We recently described the cikA (circadian input kinase A) gene, whose product supplies environmental information to the circadian oscillator in the cyanobacterium Synechococcus elongatus PCC 7942. CikA possesses three distinct domains: a GAF, a histidine protein kinase (HPK), and a receiver domain similar to those of the response regulator family. To determine how CikA functions in providing circadian input, we constructed modified alleles to tag and truncate the protein, allowing analysis of each domain individually. CikA covalently bound bilin chromophores in vitro, even though it lacks the expected ligand residues, and the GAF domain influenced but did not entirely account for this function. Full-length CikA and truncated variants that carry the HPK domain showed autophosphorylation activity. Deletion of the GAF domain or the N-terminal region adjacent to GAF dramatically reduced autophosphorylation, whereas elimination of the receiver domain increased activity 10-fold. Assays to test phosphorylation from the HPK to the cryptic receiver domain, which lacks the conserved aspartyl residue that serves as a phosphoryl acceptor in response regulators, were negative. We propose that the cryptic receiver is a regulatory domain that interacts with an unknown protein partner to modulate the autokinase activity of CikA but does not work as bona fide receiver domain in a phosphorelay.

The wide variety of organisms in which biological clocks have been described share the important feature that their endogenous clocks can be set to “local time” by external environmental signals such as light and temperature (1–4). Whereas light perception to reset the circadian clock in mammals, mice, and Drosophila is likely mediated via opsins and cryptochromes (5–7), plants such as Arabidopsis thaliana receive clock-setting information through cryptochromes and phytochromes that work synergistically (8). Cryptochromes are blue light photoreceptors that most likely evolved from DNA photolyases. They bind two chromophores, flavin-adenine dinucleotide, and a pterin (methenyltetrahydrofolate) or a deazaflavin (7,8-didehydro-8-hydroxy-5-deazariboflavin). The latter functions in light perception by transducing excitation energy to flavin-adenine dinucleotide, which then catalyzes an electron transfer reaction in the DNA photolyase (5, 9, 10). Plant phytochromes are red/far-red photoreceptors that bear linear tetrapyrrole (bilin) chromophores (11, 12) attached to an N-terminal sensory domain known as a GAF domain (13, 14). Plant phytochromes also have sequence similarity to histidine-protein kinases (HPKs) but instead possess serine/threonine (S/T) kinase activity (15, 16). Members of the phytochrome family have also been identified in prokaryotes, including cyanobacteria (17–19), purple photosynthetic bacteria (20, 21), and nonphotosynthetic eubacteria (22). Many of these proteins exhibit a similar domain structure to plant phytochromes with an N-terminal bilin-binding domain that is associated with an HPK module (23). The HPK domain of cyanobacterial Cph1 has been shown to have authentic HPK activity (19).

We recently described the cikA gene of the cyanobacterium Synechococcus elongatus PCC 7942, which encodes a phytochrome-related protein with similarity to HPKs of bacterial two component signal transduction systems (24–26) and is involved in signal perception for resetting the circadian clock in response to environmental cues (27). CikA was first identified from a Tn5 mutant that has subtle alterations in light-responsive regulation of the photosystem II gene psbAII (28). The mutant has an expression level of a psbAII::luxAB reporter fusion strain that is 50–80% of that in wild type (WT) under low light conditions and exhibits an exaggerated increase in expression upon exposure to higher light intensities. However, a more striking circadian phenotype was observed, because both the period length and amplitude of circadian rhythms of different luxAB reporter strains (kaiB::luxAB, purF::luxAB, and kaiA::luxAB) were reduced (27, 29, 30). In addition, the relative phasing of expression (timing of circadian peaks) was altered for some genes. The additive effect on period length reduction of cikA inactivation in both short and long period kai mutants of S. elongatus suggested that CikA and the Kai proteins have independent, nonoverlapping functions. A lack of phase resetting in the CikA-free mutant in response to a 5-h
dark pulse that resets WT revealed that CikA functions as a sensor component on the input pathway of the circadian clock. The deduced amino acid sequence of cikA reveals it to be a member of the extended phytochrome family (27). CikA exhibits a typical bacteriophytochrome domain structure with an N-terminal GAF domain (13) and a C-terminal histidine kinase domain (26). The C-terminal region of CikA has similarities to receiver domains of response regulators (RR) such as PhoB. However, the mode of action of this RR domain may be different, because the conserved Asp residue necessary for phosphoryl transfer is missing (24–27). In this regard, the CikA structure is similar to the family of so-called pseudo-response regulators (APRR) that has been identified in A. thaliana (35–38). APRRs also possess N-terminal CONSTANS “Myb-related” motifs, which regulate transcriptional activity in eukaryotes, in addition to their pseudo-receiver domains. In contrast to the authentic response regulator proteins of A. thaliana, APRRs lack two of three invariant residues of receiver domains, which apparently prevents them from accepting phosphoryl groups from A. thaliana histidine kinases in vitro (35). APRR mutants also exhibit significant circadian phenotypes, such as delayed flowering and shortened periods of several rhythmic markers, including expression of the CAB (chlorophyll a/b-binding protein) gene (37, 38).

