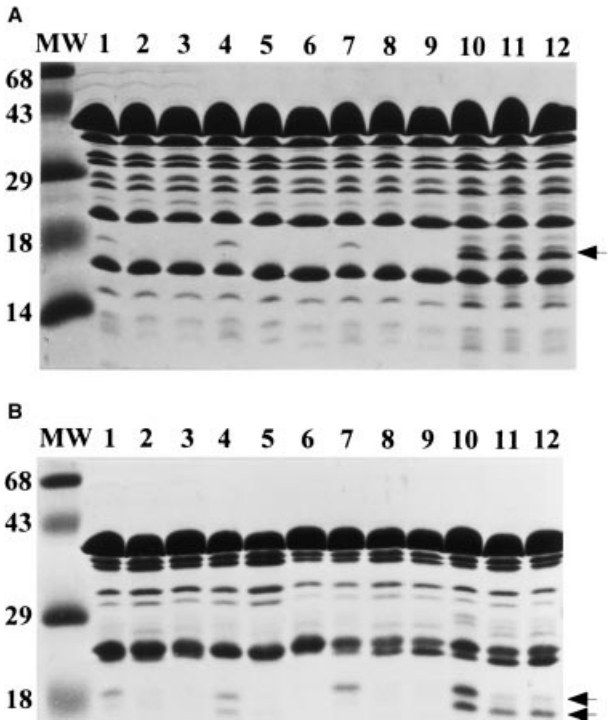

Fig. 7. Limited proteolysis of CgtA and CgtA mutant proteins. CgtA (lane 1–3), T192A (lane 4–6), T193A (lane 7–9), and T192A,193 A (lane 10–12) proteins were precomplexed with GDP (lane 2, 5, 8, and 11) or GTP (lane 3, 6, 9, and 12) and then incubated with trypsin (TPCK treated) (panel A), or chymotrypsin (TLCK treated) (panel B) at 30°C for 20 min Lane 1, 4, 7, and 10 were controls without nucleotide. Proteolysis reactions were stopped with PMSF and subjected to 20% SDS–PAGE. Parallel digestions of wild-type CgtA were separated and transferred on PVDF membranes. Bands corresponding to those in lane 1 at positions marked by arrows were excised and subjected to N-terminal sequencing. The ~18KD band from trypsin digestion starts with 'MEFLDQ…', and the two ~18KD bands from chymotrypsin start with 'MEFLDQ…' and 'LAAASA…' respectively.

significantly autophosphorylated on either a serine or threonine residue in the switch I domain (Sood *et al.*, 1994). In general, activated monomeric GTP-binding proteins are significantly autophosphorylated (see Bos, 1989). The autophosphorylation appears to be a consequence of the juxtaposition of the conserved amino acid to the γ-phosphate of GTP. We show here that purified CgtA is capable of autophosphorylation by [γ-^{32}P]GTP (Fig. 8A). The phosphorylation is base labile (Fig. 8B), indicating that CgtA is phosphorylated on either a serine or threonine residue. Given the potential position of the conserved threonine 192 or 193 near the γ-phosphate on GTP, we examined whether either of these residues was the site of autophosphorylation in CgtA. Purified T192A, T193A and T192AT193A mutant proteins were autophosphorylated at levels comparable to wild-type protein (Fig. 8C), demonstrating that neither T192 nor T193 is involved in autophosphorylation.

Discussion

To assay the consequences of depleting *C. crescentus* cells of CgtA, we constructed a strain, JM1108 (P_{xyl}*::cgtA*), in which *cgtA* expression was under a repressible/inducible promoter. After repression of *cgtA* the level of CgtA protein only decreased after several hours, indicating that the CgtA protein is relatively stable. Repression resulted in a decrease but not complete elimination of CgtA protein. This low level of CgtA protein is apparently sufficient to mediate a weak but sustained growth, as cells grew slowly in PYE+Glu and viability decreased modestly.

We used the slow growth of JM1108 on PYE+Glu to assay the ability of 17 different amino acid substitutions in the switch I and switch II domains of CgtA. In the well-characterized GTP-binding proteins, this region is involved in protein–protein interactions with effector and regulatory proteins. The switch I and switch II domains of the Obg/GTP1 proteins are remarkably conserved (Fig. 2). To examine the *in vivo* requirement for these amino acids on CgtA function, we made a series of alanine substitutions. Individual substitution of many of these residues did not perturb the ability of CgtA to function *in vivo*. We conclude that either individual amino acid residues are not important for CgtA function or, if this conserved region is involved in critical contacts with interacting proteins, multiple amino acids must contribute to the overall stability of these interactions: altering any single amino acid does not perturb these contacts. Similar conclusions have been reached

