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The N-terminal domain of the *Caulobacter crescentus* CgtA protein does not function as a guanine nucleotide exchange factor☆

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Abstract The *Caulobacter crescentus* GTP binding protein CgtA 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 rapid exchange rate constants for either nucleotide. One possible explanation for the observed rapid guanine nucleotide exchange rates is that CgtA is a bimodal protein with a C-terminal GTP binding domain and an N-terminal guanine nucleotide exchange factor (GEF) domain. In this study we demonstrate that although the N-terminus of CgtA is required for function in vivo, this domain plays no significant role in the guanine nucleotide binding, exchange or GTPase activity.

Key words: CgtA; Obg; GTP binding domain; Guanine nucleotide exchange; GTP hydrolysis; *Caulobacter crescentus*

1. Introduction

The bacterial Obg proteins are GTP binding proteins [1–3] that appear to play critical roles in regulating DNA replication and/or cell differentiation [1,4]. Recently, it has been shown that the *Bacillus subtilis* Obg protein is associated with the ribosome [5], although the role that Obg plays in ribosome biogenesis or function is unknown. It has been proposed that the guanine nucleotide occupancy of the Obg proteins is directly controlled by the relative cellular levels of GTP and GDP [3,4,6,7] and not by the action of regulatory proteins such as guanine nucleotide exchange factor (GEFs) or guanine nucleotide activating proteins (GAPs). Thus, the Obg-like proteins would be turned on (in the GTP bound state) during growth conditions and off (in the GDP bound state) under starvation conditions and may function by communicating changes in the GTP pool to downstream pathways. GTP binding proteins regulated in this manner would be expected to oscillate rapidly between the GTP and GDP bound forms. Consistent with this model, we have shown that the *Caulobacter crescentus* Obg protein, CgtA, displays a rapid exchange of either GDP or GTP, has a moderate binding affinity for guanine nucleotides and a relatively slow GTP hydrolysis rate [7].

CgtA is a 354 amino acid protein with the conserved GTP binding domain confined to the C-terminal half of the protein [2]. Although conserved among Obg-like proteins, the role of the glycine-rich N-terminal half of CgtA is unknown. Given the rapid exchange of guanine nucleotides by CgtA observed in vitro, one possibility is that the N-terminus of CgtA functions as a GEF. If true, the rapid guanine nucleotide exchange of CgtA would simply be due to the activities of the N-terminal GEF, as has been proposed for the *Escherichia coli* signal recognition particle receptor FtsY [8]. To test this hypothesis, we examined the in vivo function and in vitro biochemical properties of a truncated CgtA lacking the conserved N-terminus. We show here that although the truncated CgtA mutant fails to function in vivo, the mutant protein displays biochemical properties similar to those of the full length protein. Thus, the N-terminus of CgtA does not contribute to binding, exchange or GTP hydrolysis by CgtA. These data are consistent with a model whereby, in the absence of guanine nucleotide dissociation inhibitor, the guanine nucleotide bound state of CgtA would be controlled by the relative cellular concentration of GTP/GDP nucleotides in *Caulobacter* cells.

