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Identification of bacterial guanylate cyclases

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Abstract

The ability of bacteria to use cGMP as a second messenger has been controversial for decades. Recently, nucleotide cyclases from *Rhodospirillum centenum*, GcyA, and *Xanthomonas campestris*, GuaX, have been shown to possess guanylate cyclase activities. Enzymatic activities of these guanylate cyclases measured *in vitro* were low, which makes interpretation of the assays ambiguous. Protein sequence analysis at present is insufficient to distinguish between bacterial adenylate and guanylate cyclases, both of which belong to nucleotide cyclases of type III. We developed a simple method for discriminating between guanylate and adenylate cyclase activities in a physiologically relevant bacterial system. The method relies on the use of a mutant cAMP receptor protein, CRP_G, constructed here. While wild-type CRP is activated exclusively by cAMP, CRP_G can be activated by either cAMP or cGMP. Using CRP- and CRP_G-dependent *lacZ* expression in two *E. coli* strains, we verified that *R. centenum* GcyA and *X. campestris* GuaX have primarily guanylate cyclase activities. Among two other bacterial nucleotide cyclases tested, one, GuaA from *Azospirillum* sp. B510, proved to have guanylate cyclase activity, while the other one, *Bradyrhizobium japonicum* CyaA, turned out to function as an adenylate cyclase. The results obtained with this reporter system were in excellent agreement with direct measurements of cyclic nucleotides secreted by *E. coli* expressing nucleotide cyclase genes. The simple genetic screen developed here is expected to facilitate identification of bacterial guanylate cyclases and engineering of guanylate cyclases with desired properties.

Keywords

cGMP; cAMP; nucleotide cyclase; second messenger; signal transduction; *Escherichia coli*

INTRODUCTION

Cyclic adenosine 3',5'-monophosphate (cAMP) and cyclic guanosine 3',5'-monophosphate (cGMP) are the most important nucleotide messengers in eukaryotes. Bacteria also use cAMP to regulate diverse processes that include utilization of alternative sugars (catabolite repression), motility, virulence, etc. In contrast to cAMP, the existence and role of cGMP in

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bacteria has remained controversial for decades (1,2). Several studies claimed validity to the presence of cGMP signaling in bacteria, only to be rejected by subsequent rigorous testing.

Two recent reports have provided evidence of guanylate cyclases (cGMP synthases) in the proteobacterial species *Rhodospillium centenum* and *Xanthomonas campestris* (3,4). These reports documented intracellular cGMP and identified protein targets of cGMP-dependent signaling. In addition, in both studies, guanylate cyclases were overexpressed in *E. coli*, purified, and their activities were measured *in vitro*. However, enzymatic activities were low, which raised questions about the functionality of these enzymes *in vivo*. *R. centenum* GcyA and *X. campestris* GuaX are large multidomain proteins. At present, conditions required for their full activities are not yet known. Further, protein sequences of the substrate-binding sites in these bacterial guanylate cyclases are different from the sequences of eukaryotic guanylate cyclases (3,4), which suggests that these classes of enzymes have evolved from adenylate cyclases in parallel pathways. However, the low number of bacterial guanylate cyclases does not yet allow reliable distinction between guanylate and adenylate cyclases based on protein sequences.