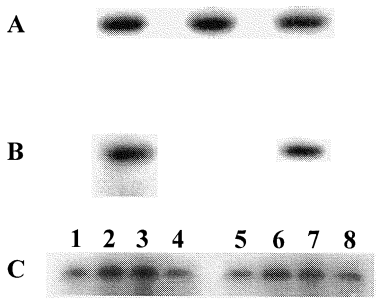

Fig. 8. Autophosphorylation of CgtA occurs on a serine or threonine other than T192 or T193.
A. CgtA protein was incubated with [γ-32P]GTP as described in *Experimental procedures*. Samples were then separated using SDS–PAGE and detected by autoradiography.
B. To determine the acid/base stability of the phosphorylation, parallel gel strips were incubated in buffer (left), 1 N NaOH (middle) or 0.2 N HCl (right), washed in 40% isopropanol and exposed to autoradiography.
C. CgtA protein was incubated with [γ-^{32}P]GTP with (lanes 5–8) or without (lanes 1–4) the addition of 2 mM KH_2PO_4, separated using SDS–PAGE and detected by autoradiography. Lanes 1 and 5, CgtA; lanes 2 and 6, T192A; lanes 3 and 7, T193A; lanes 4 and 8, T192AT193A. The corresponding gel strips were excised from a parallel SDS–PAGE, and their relative level of radioactivity was determined to be 0.8, 0.9, 0.8, 0.6 for lane 1–4 respectively.

in interpreting other alanine scan mutagenesis data (Thukral *et al*., 1991; King *et al*., 1999). We did, however, find that alteration of F191 led to a modest defect in CgtA function, whereas alteration of T193 resulted in a non-functional protein.

The CgtA protein displayed a low level of autophosphorylation (Fig. 8). A similar low level of autophosphorylation has been reported for the *B. subtilis* Obg protein and it was proposed that the phosphorylation site is histidine 189 (Welsh *et al*., 1994). However, this histidine residue is not conserved among the other members of the Obg family, including CgtA (Fig. 2). In this study, we showed that the autophosphorylation of CgtA was base sensitive (Fig. 8), providing evidence that CgtA is autophorphorylated on a serine or threonine residue. In addition to binding to interacting proteins, the switch I domain of Ras also plays a role in the binding of GTP through a critical threonine residue that forms a co-ordinate bond with the magnesium ion (Milburn *et al*., 1990; Pai *et al*., 1990). All members of the Obg family have two consecutive threonines in this region (T192 and T193 in CgtA) and we show here that T193 plays a critical role in binding to GTP and in GTPase activity. Therefore, we examined whether T193 was the site of autophosphorylation on CgtA. However, this is not the case, as autophosphoryation of CgtA is not perturbed in the T192A, T193A or T192AT193A mutant proteins. These results demonstrate that a different threonine or serine residue is the site of autophosphorylation. As structural data for these proteins become available, appropriate amino acid candidates will be identified.

In the Obg/GTP1 proteins, two threonines (T192 and T193) are potential candidates for this critical residue and we have shown that T193A does not function *in vivo*. To determine whether the lack of function *in vivo* correlated with a perturbation of guanine binding or hydrolysis *in vitro*, we examined the biochemical properties of proteins with the single and double threonine substitutions.

Although the ability of the mutant alleles to function *in vivo* differed, T192A, T193A and T192AT193A proteins all displayed a reduction in GTP binding as determined by UV cross-linking to [γ-^{32}P]GTP. A more rigorous examination of the binding affinity of these mutant proteins to GDP and GTP revealed that, compared with the wild-type protein, the T192A mutant displayed a 2.5-fold and sevenfold lower affinity for GDP and GTP respectively. The T193A mutant bound GDP with a fivefold lower affinity than wild-type CgtA and GTP binding was reduced 22-fold. The guanine nucleotide-binding equilibrium constants for the T192AT193A mutant resembled those of the T193A mutant. Therefore, substitution of either T192 or T193 for alanine modestly reduced binding to GDP. The T193A mutant protein was more severely impaired for binding GTP and this difference in GTP equilibrium binding could affect the ability of this mutant protein to function *in vivo*. Furthermore, these data demonstrate that a slightly impaired affinity for GDP (up to 2.5-fold) or GTP (up to sevenfold) does not affect the ability of these proteins to function *in vivo* under the growth conditions assayed.

