Natural and Engineered Photoactivated Nucleotidyl Cyclases for Optogenetic Applications

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Cyclic nucleotides, cAMP and cGMP, are ubiquitous second messengers that regulate metabolic and behavioral responses in diverse organisms. We describe purification, engineering, and characterization of photoactivated nucleotidyl cyclases that can be used to manipulate cAMP and cGMP levels in vivo. We identified the blaC gene encoding a putative photoactivated adenyl cyclase in the Beggiatoa sp. PS genome. BlaC contains a BLUF domain involved in blue-light sensing using FAD and a nucleotidyl cyclase domain. The blaC gene was overexpressed in Escherichia coli, and its product was purified. Irradiation of BlaC in vitro resulted in a small red shift in flavin absorbance, typical of BLUF photoreceptors. BlaC had adenyl cyclase activity that was negligible in the dark and upregulated by light by 2 orders of magnitude. To convert BlaC into a guanylyl cyclase, we constructed a model of the nucleotidyl cyclase domain and mutagenized several residues predicted to be involved in substrate binding. One triple mutant, designated BlgC, was found to have photoactivated guanylyl cyclase in vitro. Irradiation with blue light of the E. coli cya mutant expressing BlaC or BlgC resulted in the significant increases in cAMP or cGMP synthesis, respectively. BlaC, but not BlgC, restored cAMP-dependent growth of the mutant in the presence of light. Small protein sizes, negligible activities in the dark, high light-to-dark activation ratios, functionality at broad temperature range and physiological pH, as well as utilization of the naturally occurring flavins as chromophores make BlaC and BlgC attractive for optogenetic applications in various animal and microbial models.

The ability to activate or inactivate signal transduction pathways in vivo, during normal or disease conditions, in specific tissues and at desired times could provide unprecedented insights into cellular regulatory networks. However, the number of pharmaceutical activators or inhibitors of signaling proteins is limited, their target specificities are imperfect, and the spatiotemporal resolution of their action is low. A recently emerged optogenetic method can supplement pharmaceutical approaches (1, 2). Optogenetics involves introduction of genes encoding natural or engineered photoactivated proteins with desired activities into cells and tissues of model organisms.

In naturally photoactivated proteins, photons absorbed by photoreceptor protein domains induce conformational changes that are propagated to change activities of the output domains (3). Light is unique in that it can control protein activities in a reversible manner and with high spatiotemporal resolution. Visible light, particularly at low intensities, is practically harmless. The spatiotemporal resolution that can be achieved by using photoregulated proteins is limited only by the width of a laser beam, which can be focused with a subcellular precision. We are at the dawn of an exciting era when optogenetic tools will become common in biomedical applications (4).

Optogenetic applications using rhodopsin-based photoreceptors that use retinal chromophores have thus far been most successful. A subclass of rhodopsins, channelrhodopsins, that function as photoactivated ion channels, have been used to regulate neuronal activity in different animal models (5–10, 12–16).

The flavin-containing photoreceptors of LOV4 (17, 18) and BLUF (19, 20) families are also attractive for optogenetic applications because of their relatively small size (100–140 amino acids), solubility, and spontaneous incorporation of flavin chromophores into apoproteins. Because flavins are present in all cell types, there is no need for chromophore delivery to target tissues. The biggest limitation of LOV- and BLUF-based photoactivated proteins is that blue light, which they absorb, has intrinsically low tissue penetration capacity. Therefore, external light sources can be used only for small model animals (e.g. Drosophila) (8), whereas surgically implanted photoemitting devices have to be used for larger animals (e.g. mice) (16).