To facilitate the identification of new bacterial guanylate cyclases, we developed an *E. coli*-based reporter system that does not require protein purification. The key component of the system is an engineered mutant cAMP receptor protein, CRP (also known as catabolite activator protein, CAP) (5,6) that can be activated by either cAMP or cGMP. This mutant protein, designated CRP_G, along with the wild-type CRP, allows one to monitor guanylate cyclase activity in *E. coli* via CRP- and CRP_G-dependent *lacZ* gene expression. The heterologous nucleotide cyclases expressed in the *E. coli* mutant lacking its native adenylate cyclase, Cya, can be assigned to adenylate cyclases if they activate *lacZ* expression via both CRP and CRP_G. If they activate *lacZ* expression via CRP_G only, they represent guanylate cyclases. This system is expected not only to facilitate characterization of cGMP-dependent signal transduction in bacteria but also to provide an *E. coli*-based screening platform for engineering guanylate cyclases with desired properties (for example, light-activated guanylate cyclases for optogenetic applications [7,8]). In addition, we show in this study that *E. coli* secretes most cAMP as well as cGMP, therefore direct measurements of cyclic nucleotides secreted by *E. coli* (e.g., by ELISA) presents yet another method for assessing the substrate specificity of heterologous nucleotide cyclases.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown at 37°C in LB medium. Antibiotics were used at the following concentrations (μg/mL): ampicillin (Ap), 100; kanamycin (Km), 25; spectinomycin (Sp), 100; tetracycline (Tc), 12. Cyclic nucleotides, cAMP or cGMP (Sigma), were added to the LB agar medium to a final concentration of 0.1 mM.

The BL21 [DE3] *cya crp* double mutant was constructed by a one-step gene inactivation method (9) using the BL21 [DE3] *cya* strain described earlier (8). *lacZ* expression in BL21

[DE3] *cya crp* expressing CRP or CRP_G was assessed on LB plates supplemented with Ap, Tc, X-gal (40 µg/mL) and IPTG (50 µM) after 36 h incubation at 37 °C.

Plasmid construction

The triple, R123N T127C S128T, CRP mutant (designated CRP_G) was constructed by site-directed mutagenesis using the QuikChange kit (Agilent Technologies). Plasmid pHY26-1 (10) containing the wild-type *crp* gene was used as template to yield pHYC445 that contains *crp_G*. The *crp* and *crp_G* genes were PCR-amplified from pHY26-1 and pHYC445, respectively, and cloned into XbaI and EcoRI sites of the vector pRK415 (11). The resulting plasmids pRKcrp and pRKcrp_G express, respectively, *crp* and *crp_G* from the *lac* promoter.

All nucleotide cyclases tested here were cloned into pET23a(+). The adenylate cyclase domain (aa 591-857) of the CyaB1 protein from *Nostoc* sp. PCC 7120 and the full-length *guaB* gene were synthesized by GeneArt Strings (Life Technologies) and assembled with pET23(+) using Gibson assembly (NEB). The *gcyA* gene (rc1-3783) from *Rhodospirillum centenum* was amplified by PCR from the plasmid kindly provided by Dr. C. Bauer (3). The *guaA* (AZL_d00190) and XC_0250 (hereby designated *guaX*) genes were amplified from genomic DNA of *Azospirillum* sp. B510 and *Xanthomonas campestris* kindly provided by Drs. S. Sato and R. Ryan, respectively.

Overexpression and purification of CRP proteins

The His-tagged CRP and CRP_G were overexpressed in strain UQ3811 and purified using a nickel-nitrilotriacetate column according to the manufacturer's instruction (Novagen), as described earlier (10). The final purity of both proteins was >95% (based on SDS-PAGE).

Responsiveness of CRP and CRP_G to cAMP and cGMP

To determine how effectively the CRP and CRP_G proteins responded to cGMP and cAMP, the *in vitro* DNA-binding activities of CRP and CRP_G were measured with varying concentrations of each cyclic nucleotide as described previously (12,13). *In vitro* DNA-binding assays were carried out using a fluorescence polarization method. The target DNA was a 26-mer CRP consensus sequence (5'-GTAAATGTGATGTACATCACATGGAT-3') labeled with a fluorescent dye, Texas Red, as described previously (10). When the DNA-protein interaction occurs, fluorescence polarization is increased, which reflects slowed Brownian motion of the labeled DNA by the bound protein. Assays were performed in 50 mM Tris-HCl (pH 8.0), 150 mM KCl, and 1 mM EDTA with a probe concentration of 5 nM in the presence of 6.4 µM salmon sperm DNA (nonspecific competitor). The concentration of each protein was 150 nM.