Wild-type CgtA displays a rapid exchange of both GDP and GTP. The T192A, T193A and T192AT193A CgtA mutant proteins had dissociation rate constants for mant-GDP similar to that of wild-type protein. The mant-GTP dissociation rate constants of the T192A, T193A and T192AT193A mutants were 5.5- to sevenfold faster than that seen for the wild-type protein. Given that the T192A mutant functions *in vivo*, this modest increase in the dissociation rate constant does not significantly impair protein function, and it is unlikely that the change in the dissociation rate constant contributes to the inability of the T193A allele to function *in vivo*.

To date, we have favoured models for CgtA function that involve CgtA acting as a sensor of cellular GTP/GDP pools, with GTP hydrolysis playing little, if no, role in CgtA function. The affinity for GTP in the T193A mutant is 22-fold lower than that of the wild-type protein, and this lower affinity could alter the timing within which the occupancy of the T193A mutant proteins switches from a GTP- to a GDP-bound state as the cells enter stationary phase. However, the studies reported here also suggest that hydrolysis may play a critical role in CgtA function. A significant decrease in hydrolysis was observed in the non-complementing T193A and T192AT193A mutant proteins. Whereas the complementing T192A mutant had a hydrolysis rate similar to wild-type CgtA ($T_{1/2}$ of 29 and 23 min respectively), the $T_{1/2}$ of hydrolysis for T193A and T192AT193A mutant proteins increased to 170 and 200 min respectively. In order for hydrolysis to play a crucial role in the function of CgtA, exchange of guanine nucleotides must be inhibited *in vivo*. We propose that, *in vivo*, rapid exchange of guanine nucleotides by CgtA is restricted at some critical time and hydrolysis does, in fact, play a role. Inhibition of guanine nucleotide exchange could be accomplished by the association of CgtA in a complex.

Recently, it has been demonstrated that the *B. subtilis* Obg protein associates with the ribosomal subunit L13 (Scott *et al*., 2000). One possible scenario is that when Obg is associated with the ribosome the exchange of guanine nucleotides is inhibited. What might the role of the Obg proteins be? We have estimated that there are approximately 200 CgtA proteins in *C. crescentus* (data not shown), a number far less than the approximately 10 000 copies of L13 protein. Therefore, it is unlikely that the Obg proteins are structural components of the ribosome. More probably, these proteins play one of two

roles: either these proteins are involved in ribosome assembly or they are involved in monitoring the assembly state of the ribosomes. Current studies are underway to investigate these possibilities.

Experimental procedures

Bacterial strains and culture conditions

Escherichia coli cells were grown in Luria–Bertani (LB) Broth (LB; 10 g of tryptone, 5 g of yeast extract, 10 g of NaCl l^{-1}) or LB agar at 37°C containing antibiotics as required. *C. crescentus* strains were derived from NA1000 (a synchronizable but otherwise wild-type strain) and were grown in PYE medium (2 g peptone, 1 g yeast extract, 1 mM $MgSO_4$, 0.5 mM $CaCl_2$) (Poindexter, 1964). Plasmids were introduced into *C. crescentus* from the *E. coli* strain S17 using conjugal transfer (Ely, 1979). Growth of the cultures was monitored by measuring the absorption at 600 nm. Antibiotics were used at the following concentrations: for *C. crescentus*, oxytetracycline (Tet) 1 μg ml^{-1}, spectinomycin (Spec) 25 μg ml^{-1}, naladixic acid 20 μg ml^{-1} or kanamycin (Kan) 5 μg ml^{-1}; for *E. coli*, tetracycline 12 μg ml^{-1}, spectinomycin 100 μg ml^{-1}, kanamycin 30 μg ml^{-1} or ampicillin (Amp) at 100 μg ml^{-1}.

Construction of P_{xyl}::cgtA

The 354 bp *xylX* promoter was polymerase chain reaction (PCR) amplified (using the primers pxylose*Pst*1 (5′-GCAGCGGATCTGCAGCCAGCCGTG) and pxylose*Nco*I (5′-CTCACGCCCATGGCGTCGTCTCCC) from pCS199 (Meisenzahl *et al*., 1997). This was cloned into a modified pBluescript SK at the *Eco*RV site. We first modified pBluescript by adding a thymine residue at the *Eco*RV site (Marchuk *et al*., 1991). The resulting plasmid (pJM661) contained the *xylX* promoter with a *Nco*I restriction site at the start codon of *xylX*. A P_{xyl}*::cgtA* fusion was generated by cloning the 1.4 kb *Nco*I–*Bam*HI fragment, containing *cgtA* from pJM625 (Lin *et al*., 1999), into pJM661 to create pJM688. Finally, the 1.9 kb P_{xyl}*::cgtA*, containing *Pst*I–*Bam*HI, was cloned into the integrative plasmid pBGST18 (Tsai and Alley, 2000) to create pJM778.