2. Materials and methods

2.1. In vivo assay for $cgtA^{160-354}$ function

Wild type *cgtA* on a 2.25 kb *Pst*I–*Hin*dIII fragment was cloned into a pMR20 vector to generate pJM625. pJM1759 is a pMR20 vector containing the *cgtAK2E* allele and an engineered *Nco*I site upstream of the initiator methionine. The *cgtAK2E* allele was generated by site directed mutagenesis using primer 5′-GGACCCCATGGAATTC-TTGGACCA of *cgtA* in the pAlter1 vector (Promega) and the mutagenized 2.25 kb *Pst*I–*Hin*dIII fragment was inserted into pMR20. pJM1761 contains the truncated $cgtA^{160-354}$ expressed from the native *cgtA* promoter on pMR20. pJM1761 was created by generating a *Nco*I site directly upstream of amino acid 160 by site directed mutagenesis using primer 5′-CGCCTGAAGCCCATGGCCGATGTC. The 0.91 kb *Nco*I–*Hin*dIII fragment was inserted into the *Nco*I–*Hin*d-III sites of pJM1759 resulting in a substitution of the *cgtA* coding region with truncated *cgtA*. To assay for in vivo function of these *cgtA* alleles, pJM1759, pJM1761 and pJM625 were introduced, separately, into JM1108, a *C. crescentus* strain in which chromosomal *cgtA* expression is exclusively under the control of the P_{xyl} promoter (Skidmore and Maddock, unpublished). The ability of these alleles to function was assayed on PYE agar plates containing 0.2% xylose (PYE+Xyl) or 0.2% glucose (PYE+Glu) containing 2 μg/ml oxy-tetracyclin and 20 μg/ml kanamycin.

2.2. Protein purification

The C-terminal 160 amino acids of CgtA were expressed and purified from *E. coli*. The 0.91 kb *Nco*I–*Hin*dIII fragment containing $cgtA^{160-354}$ was cloned into the pET28a expression vector (Novagen) to create pJM865. Mid-log *E. coli* BL21(DE3) cells harboring pJM865 were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (Gibco BRL) for 3 h at 37°C in Luria broth (10 g peptone, 5 g yeast extract,

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10 g NaCl per l) containing 30 mg/l kanamycin. The expression of $CgtA^{160-354}$ protein was monitored by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

The $CgtA^{160-354}$ protein was purified as described for the full length CgtA [7] except the Cibaron Blue chromatography step was omitted. Briefly, 1 l of induced cells was pelleted (6000×*g*, 5 min, 4°C), resuspended in 50 ml TDG (50 mM Tris–HCl, pH 8, 1 mM dithiothreitol and 10% glycerol) supplemented with 1 mM phenylmethylsulfonyl fluoride, and lyzed in a French pressure cell. The cell extract was then clarified (28 000×*g* for 30 min, 4°C), loaded on a 50 ml Toyopearl DEAE-650M column (TosoHass), washed and eluted by a 0–200 mM KCl (200 ml) gradient. The $CgtA^{160-354}$ fractions were pooled, concentrated to less than 2 ml by ultrafiltration through YM-10 cellulose membrane (Amicon), applied to a Superdex 75 (Amersham-Pharmacia) gel filtration column and eluted with TDG containing 100 mM KCl. Protein purity was examined by SDS–PAGE and the concentration of $CgtA^{160-354}$ was determined by UV absorption in 6.0 M guanidium hydrochloride, 0.02 M phosphate buffer, pH 6.5 [9], using the extinction coefficient predicted by ProtParam (http://www.expasy.ch/tools/protparam.html).

2.3. Equilibrium binding assays

Guanine nucleotide equilibrium binding constants (K_D) were determined by an equilibrium centrifugal ultrafiltration assay [7,10] at 30°C in 1× binding buffer (50 mM Tris–HCl (pH 8.0), 50 mM KCl, 2 mM dithiothreitol, 5 μM ATP, 1 mM EDTA and 10% (w/v) glycerol) supplemented with 12 mM $MgCl_2$ unless otherwise indicated. [8-^{3}H]GDP (10^6 dpm/nmol; Amersham) and [α-^{32}P]GTP (4×10^6 cpm/nmol; Amersham) were used as ligands in the GDP and GTP binding assays, respectively. Assays were performed in triplicate and the data were analyzed by hyperbolic curve fitting.

2.4. Fluorescence measurements

Fluorescent guanine nucleotides, *n*-methyl-3′-*O*-anthranoyl-GDP (mant-GDP) and -GTP (mant-GTP) were synthesized and purified, as described [7,11]. Unless otherwise indicated, the fluorescence of the mant-nucleotides was monitored at 30°C in 1× binding buffer supplemented with 12 mM $MgCl_2$ at an excitation wavelength of 361 nm (slit width of 1.5 nm) and an emission wavelength of 446 nm (slit width of 15 nm). Excitation spectra were generated from 1 μM unbound mant-GDP or mant-GTP or 1 μM mant-nucleotide bound to 25 μM $CgtA^{160-354}$ protein at 30°C.