Detection of secreted cyclic nucleotides

cAMP and cGMP production in the BL21[DE3] mutants that express the respective nucleotide cyclases were assessed by specific cAMP and cGMP ELISA kits (Enzo Life Sciences) according to the instructions of the manufacturer. Briefly, overnight cultures were transferred to fresh M9 minimal medium at an A₆₀₀ of 0.02 and protein expression was induced by the addition of 0.1 mM IPTG. Growth was continued at 37°C until the cells reached stationary phase (A₆₀₀, 3~4) and cell-free supernatants were collected by

centrifugation at 4°C. Since *E. coli* secretes most cAMP and cGMP into the medium (7,14), extracellular cyclic nucleotide levels were determined in cell-free supernatants.

RESULTS AND DISCUSSION

Construction and properties of the cAMP- and cGMP-responsive CRP mutant

To construct the CRP mutant that can be activated by cGMP, we combined three amino acid substitutions (R123N, T127C and S128T) in the cAMP-binding pocket, each of which has been shown earlier to improve cGMP responsiveness (13,15,16). We expected that mutations in all three residues would have a cumulative effect. R123N was suggested to help properly position cGMP in the binding pocket by enabling a new H-bond to Asn (15). The exact role of T127C and S128T replacements, which increased CRP responsiveness to cGMP, is unknown (16). It is noteworthy that the residue corresponding to Thr128 of CRP in the *Pseudomonas aeruginosa* CRP homolog, Vfr, is also important for cGMP responsiveness (13).

We overexpressed and purified, using nickel affinity resin, the triple mutant (R123N, T127C, S128T), designated CRP_G. Figure 1 shows that the DNA binding by CRP_G was stimulated equally well by cAMP and cGMP (Fig. 1). This is in contrast to DNA binding by wild-type CRP, which was increased only by cAMP but not cGMP. Further, the affinity of CRP_G to DNA in the presence of cAMP and cGMP was approximately the same as the affinity to DNA of CRP in the presence of cAMP (Fig. 1). Therefore, in accord with our expectation, the three individual substitutions in the cAMP-binding pocket had cumulative effect on improving cGMP-dependent DNA binding by CRP.

Discrimination between cAMP and cGMP by wild-type CRP is complex because both molecules are known to bind with approximately equal affinities (17,18), yet, unlike cAMP, which stimulates DNA binding, cGMP inhibits it (19). Interestingly, cAMP binds to CRP in the *anti* conformation (20,21), whereas cGMP binds in the *syn* conformation (12,19,21). The structure of the cGMP-binding protein XC_0249 from *Xanthomonas campestris* shows that it too binds cGMP in the *syn* conformation (4,22). These observations are consistent with the dominant conformations of cAMP and cGMP in aqueous solutions *in vitro*, which are 95:5 *syn/anti* for cGMP and 30:70 *syn/anti* for cAMP (23). Based on these considerations, we hypothesize that CRP_G binds cGMP in the *syn* conformation as does wild-type CRP, but the *syn*-cGMP binding of CRP_G leads to an active form that may be locally different but globally similar to the active form of the wild-type CRP in the presence of cAMP. A crystal structure of the CRP_G protein in the presence of cGMP is needed to understand the cGMP-activation of DNA binding by CRP_G. However, regardless of the activation mechanism, equal responsiveness of CRP_G to cAMP and cGMP presented an opportunity to develop an *E. coli*-based system that can discriminate between adenylate and guanylate cyclase activities.