The BLUF-domain containing photoactivated adenyl cyclase from Euglena gracilis, PAC (21), proved to be a powerful analytical tool to control cAMP levels in Xenopus oocytes and neurons of Drosophila and the mollusk Aplysia (22, 23). PAC proved useful despite the large size of its subunits (>1000

The abbreviations used are: LOV, light, oxygen, and voltage-sensing protein domain of the PAS superfamily; BlaC, bacterial light-activated adenyl cyclase; BglC, bacterial light-activated guanylyl cyclase; BLUF, sensor of blue light using FAD; MBP, maltose-binding protein; PAC, photoactivated adenyl cyclase from E. gracilis; PDB, Protein Data Bank.

Grant RR016474-09.
The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1, Figs. S1–S3, and additional references.

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3 This work was supported in part by National Institutes of Health 2P20 Grant RR016474-09.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 285, NO. 53, pp. 41501–41508, December 31, 2010 © 2010 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.


**Photoactivated Adenylyl and Guanylyl Cyclases**

Adenyl cyclase and guanylyl cyclase, enzymes that catalyze the synthesis of cyclic nucleotides (cAMP and cGMP), are essential for a variety of cell processes. These enzymes are involved in regulating gene expression, ion transport, cell growth, differentiation, and survival. By using light as a signal, these enzymes provide an attractive tool for studying intracellular signaling pathways.

### EXPERIMENTAL PROCEDURES

**Microbiological Methods**—Escherichia coli BL21(DE3) and DH5α and their derivatives were routinely grown in LB medium (28). For light-dependent experiments, cells were grown at 30 °C on MacConkey agar (28) supplemented with 1% lactose. Irradiation was provided by light-emitting diode panels, either the All-blue (emission 465 nm) or All-red (635 nm) LED Grow Light panel 225 (30.5 cm square; LED Wholesalers, CA). Light was administered at an irradiance of ~1 W m⁻² for 48 h using the following regimen: 5-s light, 120-s dark.

**Recombinant DNA Techniques**—The mutation in the adenylyl cyclase gene (E. coli BL21(DE3) was constructed by a one-step gene inactivation method described by Datsenko and Wanner (29). The **Beggiatoa** sp. PS *blaC* gene (locus_tag BGP_1043; GI:153870309) (30) was synthesized by BioBasics, Inc. with the codon usage optimized for *E. coli*. The gene was cloned into pBAD/Myc-His-B (Invitrogen) to generate the plasmid pBAD-*blaC* for arabinose-inducible expression in *E. coli*. For overexpression and protein purification purposes, *blaC* was cloned into the modified in-house vector pMal-c2x (NEB Biolabs) to generate a maltose-binding protein (MBP)-*blaC* fusion (plasmid pMal-*blaC*). Site-directed mutagenesis using the QuickChange kit (Stratagene) was performed on *blaC*-containing plasmids to generate a guanylyl cyclase.

**Protein Overexpression and Purification**—The BlaC protein and its derivatives were purified as C-terminal fusions to the MBP-His₈ tag using amylose affinity chromatography according to specifications of the manufacturer (NEB Biolabs). Protein purification was performed under red light to avoid flavin photobleaching. The overnight cultures of *E. coli* DH5α [pMal-*blaC*]-expressing MBP-His₈_BlaC or DH5α [pMal-*blgC*]-expressing MBP-His₈_BlgC were grown to A₆₀₀ 0.6 in LB supplemented with 100 μg of ampicillin ml⁻¹ at 30 °C. The cultures were transferred to 18 °C, and isopropyl 1-thio-β-d-galactopyranoside was added to a final concentration of 0.5 mM to induce gene expression. Following a 16-h incubation, bacteria were collected by centrifugation at 5,000 × g for 15 min, washed, and resuspended in the amylose column binding buffer (50 mM Tris-HCl, pH 8.0, 350 mM NaCl, 10 mM MgCl₂, 0.5 mM EDTA, 10% glycerol). Cells were disrupted using a French pressure cell, and cell debris was removed by centrifugation at 35,000 × g for 45 min at 4 °C. Two milliliters (bed volume) of amylose resin (NEB Biolabs) pre-equilibrated with the binding buffer was added to the soluble cell extract derived from a 1.5-liter culture and agitated for 1 h at 4 °C. The mix was loaded onto a column, and the resin was washed with 200 ml of column binding buffer. Fractions were eluted with 12 ml of binding buffer containing 10 mM maltose. The protein was either used immediately or stored at −80 °C in 20% v/v glycerol (final concentration). Protein concentrations were measured using a Bradford protein assay kit (Bio-Rad) with bovine serum albumin as the protein standard. Proteins were analyzed using SDS-PAGE.