To determine whether CikA is an input photoreceptor to the cyanobacterial circadian clock and to clarify its mode of action, we made a variety of plasmid constructs for expression of truncated and full-length and truncated CikA variants in S. elongatus. We also made a variety of plasmid constructs for expression of His6- and Trx-tagged CikA and truncated versions of CikA in E. coli (42, 43). The assays to test for transphosphorylation from His6-tagged proteins. A PCR-derived fragment using primers AM0558 and F0748, encoding the entire cikA gene except the first methionine, was cloned into SacI/SalI-digested pQE30/80L expression vector and used to transform E. coli SG13009. The cells were grown to OD600 nm = 0.5, induced with 0.9 mM IPTG, and grown for an additional 3–5 h at 25°C to minimize the formation of insoluble protein. All of the cells were harvested, washed and passed twice through a prechilled French press at 137.9 MPa. This extract was clarified by centrifugation for 30 min at 12,000 × g to prepare a lysate for affinity purification on nickel-nitrilotriacetic acid (Ni-NTA) matrix (Qiagen). For expression of truncated versions of CikA, we used the pET overexpression system (Novagen), generating fusion proteins that carry an N-terminal thioredoxin domain (Trx) for enhanced solubility as well as N- and C-terminal His6 tags for purification by a Ni-NTA matrix (Table II).

**Expression and Purification of CikA**—To determine whether CikA is an input photoreceptor to the cyanobacterial circadian clock and to clarify its mode of action, we made a variety of plasmid constructs for expression of truncated and full-length and truncated CikA variants in S. elongatus and in E. coli. After applying a 12-h dark pulse to the cultures to synchronize the circadian clock, the psbA::luxAB and psbC::luxAB reporter strains were incubated in continuous light, and bioluminescence was measured using a Packard TopCount luminometer (Packard Instrument Company, Meriden, CT) (29). Period length and amplitude were calculated using IKA and FPT-NLLS software (www.scripps.edu/cbksiyahareware/) (29).

**Z PET Autophosphorylation Assays**—CikA autophosphorylation assays were done as previously described (42, 43), except that gels were dried for exposure to phosphorimaging plates in most cases. Briefly, the standard phosphorylation buffer stock to which purified protein was added was 50 mM Tris, pH 7.5, 100 mM KCl, 2 mM dithiothreitol, 0.1 mM ATP (unlabeled), and 0.15 μM [γ-32P]ATP. Chemical stability measurements were performed as described by McCleary et al. (42, 43) with minor modifications. The assays to test for transphosphorylation from 32P-labeled CikA6HIS or Trx-ARR (Table II) to the receiver domain of CikA were done according to previously published protocols (44). The reactions were quantitated using a Fujix BAS 2000 phosphorimaging system.

**In Vitro Bilin Lyase Assays with Purified CikA**—The assays were performed according to a previously published protocol with purified phyocyanobilin, phytochromobilin, and phycocerythrin (22, 35). Biliverdin was obtained as described previously (45). After passage through a prechilled French press at 137.9 MPa, a cleared lysate was prepared by centrifugation at 100,000 × g for 60 min. CikA6HIS in the soluble protein fraction was diluted to 200 μl in Buffer 1. Protein was bound to Ni-NTA-agarose (Qiagen), washed, and eluted with Buffer 1 that contained 50 and 250 mM imidazole, respectively.