To measure nucleotide exchange rate constants, 1 μM mant-nucleotide was prebound with 2 μM $CgtA^{160-354}$. Dissociation of the protein–mant-nucleotide complex was initiated by rapid addition of excess non-fluorescent GDP (150-fold) in a fluorometer (Shimadzu RF-5301PC) equipped with a Hi-Tech SFA-20 stopped-flow apparatus. The decrease in fluorescence intensity (excitation slit width of 5 nm; emission slit width of 20 nm) was monitored at 20 ms intervals, and the data were fitted to a monophasic ($y = A + Be^{-k_d x}$; for mant-GDP and mant-GTP) or biphasic ($y = A + Be^{-k_{d1} x} + Ce^{-k_{d2} x}$; for mant-GTP) exponential decay equation. The dissociation rate constant, k_d, of each nucleotide was determined by averaging the results from a minimum of 10 trials.

The intrinsic GTPase activity of CgtA proteins was determined by monitoring the decrease in fluorescence of the protein–mant-GTP complex when bound mant-GTP is converted to bound mant-GDP. One μM mant-GTP was prebound to excess $CgtA^{160-354}$ protein (approximately 25 μM). The fluorescence was recorded over 3 h at 1 min intervals, and the data were fitted to a single turnover hydrolysis equation [7]. The rate constant (k_h) and half-life ($t_{1/2}$) were determined by averaging results from three trials.

Table 1
Biphasic dissociation rate constants of mant-GTP from CgtA and $CgtA^{160-354}$

Protein	Mg^{2+} (mM)	k_d (s^{-1})		Amplitude of k_d fast/k_d slow
$CgtA^{160-354}$	0.5	4.6 ± 0.1	0.94 ± 0.01	3.6
	12	4.0 ± 0.1	0.94 ± 0.01	0.19
CgtA	12	3.1 ± 0.1	1.02 ± 0.01	0.48

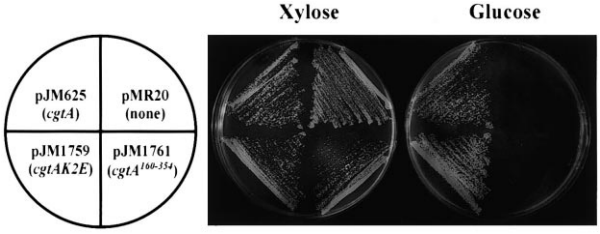

Fig. 1. CgtA protein lacking the N-terminal 159 amino acids does not function in vivo. *C. crescentus* JM1108 cells containing plasmids pMR20 (vector control), pJM625 (wild type *cgtA*), pJM1759 (*cgtAK2E*), pJM1761 ($cgtA^{160-354}$) were streaked onto PYE+Xyl and PYE+Glu plates, as indicated. Shown here are a key and representative plates incubated at 30°C for 2 days.