**Enzymatic Assays**—Enzymatic assays were performed at room temperature unless specified otherwise. A standard reaction mixture (300 μl) contained 5 μM enzyme in the assay buffer (50 mM Tris-HCl, pH 8.0, 10% glycerol, 10 mM MgCl₂, 0.5 mM EDTA). The protein was either kept in red light (All-red LED Grow Light panel) or was irradiated with blue light using the All-blue LED Grow Light panel at the approximate irradiance of 10 W m⁻² for the duration of the assay. The reaction was started by the addition of ATP or GTP. Aliquots (50 μl) were withdrawn at different time points and boiled for 5 min. The precipitated protein was removed by centrifugation at 15,000 × g for 5 min. The supernatant was filtered through a 0.22-μm pore size filter (MicroSolv) and analyzed by reversed-phase HPLC.

**Nucleotide Detection**—Nucleotide mixtures from enzymatic assays were separated and analyzed by the reversed-phase HPLC (Summit HPLC System, Dionex, Sunnyvale, CA), 15 × 4.6-cm Supelcosil LC-18-T column (Sigma) using a gradient of the phosphate–methanol buffer system that was slightly modified from what was described earlier (31).

**Intra- and extracellular cAMP and cGMP levels in *E. coli* cells that expressed BlaC or BlgC were analyzed by using an ELISA kit (Biomol) according to the instructions of the manufacturer. For these assays, exponentially (*A₆₀₀ 0.6*) grown cultures were induced by the addition of 0–1% (pBAD-*blaC*) or 1% (pBAD-*blgC*) arabinose. After induction, cultures were grown in the dark for an additional 16 h. Cells were subsequently collected by centrifugation, resuspended in 200 μl of phosphate-buffered saline (28), placed on the top of a plastic surface, and either irradiated with blue light (10 W m⁻²) for 10 min or kept in the dark at room temperature. Cells were collected by centrifugation. Cell-free supernatant was used for analysis of extracellular cyclic nucleotides, whereas whole cell extracts obtained by sonication of precipitated cells were used for analysis of intracellular cyclic nucleotides.

**Spectroscopy**—Electronic absorption spectra were recorded with a UV–1601 PC UV-visible spectrophotometer (Shimadzu) at room temperature. Protein solutions (100 μl) in 10-mm light path quartz cuvette were irradiated directly in the spectrophotometer from the top of the cuvette. The light
Photoactivated Adenylyl and Guanylyl Cyclases

The BlaC Protein from Beggiatoa sp. PS Is a Blue Light-activated Adenylyl Cyclase—In the genome of the uncultured Gammaproteobacterium Beggiatoa sp. PS we found a gene (locus_tag BGP_1043) that could encode BlaC. The BlaC protein contains two domains: BLUF (PF04940) (38), representing sensors of blue light using FAD, and guanylate_cyc (PF00211) representing an adenylyl or guanylyl cyclase catalytic domain (Fig. 1A). The latter, catalytic domain is also known as cyclase homology domain (25, 26). Because the ability of bacteria to produce cGMP has never been convincingly demonstrated, we presumed that BlaC possesses adenylyl cyclase activity. The protein size, 350 amino acids, is much smaller than the sizes of α or β subunits of PAC from E. gracilis, which are approximately 1000 amino acids. This makes BlaC an attractive alternative to PAC, which is currently used in optogenetic applications involving manipulations of the cAMP levels (22, 23).