A C-terminal deletion of pJM778 was generated by removing the 0.9 kb *Sal*I–*Sma*I fragment. After treatment with T4 DNA polymerase, the DNA was self-ligated to create pJS10. pJS10 was introduced into wild-type *C. crescentus* using conjugation and the resulting integrants were selected on PYE-xylose Tet Nal plates. Two glucose-sensitive colonies (out of 120 tested) were identified, and the integration at the chromosomal *cgtA* locus was confirmed using PCR with primers pxylosePST1 and CgtAENDRev (5′-CGGCGTCCAGCCTCCGGGGGTCTCGTC). The resulting strain used in this study was JM1108.

Generation of cgtA *mutant* alleles

A 2.25 kb *Pst*I–*Hind*III *cgtA*-containing fragment was subcloned into pAlter-1 (Promega) and subjected to Altered Sites II mutagenesis as recommended by the manufacturer. Plasmid DNA was isolated using either a Qiagen or a Wizard (Promega) kit. Oligos (Gibco) were treated with polynucleotide kinase (NEB). After the synthesis reactions, electrocompetent ES1301 *mutS* cells were transformed and allowed to outgrow in LB for 30 min. The pool of transformants was grown in LB Amp overnight, plasmid DNA isolated and transformed into JM109 cells. Ampicillin-resistant transformants were screened for sensitivity to tetracycline. Single-stranded DNA was isolated and mutations identified using sequence analysis. The *cgtA* alleles were then subcloned as 2.25 kb *Pst*I–*Hin*dIII fragments into pMR20 (Stephens *et al*., 1997).

Immunoblot analysis

Equivalent OD amounts of cells were loaded onto Novex NuPage 10% SDS gels, run with 1× MOPS under denaturing conditions, and electroblotted to nitrocellulose (Schleicher and Schuell) using a Hoeffer semidry transfer apparatus. The membranes were probed with a 1:5000 dilution of polyclonal rabbit anti-CgtA, washed with phosphate–buffered saline containing 0.05% Tween (PBST). Samples were probed with a 1:20 000 dilution of goat anti-rabbit conjugated to horseradish peroxidase and detected by fluorography using ECL (Amersham Pharmacia Biotech) as recommended by the manufacturer.

Overexpression and purification of CgtA proteins

The mutant *cgtA* alleles were cloned into pET28 by a replacement of the 0.9 kb *Bgl*II–*Xho*I fragment of pJM625 [wild-type *cgtA* in pET28; (Lin *et al*., 1999)]. CgtA proteins were overexpressed and purified as has been described (Lin *et al*., 1999) with the following modifications: 1 l of mid-log *Escherichia coli* BL21DE3, containing plasmid pJM746 (T192A), pJM747(T193A) or pJM1071(T192AT193A), was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (GIBCO) for 3 h at 37°C, and the level of CgtA overexpression was determined using SDS–PAGE. Cells were pelleted (6000 *g*, 5 min, 4°C), resuspended in 50 ml of TDG [50 mM Tris-HCl, pH 8, 1 mM DTT, 10% glycerol (w/v)] supplemented with 1 mM PMSF and lysed by two passages through a French pressure cell. The resulting cell extract was then clarified (28 000 *g* for 30 min, 4°C), loaded on Cibacron Blue column and eluted with linear gradient (0–1 M KCl) in 300 ml of TDG. Fractions containing mutant CgtA proteins were pooled and subjected to Toyopearl DEAE-650 M (TosoHass) anion-exchange followed by Superdex 75 (Amersham-Pharmacia) gel filtration, as has been described (Lin *et al*., 1999). The purity of the proteins was examined by SDS–PAGE and their concentration was determined using the Bradford assay (Bio-Rad) using bovine serum albumin standards.

UV cross-linking

Purified CgtA proteins (10 μM) were incubated with 5 μCi of [γ-^{32}P]GTP (2 μM, 5000 Ci $mmol^{-1}$, Amersham-Pharmacia) in 20 μl of 1× binding buffer [50 mM Tris-HCl, pH 8.0, 50 mM KCl, 2 mM DTT, 5 mM ATP, 1 mM EDTA, glycerol (10% w/v) with 12 mM $MgCl_2$]. Samples were preincubated on ice for

5 min and the bound nucleotide was UV cross-linked to the CgtA proteins as has been described (Lin *et al*., 1999). The protein–nucleotide complexes were separated from the free nucleotides using SDS–PAGE and detected by autoradiography. Corresponding gel strips were also removed from a parallel SDS–PAGE and their relative level of radioactivity was determined using scintillation counting.