3. Results and discussion

3.1. The $cgtA^{160-354}$ allele does not function in vivo

We have constructed a strain of *C. crescentus*, JM1108, in which *cgtA* expression is exclusively under the control of the P_{xyl} promoter (Skidmore and Maddock, unpublished). The activity of P_{xyl} promoter is strictly dependent on xylose, being fully induced in PYE+Xyl and repressed when xylose is removed from the growth medium (PYE+Glu; [12]). Repression of P_{xyl}::*cgtA* results in a reduction, but not an elimination of CgtA protein levels, and impaired cell growth (Skidmore and Maddock, unpublished). The slow growth of JM1108 in glucose can be exploited to assay for CgtA function by complementation with mutant alleles of *cgtA* on episomal plasmids. JM1108 cells containing a plasmid-borne wild type *cgtA* allele (pJM625) displayed robust growth regardless of whether the chromosomal copy of *cgtA* was expressed (PYE+Xyl) or not (PYE+Glu)(Fig. 1). Xylose independent growth was also observed for JM1108 harboring a *cgtAK2E* mutant (pJM1759) indicating that this amino acid substitution did not affect CgtA function. In contrast, JM1108 cells containing the empty pMR20 vector only or the $cgtA^{160-354}$ allele (pJM1761) grew well when the chromosomal *cgtA* allele was expressed (PYE+Xyl) but poorly when it was not (PYE+Glu) (Fig. 1). These data indicate that the N-terminus of CgtA is critical for proper CgtA function, either because $CgtA^{160-354}$ is not sufficient for function, or because the N-terminus of CgtA is necessary for correct protein folding or stability.

3.2. $CgtA^{160-354}$ binds guanine nucleotides with similar affinity to wild type CgtA

Induction of $CgtA^{160-354}$ from pJM865 resulted in the accumulation of a prominent ~20 kDa protein (data not shown). Unlike the wild type CgtA protein, the truncated protein does not bind Cibacron Blue 3GA agarose (Sigma) and therefore we purified $CgtA^{160-354}$ by DEAE anion-exchange followed by Superdex Gel-filtration. The identity of $CgtA^{160-354}$ was verified by N-terminal sequence analysis and shown to begin, as predicted, with the amino acid sequence ADVGLV.

$CgtA^{160-354}$ binds radiolabeled GDP and GTP with moderate affinity as determined by equilibrium centrifugal ultrafiltration. At 30°C in 12 mM Mg^{2+}, the equilibrium binding constants, K_D, for GDP and GTP were 1.5 ± 0.2 and 1.1 ± 0.3 μM, respectively, compared with 0.52 ± 0.03 and 1.11 ± 0.13 μM as previously reported for full length CgtA [7]. Thus, the affinity of $CgtA^{160-354}$ for GTP was similar to that of

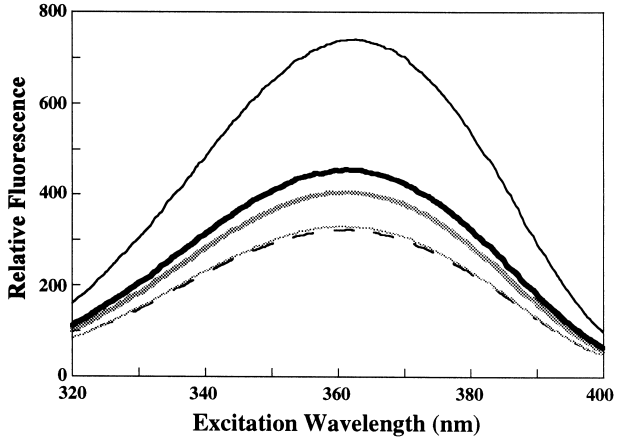

Fig. 2. The CgtA$^{160-354}$ protein binds to mant-nucleotides. Shown is the excitation spectra (emission at 446 nm) of 1 mM free mant-GTP and mant-GTP bound to 25 μM CgtA$^{160-354}$ at 30°C. Shown are the relative excitation spectra of mant-GDP (dashed line), CgtA$^{160-354}$–mant-GDP without (thick gray line) or with 12 mM Mg^{2+} (thick black line), and CgtA$^{160-354}$–mant-GTP without (thin gray line) or with 12 mM Mg^{2+} (thin black line). The spectrum of the free mant-GTP overlays that of the free mant-GDP (data not shown).

the full length protein and the affinity for GDP was approximately three-fold weaker than that of wild type CgtA. These data show that the N-terminus of CgtA does not play a significant role in the association of CgtA with guanine nucleotides. Moreover, these data are consistent with the observation that a temperature sensitive mutation in the N-terminus of the *B. subtilis* Obg protein had the same affinity for guanine nucleotides as the wild type Obg [6].