To test whether BlaC had the predicted function, we constructed the BL21(DE3) cya mutant, which lacks a native adenyl cyclase, and expressed the blaC gene from an arabinose-inducible promoter (plasmid pBAD-blaC). To improve protein expression, the blaC gene was synthesized based on the optimum E. coli codon usage. The lack of cAMP results in the inability of the BL21(DE3) cya mutant to up-regulate expression of the cAMP-CRP-dependent operons and prevents this strain from growing on minimal medium containing lactose. Expression of blaC restored growth of BL21(DE3) cya on MacConkey agar containing lactose and the inducer, 0.2% L-arabinose, only if the strain was grown in the light but not in the dark (Fig. 1B). The growth of BL21(DE3) cya expressing blaC could also be restored upon intermittent illumination with blue light emitted by a LED panel. Exposure to red light did not restore growth (not shown). This test confirmed the prediction that BlaC functions as a blue light-activated adenyl cyclase.

To demonstrate that BlaC can be used for high precision regulation of cAMP synthesis in vivo, we spread a lawn of BL21(DE3) cya [pBAD-blaC] cells on a Petri dish containing MacConkey agar plus lactose and covered the plate with thick black paper, leaving an image in the center of the plate exposed to intermittent irradiation with blue light. As expected, only cells exposed to blue light were able to grow (Fig. 1C). Thus, BlaC can be used to control cell behavior (E. coli growth in this instance) by light with high spatial resolution.

Purification and Biochemical Characterization of BlaC—We overexpressed BlaC in E. coli as an N-terminal His$_6$ fusion and as a C-terminal fusion to the MBP-His$_6$ tag. The His$_6$-BlaC fusion proved to be insoluble, regardless of overexpression conditions, and its purification was not pursued further. However, the MBP-His$_6$-BlaC was partially soluble, particularly when induced at low temperature (18 °C). We have purified this protein to >95% purity (Fig. 2A). Because removal of the MBP-His$_6$ tag resulted in protein aggregation, all subsequent characterization was done using the MBP-His$_6$-BlaC fusion (subsequently called BlaC for simplicity).

The protein, as purified, contained a mixture of flavins, FAD and FMN, as observed earlier (39). By extracting the cofactors and comparing protein and flavin amounts, we estimated that ~90% of the protein was in the holoform (not shown). Subsequent protein characterization was performed using proteins “as purified.”

The adenylyl cyclase activity was assayed using 5 μM protein at pH 8.0 at room temperature. Under these conditions, adenylyl cyclase activity in the dark was undetectable for ~30 min. Irradiation with blue light (10 W m$^{-2}$) increased activity by >100-fold (Fig. 2B). Precise calculation of the photoactivation fold was impossible because of the sensitivity of cAMP detection. The activity assays at different concentrations of
the substrate, ATP, yielded $K_m$ of 0.5 mM and $V_{max}$ of 57 nmol (mg of protein)$^{-1}$ min$^{-1}$ (Fig. 2C). Adenylyl cyclase activity was dependent on the intensity of blue light irradiation. At the highest irradiation levels used here, 10 W m$^{-2}$, the maximum enzymatic activity was not reached (Fig. 2D). The pH optimum for BlaC was found to be 9.5 (Fig. 2E). The temperature optimum of BlaC was found to be 45 °C (Fig. 2F). Such a high temperature optimum was somewhat unexpected for a bacterium isolated from the 4-m depth in the Baltic Sea (30). Under room temperature, BlaC had approximately one-fourth of its maximal activity. The small size, lack of activity in the dark, high photocactivity fold, relatively high specific enzymatic activity, and functionality in the range of physiological pH and temperatures compatible with various microbial and animal models make BlaC particularly attractive for optogenetic applications.

Photochemical Properties of BlaC—The electronic absorption spectrum of BlaC had a typical flavin spectrum. Upon irradiation, the spectrum underwent a 9-nm red shift, which is within the 5–10-nm range observed in BLUF domain photoreceptors characterized earlier (Fig. 3A).