**Assay of Biomollients in 96-well Microtiter Plates**—Inocula of AMC913, AMC914, AMC1006, and the corresponding control strains were streaked from solid medium onto 300-μl BG-11 M agar pads in 96-well plates. After applying a 12-h dark pulse to the cultures to synchronize the circadian clock, the psbA::luxAB and psbC::luxAB reporter strains were incubated in continuous light, and bioluminescence was measured using a Packard TopCount luminometer (Packard Instrument Company, Meriden, CT) (29). Period length and amplitude were calculated using IKA and FPT-NLLS software (www.scripps.edu/cbksiyahareware/) (29).

**RESULTS**

**Complementation of a cikA Mutant with an Affinity Tagged Allele**—We constructed a 6-histidine tag-encoding full-length allele of cikA (cikA_att) in the IPTG-inducible expression vector pQE30/80L to express, purify, and characterize recombinant CikA in both E. coli and S. elongatus (Table II). To verify that the tagged allele has equivalent functions to WT CikA, the *cikA_att* gene was introduced into the *S. elongatus* CikA-free...
### CikA Activities

#### Table I

| Strains | Plasmids for transformations | Marker | Characteristics of plasmids and strains | Source |
|---------|-----------------------------|--------|----------------------------------------|--------|
| S. elongatus |                           |        |                                        |        |
| PCC 7942 |                            |        |                                        |        |
| AM064 | pAM1583, pAM1850 | Km, Cm | basic wild type strain | Lab collection |
| AM068 | pAM1583, pAM1850, pAM2152 | Km, Cm, Gm | cikA, pshA::luxAB NS2.2, pshB::luxCDE NS2.1 | Lab collection |
| AM069 | pAM2195 | Cm | pshB::luxABCDCE, NS2.2 | Lab collection |
| AM071 | pAM1583, pAM2256, pAM2247 | Cm, Km, Gm, Sp | cikA, pshA::luxAB NS2.2, pshB::luxCDE NS2.1, cikA<sub>mut</sub> NS1 | This study |
| AM074 | pAM1583, pAM1850, pAM2152 | Cm, Km, Gm | cikA, pshA::luxAB, pshB::luxCDE, cikA<sub>mut</sub> NS1; promoter structure: P<sub>laq</sub>-OP-P<sub>plac</sub>-OP-cikA<sub>mut</sub> | This study |
| AM075 | pAM2195, pAM2448 | Cm, Km, Sp | cikA, pshA::luxAB, pshB::luxCDE, cikA<sub>mut</sub> NS1; promoter structure: P<sub>laq</sub>-OP-P<sub>plac</sub>-OP-cikA<sub>mut</sub> | This study |
| AM077 | pAM1580 | Cm | pshA::luxCDE NS2.1 | This study |
| AM079 | pAM1580, pAM2256 | Cm, Km | cikA, pshA::luxAB NS2.2, pshB::luxCDE NS2.1 | This study |
| AM081 | pAM1580, pAM2256, pAM2448 | Cm, Km, Sp | cikA, pshA::luxAB NS2.2, pshB::luxCDE NS2.1, cikA<sub>mut</sub> NS1; promoter structure: P<sub>laq</sub>-OP-P<sub>plac</sub>-OP-cikA<sub>mut</sub> | This study |
| AM083 | pAM1580, pAM2256, pAM2448, pAM2761 | Cm, Km, Sp | cikA, pshA::luxAB NS2.2, pshB::luxCDE NS2.1, cikA<sub>mut</sub> NS1; promoter structure: P<sub>laq</sub>-OP-P<sub>plac</sub>-OP-cikA<sub>mut</sub> | This study |