CRP/CRP_G reporter-based discrimination of adenylate and guanylate cyclases

To design a system for discriminating between adenylate and guanylate cyclase activities, we constructed a BL21 [DE3] *cya crp* double mutant that lacks genes encoding a native adenylate cyclase, Cya, and CRP. The *crp* and *crp_G* genes were cloned in vector pRK415

downstream of a *lac* promoter and introduced into this double mutant. First, we tested the ability of the *crp* and *crp_G* genes to activate the chromosomal *lacZ* gene expression in the presence of exogenously added cAMP or cGMP. As expected, CRP activated *lacZ* expression (judged by the appearance of blue color) in the presence of cAMP but not cGMP, whereas CRP_G activated *lacZ* expression in the presence of both cGMP and, to a lesser extent, cAMP (Fig. 2A). We do not understand the apparent preference of CRP_G for cGMP versus cAMP in *E. coli* in light of the approximately equal affinities for these cyclic nucleotides *in vitro* (Fig. 1). However, this fortunate circumstance only improves the discriminating capacity of the CRP_G/CRP system.

Next, we tested known adenylate and guanylate cyclases. The adenylate cyclase domain of CyaB1 from *Nostoc* sp. is known to possess adenylate cyclase activity (24). We introduced this domain into the BL21 [DE3] *cya crp* strains carrying either CRP or CRP_G and observed that it activated *lacZ* expression in both test strains (Fig. 2B). In contrast, the full-length proteins *R. centenum* GcyA and *X. campestris* GuaX activated *lacZ* expression only in the presence of CRP_G but not CRP (Fig. 2B). This indicates that *E. coli* cells expressing GcyA or GuaX accumulated mostly cGMP but not cAMP, which is consistent with the original reports (3,4).

To further test the utility of the CRP/CRP_G system, we cloned two genes encoding putative nucleotide cyclases of unknown substrate specificity. One of these, AZL_d00190, hereby designated *guaA*, originates from plasmid pAB510d present in *Azospirillum* sp. B510. This protein was suggested to have guanylate cyclase activity (3). Another enzyme, hereby designated CyaA, from *Bradyrhizobium japonicum* USDA 6 (YUI_RS0121825) had unknown substrate preference. The CRP/CRP_G test showed that GuaA is a guanylate cyclase, while CyaA is an adenylate cyclase (Fig. 2B). Note that in our assay, expression of nucleotide cyclase and CRP/CRP_G are controlled by IPTG concentration, which can be increased to enhance assay sensitivity (in case of low activity nucleotide cyclases) or decreased (to better discriminate between adenylate and guanylate cyclase activities of super-active cyclases).

Measurements of cNMPs secreted by *E. coli* expressing nucleotide cyclases

In search of an independent means for verifying the performance of the CRP/CRP_G reporter system, we exploited the fact that both cAMP (14) and cGMP (7) are actively secreted by *E. coli* into the medium, although secretion efficiencies appear to differ dependent on which nucleotide is secreted (7). We measured secreted cNMPs in the BL21 [DE3] *cya crp* mutant expressing the nucleotide cyclases tested by the CRP_G/CRP system. The results of analysis of secreted nucleotides via ELISA that involves the cAMP- and cGMP-specific antibodies (Table 2 and Fig. 2C) were in excellent agreement with the results of the *E. coli* CRP/CRP_G reporter system (Fig. 2B). All strains expressing guanylate cyclases secreted >10²-fold higher amounts of cGMP than cAMP, while strains expressing adenylate cyclases secreted 10-10²-fold higher amounts of cAMP than cGMP. Such large differences make the distinction between these enzymes unambiguous. Importantly, the absolute levels of cNMPs secreted by cells expressing different cyclases varied widely, by orders of magnitude. The absolute amounts of secreted cNMPs may reflect differences in the expression levels,

solubility of the heterologous cyclases as well as differences in their relative enzymatic activities. Despite such variation, the distinction between two classes of nucleotide cyclases was clear in the ELISA test as well as in the CRP_G/CRP system reporter system.