After the light switch-off, flavin absorption recovery completely to the preirradiation dark state following single-exponential kinetics. At room temperature, half of the protein recovered back to the dark state by ~16 s (Fig. 3B). The fast return to the dark, inactive state is another important factor for an optogenetic probe that allows one to induce short, physiologically relevant cAMP impulses.

Importantly, prolonged exposure of BlaC to blue light at the highest intensity used here, 10 W m$^{-2}$, resulted in only a minor loss of the adenylyl cyclase activity (Fig. 3C). A high level of tolerance to continuous irradiation is yet another attractive feature of BlaC.

Conversion of BlaC into a Guanylyl Cyclase—The use of photoactivated guanylyl cyclases could greatly improve our understanding of cGMP signaling pathways. As no such enzymes currently exist, we set out to convert BlaC to a photoactivated guanylyl cyclase by changing its substrate specificity from ATP to GTP. A similar conversion, albeit in the opposite direction, from guanylyl to adenylyl cyclase, has been achieved earlier (40) by replacing as few as two amino acid residues.

To identify residues in BlaC involved in substrate binding, we performed a bioinformatics analysis of sequences and structures of adenylyl and guanylyl cyclase domains. An emphasis was placed on adenylyl or guanylyl cyclase domains from multidomain proteins of various architectures. This approach diminished conservation in the residues involved in inter- or intramolecular interactions while retaining the conserved residues essential for enzymatic activity.

We constructed a dataset of class III nucleotidyl cyclases consisting of three subsets: eukaryotic guanylyl cyclases (6 sequences), bacterial adenylyl cyclases (9 sequences), and a single putative bacterial guanylyl cyclase domain (Cya2 from Synechocystis sp. PCC 6803) (41). Each group has a representative three-dimensional structure, i.e. PDB 1WC5, 2W01, and 3ET6, respectively. The sequences of these proteins were aligned using MUSCLE (Fig. 4A and supplemental Fig. S1). A phylogenetic tree was constructed to confirm the subsets using PhyML (supplemental Fig. S2).

The multiple sequence alignment was analyzed to identify residues potentially involved in a functional shift between the
different subsets, e.g. residues that change substitution rate between the eukaryotic guanylyl cyclases and the bacterial adenylyl cyclases, or residues that change their physicochemical character between subsets. This analysis led us to three such residues in BlaC, i.e. Lys\(^{197}\), Asp\(^{265}\), and Thr\(^{267}\) (Fig. 4A).

All adenylyl cyclases in the selected subset have Lys at position equivalent to position 197 in BlaC, whereas all guanylyl cyclases sequences have Glu. At position 265, all adenylyl cyclases sequences have Asp or Glu, whereas all eukaryotic guanylyl cyclases have Arg, and the putative bacterial guanylyl cyclase has Lys. At position 267, all adenylyl cyclases have Thr, all eukaryotic guanylyl cyclases have a Cys, and the putative bacterial guanylyl cyclase has Gly.

We verified the significance of sequence analysis by performing structural analysis. The structure of a bacterial adenylyl cyclase with a bound ATP analog (1WC5) provided us with information about the residues located in the adenosine-binding pocket. A homology model of BlaC was built based upon that structure (Fig. 4B and supplemental Fig. S3). Importantly, the residues, whose importance was suggested by sequence analysis, were found to be located in the adenosine-binding pocket of BlaC (Fig. 4, C and D).

In addition to these three residues, position Ala\(^{277}\) (Fig. 4A), which is either a Ser or a Tyr in guanylyl cyclases, was added to the set of potential sites for site-directed mutagenesis. Based upon this analysis, the following individual mutations and combinations of mutations were engineered into the BlaC protein: K197E, D265K, D265R, T267G, T267C, and A277Y. All mutant proteins were overexpressed and purified, and their adenylyl and guanylyl cyclase activities were tested in vitro.