| E. coli |               |        |                                        |        |
|---------|----------------|--------|----------------------------------------|--------|
| AM1843 | pREP4 | Km | Qiagen E. coli overexpression strain SG13009; contains pREP4 plasmid | Qiagen |
| AM2255 | p322-Ptec | Ap | Plasmid used to overexpress genes from P<sub>laq</sub>; includes laq | H. Iwasaki (Nagoya University) |
| AM2314 | pAM2314 | Sp | basic NS1 cloning vector | Lab collection |
| AM2420 | pAM2420 | Ap, Km | PCR amplified cikA fragment (AM0558 and F0748), encoding full-length CikA<sub>mut</sub> cloned into SacI–SalI-digested pQE30<sup>Δ</sup>; also carries pREP4 | This study |
| AM2428 | pAM2428 | Sp | NS2 overexpression cloning vector; blunt end BgII fragment from pAM255 cloned into Smal-I cut pAM2314; unique Smal site downstream of P<sub>laq</sub> promoter | This study |
| AM2448 | pAM2448 | Sp | XhoI–HindIII fragment from pAM2420, encoding CikA<sub>mut</sub> plus T5 promoter and lacI operator cloned into Smal site of pAM2428; used for complementation of cikA in pshA::luxAB pshB::luxCDE reporter background (AM0568) | This study |
| AM2477 | pAM2477 | Sp | Blunted MfeI–HindIII fragment from pAM2420 cloned into Smal-digested pAM2428 (NS1) for complementation of cikA mutation in a pshA::luxAB pshB::luxCDE reporter background | This study |
| AM2478 | pAM2478 | Ap, Km | cikA SacI–SalI fragment from pAM2420, encoding full-length CikA<sub>mut</sub> cloned into SacI–SalI cut pQE30<sup>Δ</sup>, pREP4 (laq) | This study |
| AM2760 | pAM2760 | Ap | PCR-amplified 1.8-kb cikA fragment (AM0558–AM0543) cloned into SacI–XhoI cut pET32a<sup>Δ</sup> (+), encoding ΔRR | This study |
| AM2761 | pAM2761 | Ap | PCR-amplified 0.5-kb cikA fragment (AM0542–F0748) cloned into BamHI–SalI cut pET32c<sup>Δ</sup> (+), encoding Trx-RR | This study |
| AM2763 | pAM2763 | Ap | Novagen<sup>Δ</sup> strain for protein overexpression; contains chromosomal copy of T7 polymerase gene under control of IPTG inducible promoter; suitable as host for vectors like pET22a<sup>Δ</sup>-c(+) with T7 lac promoter | Novagen<sup>Δ</sup> |
| AM2803 | pAM2803 | Ap | PCR-amplified cikA fragment (AM0558–AM0559) cut with SacI–XhoI and cloned into pET32a<sup>Δ</sup> (+), encoding ΔHPK | This study |
| AM2804 | pAM2804 | Ap | PCR-amplified cikA fragment (AM0558–AM0560) cut with SacI–XhoI and cloned into pET32a<sup>Δ</sup> (+), encoding ΔHPK | This study |
| AM2932 | pAM2932 | Ap | 2.2-kb NruI–HindIII fragment of pAM2478 cloned into pAM2798 at NruI–HindIII sites, encoding Trx-CikA | This study |
| AM2933 | pAM2933 | Ap | 1.6-kb BgIII–BamHI fragment of pAM2478 cloned into pAM2761 at BgIII–BamHI sites, encoding ΔN | This study |
| AM2934 | pAM2934 | Ap | pAM2932 derivative from which GAF domain has been removed at positions 1051–1515 of pAM2932 by the method of Taylor and Eckstein (see “Experimental Procedures”) with AMO693 and AMO694, encoding ΔGAF | This study |
| AM2935 | pAM2935 | Ap | pAM2084 derivative from which a 0.6-kb BgIII fragment was removed and the remaining plasmid self-ligated, encoding Trx-GAF | This study |
| AM2936 | pAM2936 | Ap | pAM2478 derivative produced by the method of Taylor and Eckstein (see “Experimental Procedures”) with AMO697 and AMO698, containing a point mutation (Ala to His) at amino acid number 407 in CikA<sub>HIS</sub> (corresponds to WT His<sup>407</sup>) | This study |
| AM2937 | pAM2937 | Ap | 2.2-kb NruI–HindIII fragment from pAM2936 cloned into pAM2798 at NruI–HindIII sites, encoding Trx–H393A | This study |
light-regulated relative phasing of circadian peaks for some genes, such as the AMC669, to assess the circadian phenotypes. The cikA6HIS, and luxAB::psbAI::psbAII::cikA