CONCLUSIONS

The triple, R123N T127C S128T, mutant of the cAMP receptor protein, CRP_G, binds DNA *in vitro* equally well in the presence of either cAMP or cGMP. In *E. coli*, CRP_G activates *lacZ* gene expression in response to both cyclic nucleotides but shows preference to cGMP. The *E. coli* CRP/CRP_G reporter system developed here presents a reliable tool for distinguishing between adenylate and guanylate cyclase activities of nucleotide cyclases without the need of enzyme purification. We expect this system to facilitate (i) characterization of cGMP-dependent signal transduction in bacteria and (ii) engineering of guanylate cyclases with desired properties.

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Figure 1. DNA-binding of CRP and CRP_G at various concentrations of cAMP and cGMP. *In vitro* DNA binding was measured by fluorescence anisotropy.

Figure 2. The CRP/CRP_G reporter system for detecting adenylate and guanylate cyclase activities. **A**, The BL21 [DE3] *cya crp* strains expressing wild-type CRP from pRKcrp (A) or CRP_G from pRKcrp_G (G) were grown with or without the addition of cAMP or cGMP. The strain expressing CRP developed blue color only in the presence of cAMP whereas the strain expressing CRP_G developed blue color in the presence of either cAMP or cGMP. **B**, Heterologous nucleotide cyclases were introduced into the BL21 [DE3] *cya crp* mutants expressing CRP or CRP_G. The CyaB1 (catalytic domain) and CyaA (full-length) produced blue color in both strains, while GcyA, GyaA and GyaX produced blue colonies only in the presence of CRP_G. The medium contained 50 μ M IPTG. **C**, Analysis of secreted cNMPs from the *E. coli* strains expressing nucleotide cyclases. The same strains as in panel B were used. Cyclic nucleotide levels were measured by ELISA using cAMP- and cGMP-specific

antibodies. Average data and standard deviations from three independent experiments are shown.

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Table 1

Strains and plasmids used in this study.

Strain	Description	Source or Reference
DH5 α	Strain used for cloning	New England Biolabs
UQ3811	Strain used for CRP protein purification	10
BL21 [DE3] <i>cya crp</i>	<i>fhuA2 [lon] ompT gal (λDE3) [dcm] hsdS cpr^r. Km^r cyaA^r. Sp^r</i>	New England Biolabs
Plasmid		
pET23a(+)	Bacterial expression vector, Ap ^r	Invitrogen
pETcyaA	pET23a(+) encoding <i>B. japonicum</i> USDA6 CyaA	This study
pETcyaB1	pET23a(+) encoding aa 591-857 (catalytic domain) of <i>Nostoc</i> sp. CyaB1	This study
pETgcyA	pET23a(+) encoding <i>R. centenum</i> GcyA	This study
pETguaA	pET23a(+) encoding <i>Azospirillum</i> sp. B510 GuaA	This study
pETguaX	pET23a(+) encoding <i>X. campestris</i> GuaX	This study
pHY26-1	Vector expressing His-tagged <i>E. coli</i> CRP	10
pHYC445	Vector expressing His-tagged CRP triple (R123N, T127C, S128T) mutant, CRP _G	This study
pRK415	Broad-host range vector, IncP, Tc ^R	11
pRK-CRP	pRK415 encoding <i>E. coli</i> CRP under the <i>lac</i> promoter	This study
pRK-CRP _G	pRK415 encoding CRP _G under the <i>lac</i> promoter	This study

Table 2

Cyclic nucleotide production by nucleotide cyclases in strain BL21[DE3] *cya crp*.

Protein	None	CyaB1	GcyA	GuaA	CyaA	GuaX
Nucleotide, nM						
cAMP	0.8 ± 0.3	1.4 ± 0.5 × 10 ⁵	11.7 ± 4.9	78.6 ± 16.9	2.3 ± 0.3 × 10 ²	4.0 ± 0.9 × 10 ²
cGMP	1.3 ± 0.7	49.5 ± 14.1	5.2 ± 2.8 × 10 ³	3.8 ± 0.3 × 10 ⁴	28.0 ± 5.2	1.0 ± 0.3 × 10 ⁵