The single mutations, K197E or D265K, abolished adenylyl cyclase catalytic activity and did not result in any guanylyl cyclase activity (Fig. 5). Inspection of the guanylyl cyclase structures revealed that the original residues at these positions interact at the dimer interface. Hence, mutation of single residues might have resulted in electrostatic repulsion (Glu\(^{197}\) and Asp\(^{265}\) or Lys\(^{197}\) and Lys\(^{265}\)) between monomers. A double mutant, K197E/D265K, on the other hand, gained some guanylyl cyclase activity while retaining significant adenylyl cyclase activity (Fig. 5). Simultaneous mutations of both K197E and D265K were necessary for guanylyl cyclase activity as evident from analysis of the following mutants: K197E/T267G, K197E/A277Y, D265K/T267G, and D265K/A277Y (Fig. 5). The single or double mutations, T267G or A277Y, resulted in lower adenylyl cyclase activity but no gain in guanylyl cyclase activity (Fig. 5).

A combination of three mutations, K197E/D265K/T267G, resulted in a mutant protein with high guanylyl cyclase activity and residual adenylyl cyclase activity, at \(\sim 10\%\) of the guanylyl cyclase activity (Fig. 5). In contrast, a triple mutant, K197E/D265K/T267C, had lost guanylyl cyclase activity of the double mutant, K197E/D265K (Fig. 5). Addition of another mutation, H266Y, to restore the conserved three-amino acid stretch characteristic of eukaryotic guanylyl cyclases, 265DHY→RYC, proved to be nonproductive (Fig. 5). A triple mutant containing D265R as opposed to D265K, i.e. K197E/D265R/T267G, where Arg is a conserved residue in eukary-
Biochemical Characterization of BlgC—We purified MBP-His6-BlgC (subsequently referred to as BlgC) to >95% purity (Fig. 6A) and assayed its enzymatic activity in a manner similar to that of BlaC. Guanylyl cyclase activity of BlgC was undetectable in the dark and increased upon irradiation by approximately 2 orders of magnitude, i.e., the introduced mutations did not affect photoactivation properties of the protein (Fig. 6B).

The three introduced mutations drastically increased affinities of BlgC for ATP and GTP, compared with BlaC, i.e., $K_{m(GTP)}~25 \mu M$ and $K_{m(ATP)}~10 \mu M$. At relatively low substrate concentrations, $V_{max}$ for the guanylyl compared with adenylyl cyclase reaction was ~7-fold higher, i.e., 19.5 versus 2.6 nmol (mg of protein)$^{-1}$ min$^{-1}$ (Fig. 6C and D). Therefore, the predominantly guanylyl cyclase activity of BlgC is due to the higher turnover rate of GTP compared with ATP and despite the lower affinity for GTP compared with ATP.

We found that high, millimolar concentrations, GTP and ATP strongly inhibited guanylyl cyclase and residual adenylyl cyclase activities of BlgC (Fig. 6, C and D). Under our experimental conditions, adenylyl cyclase activity of BlgC in vitro was no longer detectable at 4 mM ATP (Fig. 6C). The significance of this observation for the performance of BlgC in vivo is discussed in the following section. The pH and temperature dependence of the guanylyl cyclase activity of BlgC were similar to those of BlaC (Fig. 6, E and F).

Blac Functions as a Photoactivated Adenylyl Cyclase in E. coli—We tested performance of BlaC and BlgC in vivo using E. coli BL21(DE3) cya as a model. We induced BlaC expression in BL21(DE3) cya [pBAD-blaC] for 16 h in the dark with 0–1% arabinose, collected cells, resuspended them in a small volume, and irradiated them with blue light at 10 W m$^{-2}$ (or kept them in the dark) for 10 min at room temperature. We measured cAMP and cGMP levels directly using a sensitive ELISA. E. coli has been reported to excrete most cAMP into the medium (42). We confirmed that, following irradiation of BL21(DE3) cya [pBAD-blaC], the extracellular cAMP concentration was 10-fold higher than the intracellular concentration (data not shown). Interestingly, extracellular and intracellular cGMP concentrations were similar, which likely reflects less efficient cGMP efflux in E. coli, compared with the cAMP efflux. Because measuring extracellular cyclic nucleotide levels proved more sensitive, we focused on these measurements.