cation of expression from reporter genes, types (27). These include alteration of period length (shortened mutant strain AMC568, which carries a psbAI::luxAB reporter gene. Previous work had shown that the insertional inactivation of cikA in strain AMC568 leads to clear circadian phenotypes (27). These include alteration of period length (shortened by about 2 h) and reduction in amplitude of the oscillation of expression from reporter genes, e.g. kaiB::luxAB, purF::luxAB, psbAI::luxAB, and psbAII::luxAB, as well as a change in the relative phasing of circadian peaks for some genes, such as the light-regulated psbA family. A construct that bears the cikA<sub>His</sub> allele (pAM2477: p<sub>Trc</sub>-OP-OP- cikA<sub>His</sub>) was introduced into strain AMC568 via a neutral site I-targeting overexpression vector pAM2428 (p<sub>r</sub>, and laqI regulation system) (Table I). We monitored bioluminescence from the complemented strain, AMC913 (pAM2477), along with the parent strain AMC568 and the WT psbAI::luxAB reporter strain AMC669, to assess the circadian phenotypes. The cikA<sub>His</sub> allele complemented the cikA phenotype with respect to period length and amplitude (compare traces from of AMC568 and AMC913 without IPTG; Fig. 1.). Based on these experiments, the following period lengths were calculated: (i) AMC568: 0.34 h, n = 8; (ii) AMC669: 25.24 ± 0.23 h, n = 8; and (iii) AMC913 without IPTG: 23.85 ± 0.35 h, n = 8. Because period length was altered in AMC913 in the absence of inducer, transcriptional repression of cikA<sub>His</sub> appears to be leaky despite the presence of twin LaqI operator sequences; the leaky expression was sufficient to complement the cikA phenotype. Induction of CikA<sub>His</sub> expression in AMC913 by adding 2 mM IPTG led to an arrhythmic phenotype of CikA<sub>His</sub> in strain AMC1006 also led to arrhythmic bioluminescence (data not shown).

TABLE I—continued

| Oligonucleotides | Sequence 5' to 3' | Characteristics | Source |
|-----------------|-------------------|-----------------|--------|
| AMO558          | GTTTTGCCGTGACCTCGAACACATC | upstream cikA primer with SacI site, replaces cikA ATG start codon in all overexpression constructs downstream cikA primer with SacI site, includes native stop codon | This study |
| F0748           | GACATTCGTCCATCGAGCACAG | with F0748 amplifies 500-bp fragment, which encodes N-terminally truncated version of CikA (CikA-Rt) in AM2761; contains mutated BamHI site for cloning into pET32a(+)′ and pET32a(+)′ sequencing primer | This study |
| AMO542          | CAGTCGGATCCCGGACAA | with AMO558 amplifies 1870-bp fragment, which encodes C-terminally truncated version of CikA in AM2780 (CikA-CBD-HKD); contains mutated XhoI site for cloning into pQE80 or pET32a(+)′ sequence site, includes | This study |
| AMO543/548      | TTTCGAGGGAGGCTAGAG | with AMO558 amplifies 1870-bp fragment, which encodes C-terminally truncated version of CikA in AM2780; contains mutagenized XhoI site for cloning into pET32a(+)′ | This study |
| AMO556          | CAGGGGCTCTCGGTATGA | with AMO558 amplifies 1870-bp fragment, which encodes C-terminally truncated version of CikA in AM2780; contains mutagenized XhoI site for cloning into pET32a(+)′ | This study |
| AMO559          | GTTCATGACTCGAGGTGGGACC | with AMO558 amplifies 1870-bp fragment, which encodes C-terminally truncated version of CikA in AM2780; contains mutagenized XhoI site for cloning into pET32a(+)′ | This study |
| AMO560          | CTGATCTTGAAGCTTACCTAGAG | with AMO558 amplifies 1870-bp fragment, which encodes C-terminally truncated version of CikA in AM2780; contains mutagenized XhoI site for cloning into pET32a(+)′ | This study |
| AMO693          | CAAATTCGTCTCAGTTCTCTAC | forward primer of fusion sequence from positions 1033–1050 and positions 1516–1533 of Trx-CikA open reading frame in pAM2932, used for a deletion of GAF domain | This study |
| AMO694          | GAAAATGCGGCTAGGGAGTAAC | reverse primer of fusion sequence from positions 1033–1050 and positions 1516–1533 in Trx-CikA open reading frame in pAM2932, used for a deletion of GAF domain | This study |
| AMO698          | GGCCACGATCGCAGCAGCACTGAGC | forward primer to introduce a point mutation of His to Ala at amino acid number 407 in CikA<sub>His</sub> reverse primer to introduce a point mutation of His to Ala at amino acid number 407 in CikA<sub>His</sub> | This study |
| AMO697          | CTGTCGGATTGCGCGCACTGCTGCC | forward primer to introduce a point mutation of His to Ala at amino acid number 407 in CikA<sub>His</sub> reverse primer to introduce a point mutation of His to Ala at amino acid number 407 in CikA<sub>His</sub> | This study |

*a* Neutral site II (GenBank<sup>TM</sup> accession U44761) cloning sites NS2.1 (BstEII) and 2.2 (BglII).