We also examined the guanine nucleotide binding properties of CgtA$^{160-354}$ with mant-GDP and mant-GTP (Fig. 2). CgtA$^{160-354}$ displayed an excitation profile similar to that of full length CgtA [7]. Binding of CgtA$^{160-354}$ to mant-GTP and mant-GDP led to an increase in mant-nucleotide fluorescence with an optimal excitation wavelength of 361 nm (Fig. 2). CgtA$^{160-354}$ bound to mant-GTP resulted in a 2.3-fold enhancement of fluorescence intensity over free mant-GTP (Fig. 2), whereas the mant-GDP fluorescence increased only 1.4-fold upon binding to CgtA$^{160-354}$. This increase in mant-nucleotide fluorescence further demonstrates that the truncated CgtA protein binds guanine nucleotides. CgtA$^{160-354}$ does, however, result in a higher fluorescence increase of the mant-GTP than does the full length CgtA (2.3- and 1.5-fold, respectively). This increase in fluorescence may reflect an increase in the hydrophobicity of the guanine nucleotide binding pocket in the absence of the N-terminus of CgtA. Like wild type CgtA, CgtA$^{160-354}$ also required Mg^{2+} for GTP but not GDP binding (data not shown).

3.3. The GTPase activity of CgtA$^{160-354}$ is equivalent to that of wild type CgtA

We used the greater fluorescence of the CgtA$^{160-354}$–mant-GTP complex relative to the CgtA$^{160-354}$–mant-GDP complex to determine the rate of hydrolysis. The intrinsic GTPase activity of CgtA$^{160-354}$ was measured by monitoring the reduction in fluorescence that accompanied the single-turnover conversion of bound mant-GTP to bound mant-GDP [7]. Curve fitting the decrease in fluorescence to a single exponential hydrolysis function gave a single turnover rate constant, k_h, of 5.3×10^{-4} s^{-1} or a $t_{1/2}$ of 22 ± 2 min. The GTPase activity of CgtA$^{160-354}$ is almost identical to that reported for wild type CgtA ($k_h = 5.0\times10^{-4}$ s^{-1}, $t_{1/2} = 23 \pm 2$ min) measured under the same conditions [7]. Thus, removal of the N-terminal 159 amino acids of CgtA has no effect on the GTPase activity of the protein.

3.4. The N-terminus of CgtA is not necessary for the rapid exchange of guanine nucleotides

The in vitro exchange of guanine nucleotides by CgtA has been previously shown to be 10^3–10^5-fold faster than that of the well-characterized eukaryotic Ras-like GTP binding proteins [7]. One model proposed to explain the rapid exchange was that CgtA is a bimodal protein with a Ras-like GTP binding domain at its C-terminus and GEF activity at its N-terminus [7]. If this is true, removal of the GEF domain should result in a slower guanine nucleotide exchange rate constant than that of the full length protein. To test this model we determined the exchange rate constant of CgtA$^{160-354}$ for both mant-GTP and mant-GDP using stopped-flow fluorospectroscopy. The dissociation of the mant-nucleotide was monitored by the fluorescence decrease that accompanied the displacement of bound mant-nucleotide by excess unlabeled nucleotide. The observed first order dissociation rate constants for mant-GDP and mant-GTP were 1.11 ± 0.01 and 1.03 ± 0.04 s^{-1}, respectively. These rates are comparable to those of full length CgtA (1.43 ± 0.04 and 1.28 ± 0.02 s^{-1}, respectively) under similar conditions [7]. Therefore, the N-terminus of CgtA does not play a role as a GEF.