The readings of the BL21(DE3) cya strain containing an empty vector corresponded to ~0.30 pmol of cAMP or cGMP/10⁶ cells (Table 1). Because BL21(DE3) cya produces neither CAMP nor cGMP, these values represent nonspecific background. Expression of BlaC at low levels (induction with 0–0.1% arabinose) in the dark did not affect the background levels (Table 1), which is consistent with the lack of adenylyl cyclase activity of BlaC in the dark in vitro (Fig. 2B). Irradiation of cells with blue light increased extracellular cAMP levels to 2.9, 13.0, or 36.3 pmol cAMP/10⁶ cells at 0, 0.05, or 0.1% arabinose, respectively (Table 1). Therefore, one can photoactivate cAMP levels in E. coli by at least 2 orders of magnitude without increasing the background (dark) cAMP concentrations.
When BlaC was expressed at higher levels (induction with 0.2 and 1% arabinose), the undesirable cAMP synthesis in the dark became measurable (Table 1). We detected no contaminating guanylyl cyclase activity of BlaC even at the highest expression levels tested (Table 1).

**BlaC** Functions as a Photoactivated Guanylyl Cyclase in *E. coli*—We tested the performance of BlgC in *E. coli* in a manner similar to that of BlaC. First, we investigated whether the residual adenylyl cyclase activity of BlgC observed in vitro (Fig. 5) was sufficient to sustain growth of BL21(DE3) cya [pBAD-blgC] on MacConkey agar plus lactose. We found that it was not sufficient, whether the plates were grown in the dark or light, even when pBAD-blgC was induced with 1% arabinose (not shown).

We then proceeded to measure cAMP and cGMP synthesis in BL21(DE3) cya [pBAD-blgC] in the dark and light. A 10-min irradiation with blue light of BL21(DE3) cya [pBAD-blgC] resulted in the extracellular cGMP levels of 4 pmol of cGMP/10⁹ cells, whereas cGMP levels in the dark remained undetectable (Table 1). Therefore, BlgC can be photoactivated in vivo by at least 12-fold.

We detected no cAMP despite the relatively high expression of BlgC (1% arabinose) and much more sensitive cAMP detection compared with the detection of cGMP. Therefore, BlgC functions as a specific, photoactivated guanylyl cyclase in *E. coli*. The lack of cGMP signaling pathways in *E. coli* prevented us from exploring how BlgC might affect cell behavior.

**DISCUSSION**

In this study, we characterized a novel, bacterial photoactivated adenylyl cyclase, BlaC, and engineered a photoactivated guanylyl cyclase, BlgC. BlaC originates from a filamentous sulfur proteobacterium, *Beggiatoa* sp. PS, where its function is unknown. Our analysis revealed that BlaC possesses several properties that make it highly desirable for optogenetic applications. BlaC has a relatively small size, 350 amino acids, and uses flavin chromophores (FAD and/or FMN) present in all cell types. The adenylyl cyclase activity of BlaC in the dark is practically nonexistent in vitro (Fig. 2B) and in vivo (E. coli) when the protein is expressed at relatively low levels (Fig. 1B and Table 1). The extent of photoactivation of BlaC can be readily adjusted by manipulating the expression level of BlaC (Table 1) as well as the intensity and duration of irradiation (Fig. 2D). Importantly, BlaC is very tolerant to prolonged exposure to light (Fig. 3C).