*b* Neutral site I (GenBank<sup>TM</sup> accession U40322).

*c* Qiagen, Valencia, CA.

*d* Novagen, Madison, WI.

**Table II**

Characteristics of variant CikA proteins

Domains in native CikA were defined as follows: (i) GAF domain: aa 184–338; (ii) HPK domain: H-box aa 393, N-box aa 498–518, D/F-box aa 538–562; and (iii) RR domain: aa 630–689.

| Name     | Strain | Encoded amino acids of native CikA | Location of His tag |
|----------|--------|-----------------------------------|---------------------|
| CikA<sub>His</sub> | AM2478 | 2–754 | N terminus |
| Trx-CikA | AM2932 | 2–754 | N terminus |
| ΔRR      | AM2760 | 2–621 | Twin tag |
| ΔTP      | AM2798 | 2–419 | N terminus |
| ΔHPK     | AM2803 | 2–390 | N terminus |
| N + GAF  | AM2404 | 2–350 | Twin tag |
| ΔN       | AM2933 | 184–754 | N terminus |
| ΔGAF     | AM2934 | 2–183, 399–754 | N terminus |
| Trx-RR   | AM2761 | 606–754 | N terminus |
| Trx-GAF  | AM2935 | 184–350 | Twin tag |
| Trx-H393A | AM2937 | 2–754 | N terminus |
Chromophore Ligation Assays with Various Substrates—We assayed in vitro ligation of bilin chromophores to full-length CikA<sub>6HIS</sub> and a number of variants to determine whether the GAF-like domain of CikA can autocatalytically attach a bilin chromophore, indicating lyase activity. Table II and Fig. 2 describe the proteins used in this study. The proteins were overexpressed in E. coli and affinity-purified by His<sub>6</sub> tags. Truncated versions of CikA required, additionally, a Trx tag at the N terminus to improve solubility, even though the full-length protein is soluble when expressed with only a His<sub>6</sub> tag. Zinc blot analysis, which detects tetrapyrroles, gave clear fluorescent signals for CikA<sub>6HIS</sub> with phytochromobilin (P<sub>B</sub>B) and PCB chromophores (Fig. 3). The fluorescence with these substrates was well above the background obtained when the protein was incubated with Me<sub>3</sub>S<sub>4</sub> alone as a control, as well as above that detected with either BV or phycocyanobilin, to which CikA<sub>6HIS</sub> does not appear to attach covalently (Fig. 3).

Assaying GAF as a Chromophore Attachment Domain—CikA<sub>6HIS</sub>. Thioredoxin-tagged CikA (Trx-CikA) and its variants were assayed for chromophore ligation with purified PCB in vitro. In this experiment, we decreased the PCB concentration to 2.0 μM to reduce nonspecific binding. As shown in Fig. 4 (A and B), recombinant proteins that included the GAF domain (CikA<sub>6HIS</sub>, Trx-CikA, ΔRR, ΔATP, ΔHPK, N+GAF, and ΔN) emitted obvious zinc-induced fluorescence, indicating the presence of protein-chromophore interaction. However, the ΔGAF variant had a reduced signal, only slightly higher than background negative controls (Fig. 4B). We concluded that GAF, but no other single motif, is important for bilin attachment. However, a Trx-GAF variant, composed of only GAF and the Trx tag, produced no signal above that obtained with a Trx-RR, in which the only the receiver-like domain of CikA is fused to Trx (Fig. 4C). These results suggest that the GAF domain we defined is not sufficient for bilin lyase activity or that the domain does not fold stably when removed from its natural protein context.

Although lyase assays showed that CikA can bind a bilin in vitro, CikA<sub>6HIS</sub> isolated from S. elongatus gave no fluorescent signal on a zinc blot (Fig. 4D) and showed no spectral evidence of a chromophore (data not shown). This negative result was obtained whether the protein was isolated from cells under normal illumination or in darkness. We reasoned that CikA might associate noncovalently with a bilin in vivo and that the co-factor might be lost during purification. However, co-expression of the protein with either PCB or BV in E. coli, under conditions in which other bacteriophytochromes form photoactive adducts (46, 47), showed no evidence of association between CikA and the chromophores (Fig. 5). We concluded from these various lines of data that CikA is unlikely to form a biliprotein complex naturally in S. elongatus.  