Equilibrium binding assays

The affinity of mutant CgtA proteins for guanine nucleotides was determined using the equilibrium centrifugal ultrafiltration assay as has been described previously (Lin *et al*., 1999). The mutant CgtA proteins display a significantly higher affinity for GDP than GTP. Therefore, the equilibrium dissociation constant, K_D, for GTP was determined using [γ-^{32}P]GTP (Amersham-Pharmacia) instead of [α-^{32}P]GTP to ensure that only the affinity of GTP was measured. [8-^{3}H]GDP (Amersham-Pharmacia) was used to determine K_D for GDP. K_D values were determined by fitting bound versus total nucleotide concentrations to a hyperbolic binding function (Lin *et al*., 1999).

Fluorescence measurements

Fluorescent mant-nucleotides were synthesized and purified as has been described (Lin *et al*., 1999). Fluorescence measurements were performed using a Shimadzu RF-5301PC spectrofluorometer equipped with a Hi-Tech SFA-20 stopped-flow apparatus. Unless otherwise indicated, all assays were performed at 30°C in 1× binding buffer. In all experiments, mant-nucleotide fluorescence was monitored with an excitation wavelength of 361 nm and an emission wavelength of 446 nm.

Excitation spectra were generated in 1× binding buffer using 1 μM mant-nucleotides in the presence or absence of excess CgtA protein (excitation slit 1.5 nm, emission slit 15 nm). To determine the rate of nucleotide exchange, 1 μM mant-nucleotide was prebound with 10 μM purified mutant CgtA protein. Dissociation of CgtA–mant-nucleotide complexes was initiated by the rapid addition of 150× GDP as a competitor. The decrease in fluorescence intensity, indicative of the mant-nucleotide changing from the bound to the free state (excitation slit width 5 nm; emission slit width 20 nm), was monitored over time. Data were collected at 20 ms intervals and curve-fitted to a monophasic (for mant-GDP) or biphasic (for mant-GTP) exponential decay equation (Lin *et al*., 1999). Unless otherwise indicated, the dissociation rate constant, k_d, of each nucleotide was determined by averaging the results from 10 trials.

To estimate the intrinsic GTPase activity of the CgtA mutant proteins, 1 μM mant-GTP was saturated with mutant CgtA protein (25 μM) and incubated at 30°C. Samples of the reaction mixture were withdrawn and subjected to the nucleotide exchange assay described above. The data were fitted to an additive two exponential function and the amplitudes of the two phases were determined. The amount of mant-GDP increases over time as a result of the hydrolysis of mant-GTP by CgtA. We likewise observed that the contribution of one of the phases to the total amplitude increased over time. Therefore, we assigned this phase to mant-GDP. The half-time of a single-turnover mant-GTP hydrolysis by mutant CgtA proteins was estimated by curve-fitting the increase of mant-GDP amplitude to a first order hydrolysis equation.

Limited proteolysis

CgtA protein (100 μg) was saturated with 10 mM GDP or GTP in 30 μl of 1× binding buffer. Proteolysis reactions were started by adding 5 ng of trypsin (TPCK treated, Sigma), or 120 ng of chymotrypsin (TLCK treated, Sigma) at 30°C. The reactions were stopped after 20 min with 1 mM PMSF in 2× loading buffer [0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% (w/v) glycerol, 0.2 M DTT, 0.02% bromophenol blue], heated (95°C, 1 min) and subjected to 20% SDS–PAGE. To identify the unique bands on the gel, parallel digestions of wild-type CgtA were also separated by SDS–PAGE. All gels were transblotted onto polyvinylidine fluoride (PVDF) membrane in 10 mM CAPS buffer (pH 11, Sigma). The PVDF membrane was then stained with Coomassie blue R250 (Acros) for 1 min and destained with methanol for 5 min. The relevant bands were excised and the N-terminal sequence was determined using Edman degradation at the University of Michigan Protein and Carbohydrate Core facility.

Autophosphorylation assays

In 20 μl of 1× binding buffer, each CgtA protein (10 μM) was incubated with 0.05 μM [γ-^{32}P]GTP at 30°C for 1 h in the presence or absence of 2 mM KH_2PO_4. Samples were then separated using SDS–PAGE and detected by autoradiography. The corresponding gel strips were excised from a parallel SDS–PAGE and their relative level of radioactivity was determined using scintillation counting. To determine the acid/base stability of the phosphorylation, parallel gel strips were incubated in 1 N NaOH or 0.2 N HCl for 1 h at 55°C, washed in 40% isopropanol and exposed to autoradiography.

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