Our photoactivation experiments in *E. coli* showed that cAMP levels can be increased by >2 orders of magnitude without undesirable dark activity (Table 1). Another advantageous property of BlaC is that the half-life of its lit state is short, i.e. 16 s at room temperature (Fig. 3B). This means that one can induce short spikes in cAMP synthesis, which is important for studying cAMP-dependent signal transduction pathways in vivo. It is also noteworthy that BlaC can operate in a range of temperatures and at physiological pH (Fig. 2, E and F), which makes it applicable for a variety of microbial and animal models.

Currently, no photoactivated guanylyl cyclases exist. To convert BlaC to a guanylyl cyclase, we reengineered its substrate-binding site. Our engineering efforts were facilitated by the available x-ray structures of eukaryotic and bacterial adenylyl and guanylyl cyclases. A combination of three mutations at the substrate-binding pocket (K197E/D265R/T267G) was sufficient to turn BlaC into BlgC, which has predominantly guanylyl cyclase activity. Interestingly, the affinity of BlgC for GTP is somewhat lower than that for ATP, whereas the rate of the guanylyl cyclase reaction is several-fold higher than that of the adenylyl cyclase reaction. Therefore, it is the kinetic factor, not substrate specificity, that determines the prevailing enzymatic activity of BlgC. Affinities comparable with those of GTP and ATP have been reported in native guanylyl cyclases, *e.g.* mammalian soluble guanylyl cyclase (43). The predominantly guanylyl cyclase activity of the catalytic domain from the putative bacterial guanylyl cyclase Cya2 also stemmed from more efficient GTP turnover (41).

Both guanylyl and residual adenylyl cyclase activities of BlgC are strongly inhibited by millimolar concentrations of ATP and GTP. The reason for this inhibition remains to be explored. In *E. coli* grown exponentially in a glucose-containing medium, intracellular ATP and GTP concentrations are ~10 mM ATP and 5 mM GTP (44). In cells grown at less than maximum growth rates, intracellular ATP and GTP levels are in the lower millimolar range (11). The inhibition by millimolar substrate concentrations explains why, even at relatively high expression of BlgC in the irradiated *E. coli* cells, cGMP formation was not detected, since cAMP synthesis was always the predominant process. While cAMP is a well-studied second messenger, guanylyl cyclase activity cannot be as easily exploited in vivo because the levels of the second messenger cGMP are generally much lower than cAMP levels in bacteria. We are currently investigating a means to increase the intracellular concentrations of cGMP, making it more feasible to induce cell behavior using BlgC as a tool.
was accumulated to modest levels (compared with the levels of cAMP produced by BlaC) (Table 1).

Photoactivation of the guanylyl cyclase activity of BlgC in E. coli was >12-fold. Given that in vitro BlgC is photoactivated by 2 orders of magnitude (Fig. 6B), similar to the photoactivation fold of BlaC (Fig. 2B), and that photoactivation of BlaC in vitro and in vivo was similar, it is likely that BlgC is also photoactivated in vivo by 2 orders of magnitude.

BlgC possesses ~10% residual adenyl cyclase activity in vitro (Fig. 5). This contaminating activity is undesirable for specific activation of cGMP-signaling pathways in vivo. Because intracellular concentrations of ATP are 2- to severalfold higher than those of GTP and because BlgC is inhibited by the physiological ATP levels stronger than by the physiological GTP levels (Fig. 6, C and D), the 10:1 guanylyl:adenylyl cyclase ratio displayed by BlgC in vitro is likely to be much higher in vivo. Consistent with this expectation, cAMP synthesis in vivo was 12-fold. Given that 10% residual adenylyl cyclase activity in vivo (Table 1).

Therefore, BlgC can be used as a specific photoactivated guanylyl cyclase in vivo. In conclusion, we suggest that both native photoactivated adenyl cyclase, BlaC, and engineered guanylyl cyclase, BlgC, can be used as optogenetic tools to manipulate cAMP and cGMP signaling pathways or to generate synthetic cAMP- or cGMP-dependent signaling cascades in various model systems.

Acknowledgment—We are thankful to Kurt Miller for critical reading of the manuscript.

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