Autophosphorylation Assays with Overexpressed and Partially Purified CikA<sub>6HIS</sub>—We performed in vitro autophosphorylation assays with affinity-purified CikA<sub>6HIS</sub> apoprotein under various conditions to test whether the protein has authentic HPK activity as predicted from its sequence. CikA<sub>6HIS</sub> showed strong autophosphorylation, with a temperature optimum around 25 °C and detectable activity at 0 °C (Fig. 6A). Maximum phosphorylation was detected after 10 min of incubation at 25 °C under these conditions (Fig. 6B). Autophosphorylation activity showed a strong dependence on substrate concentration (100% signal remaining; Fig. 6C), and cold ATP competed with [γ<sup>32</sup>P]ATP as expected for an enzymatic activity (Fig. 6D). The phosphoryl linkage remained stable under basic conditions (100% signal remaining; Fig. 6E, lane 1) and decreased after treatment with hydroxylamine (14% signal remaining; Fig. 6E, lane 2) and under acidic conditions (11% signal remaining; Fig. 6E, lane 3). This result is consistent with a phosphoryl linkage to the His<sup>393</sup> determined as an H-box by sequence alignment (27). The holoprotein generated in vitro by addition of 2 or 4 μM PCB showed the same autophosphorylation activity as the apoprotein purified from E. coli, and CikA<sub>6HIS</sub> purified directly from the cyanobacterium showed autokinase activity comparable with that observed for E. coli-derived protein (data not shown).

Influence of other CikA domains on HPK Activity—The assay conditions described for partially purified CikA did not produce robust activity when the protein was more highly purified, either as a His<sub>6</sub> or Trx fusion. The addition of 2 μg/μl (w/v) BSA to the reaction mixture allowed a linear increase of autophosphorylation of CikA variants up to 120 min at 30 °C, whereas the mixture without BSA reached a plateau at 15 min (data not shown). Therefore, the following reactions were carried out in the presence of 2 μg/μl BSA at 30 °C for 1 h.

To identify which regions of the protein influence autophosphorylation activity, we produced variants that are missing specific residues or motifs and performed in vitro autophosphorylation assays. Because truncated CikA variants were soluble only if fused to Trx, we first compared autophosphorylation of full-length CikA<sub>6HIS</sub> and Trx-CikA (Fig. 7). The addition of the Trx tag dropped autophosphorylation to 20–25%; this variant served as the control for the following mutant derivatives.

All of the recombinant proteins that have a critical deficiency
panels, zinc-induced fluorescence from CikA variants (6 variants have a second His 6 tag at the C terminal; star, mutagenized histidyl to alanyl substitution. duplex coding regions for each recombinant protein variant; black bars, protein coding sequences for each of the CikA protein; gray bars, CikA protein; DMSO, dimethyl sulfoxide as a negative control; STORM with red laser setting and is shown as an inverted image. (sCikA) with no added PCB. holoproteins with PCB is shown as an inverted image. A SERA using purified CikA 6HIS without or with PCB for chromophore ligation assays. B,4/H9021 P4/H904 E. coli (rCikA) with added PCB and CikA6HIS purified from S. elongatus as a negative control (CBB Stain). (loaded on 8% SDS-PAGE was visualized by Coomassie Brilliant Blue (CBB Stain)) to check protein concentrations and mobilities. Lower panels, zinc-induced fluorescence from CikA variants (6 µg each) with 2 µM PCB was visualized on PVDF membranes (Zn Stain). D, zinc-induced fluorescence assay from recombinant CikA6HIS produced in E. coli (rCikA) with added PCB and CikA6HIS purified from S. elongatus (sCikA) with no added PCB.

of the HPK domain (ΔHPK and Trx-RR) or a point mutation at the expected phosphoryl acceptor His residue in the kinase domain (Trx-H393A) were completely defective in autophosphorylation activity (Fig. 7, B and C). ΔGAF, which conserves an intact HPK domain (Fig. 2), also had no autophosphorylation signal (Fig. 7, B and C). Additionally, only an extremely weak signal was detected after 60 min from ΔN (Fig. 7, B and C), which retains all recognizable motifs of the protein (Fig. 2), and was lower than the detectable sensitivity at 30 min (data not shown). In sharp contrast, removal of the receiver domain enhanced autophosphorylation activity by about 2-fold over CikA6HIS, and more than 10-fold over the more comparable Trx-CikA full-length control. We confirmed the linearity of these single time point experiments by performing time courses for the active CikA variants (CikA6HIS, Trx-CikA, and ΔRR; Fig. 8A), except for ΔN, which needs longer than 60 min to visualize activity. The assays showed a linear increase of autophosphorylation as expected for kinase activity dependent on the HPK domain, as depicted for CikA6HIS in Fig. 8B.