Interestingly, the mant-GTP dissociation data fit a biphasic exponential decay equation better than a single exponential decay. At 12 mM Mg^{2+} the majority (~85%) of the dissociation is slow (0.94 ± 0.01 s^{-1}) while the remaining dissociation (15%) is fast (4.0 ± 0.1 s^{-1}). Even after a prolonged incubation at 30°C (20 min) there was no significant change in the ratio of the amplitudes of these two phases indicating that they

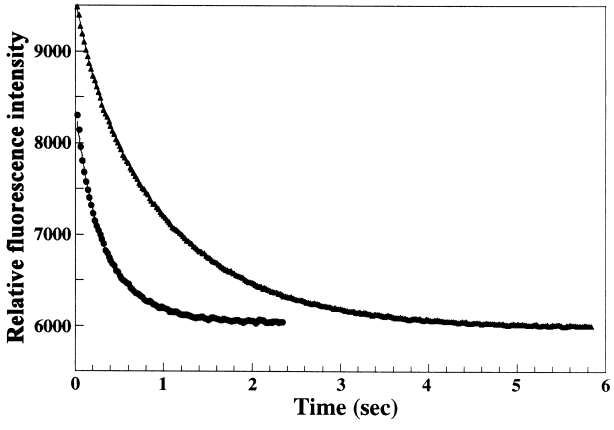

Fig. 3. CgtA$^{160-354}$ displays biphasic mant-GTP dissociation. CgtA$^{160-354}$–mant-GTP complexes were generated by prebinding 1 μM mant-GTP and 2 μM CgtA$^{160-354}$ in 0.5 mM (circles) or 12 mM Mg^{2+} (triangles). These complexes were rapidly mixed with excess GDP (150 mM) and the change in the relative fluorescence intensity over time was monitored. Shown are the pooled data from 10 trials with a biphasic fit shown by the solid line. Relative fluorescence intensity = $A + Be^{-k_{d1}x} + Ce^{-k_{d2}x}$.

were not likely due to contaminating mant-GDP (a product of hydrolysis) or due to the inactivation of $CgtA^{160-354}$. Moreover, the relative abundance of these two putative $CgtA^{160-354}$–mant-GTP complexes showed strong Mg^{2+} dependency. When the Mg^{2+} concentration in the exchange reaction was lowered to 0.5 mM, the rate constants of either phase did not change but their relative amplitude was almost reversed (Table 1 and Fig. 3). To examine whether the biphasic exchange was a unique feature of the truncated CgtA, we reexamined the dissociation of mant-GTP from wild type CgtA and found that CgtA also displayed a biphasic mant-GTP exchange profile with a similar Mg^{2+} dependence (data not shown). Since the rate constants of these two phases were very similar, we overlooked the biphasic nature of the GTP exchange in our initial analysis of CgtA. The nature of the biphasic dissociation is unknown. We have recently reported a similar biphasic dissociation of mant-nucleotides from the *E. coli* GTP binding protein Era [13]. It will be of interest to determine whether a similar rapid and biphasic dissociation is observed in other bacterial GTP binding proteins.

These data clearly demonstrate that the N-terminus of CgtA does not act as a GEF as had been proposed, but not experimentally tested, for the *E. coli* protein, FtsY [8]. Moreover, we have recently shown that the *E. coli* Era protein also displays a rapid guanine nucleotide exchange rate constant [13]. We have also observed that other bacterial GTP binding proteins share more amino acid similarity in the conserved GTP binding pocket with each other than with the well-characterized, GEF-controlled eukaryotic ras-like proteins, raising the possibility that low affinity and rapid exchange of guanine nucleotides may be a hallmark feature of these bacterial GTP binding proteins.

What might be the cellular role of the N-terminal domain? One possibility is that this domain is required for the interaction of CgtA with other cellular proteins. The interaction between these putative targets and the N-terminus of CgtA is not likely to be affected by structural differences between the GTP- and GDP-bound states, as guanine nucleotide binding appears to be mediated through the C-terminus of the protein. More likely, the N-terminus may be involved in anchoring CgtA to its cellular target via protein–protein interactions. Recently, it has been shown that the *B. subtilis* Obg protein interacts with the ribosome, specifically interacting with Rpl13 [5]. It will be of interest to see if the N-terminal domain is necessary for this interaction.

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