Absence of Internal Phosphoryl Transfer in CikA—We tested whether CikA transfers a phosphoryl group from the HPK domain to the atypical receiver domain in transphosphorylation assays. In the first strategy, we used autophosphorylated full-length CikA6HIS or ΔRR as a phosphodonor and Trx-RR as a potential acceptor. No phosphorylation of Trx-RR was observed (data not shown). Because HPKs tend to dimerize, we reasoned that if phosphoryl transfer occurs in CikA, it might involve the H-box of one monomer and the receiver domain of the other. To test this possibility, we used ΔRR as a donor that is lacking its own receiver and Trx-H393A as a potential receiver that is incapable of autophosphorylation (Fig. 9A). No phosphorylation of Trx-H393A was observed (Fig. 9B). The same negative result was obtained when the Trx-H393A sequence was modified to substitute Asp for Ala at the site that most closely aligns with Asp of bona fide receivers of two-component phosphorelay systems (data not shown).

DISCUSSION

The identification of cikA as a locus that affects circadian phasing and induction of light-regulated genes and resetting of circadian rhythms in response to a dark pulse implicates the CikA protein as important for transducing light signals from the environment, especially as an entry point to the circadian clock (27). Its structure is similar to bacteriophytochromes, suggesting a direct role as a photoreceptor. However, if CikA is a phytochrome-like photoreceptor, its properties are very different from previously reported bacteriophytochromes. CikA can form adducts with bilins in vitro that survive conditions used for SDS-PAGE, despite the absence of residues expected to be necessary for covalent linkage of a bilin (Fig. 3) (27). Mutation of the GAF domain at residues highly conserved among bacteriophytochromes (E236A, E285A, or C299A) did not abolish in vitro bilin binding (data not shown). Although the GAF domain was important for bilin lyase activity, it was not sufficient (Fig. 4C), and its absence did not completely abolish binding (Fig. 4B). This suggests either that the covalent
attachment site(s) lies outside of the GAF proper or that GAF alone does not assume the tertiary structure needed to support activation despite the existence of an intact HPK domain (Fig. 9), we conclude that the latter region of CikA may have been modified. This is consistent with the finding that a functional domain that influences kinase activity resides in this portion of the protein.

The C terminus of CikA contains a similar sequence to the receiver domain conserves other key residues of the motif, the invariable aspartyl residue that is phosphorylated by a cognate receiver domain of response regulators involved in two-component signal transduction systems. Although this cryptic receiver domain conserves other key residues of the motif, the invariant aspartyl residue that is phosphorylated by a cognate HPK in bona fide H-box works as a typical phosphoacceptor residue (42).

In vitro autophosphorylation assays with a series of truncated CikA variants identified regions that affect autokinase activity. Purified full-length CikA tagged by His6 or Trx and some of the Trx-tagged truncated variants that carry the HPK domain dramatically reduced the autophosphorylation of phosphoryl linkage after 1 h of treatment at 37 °C with 2 M NaOH on PVDF membrane but was not stable to hydroxylamine or acidic conditions. These results also support the expectation that the histidyl residue in the H-box as a typical phosphoacceptor residue (42).

As is implicit in the name of the gene cikA (circadian input kinase A), the deduced amino acid sequence predicts a well-conserved transmitter module typical of the sensor kinases of bacterial two-component signal transduction systems and is expected to supply a phosphoryl group to a specific, yet unidentified, response regulator(s) (27, 31). A histidyl residue in the region designated as the H-box is the site of autophosphorylation in HPKs. In CikA, this residue is His393, and a single point mutation at that site completely abolished the autophosphorylation with [γ-32P]ATP (H393A in Fig. 7). Additionally, the 32P-labeled protein obtained by in vitro autophosphorylation was alkali-tolerant in 3 M NaOH on PVDF membrane but was not stable to hydroxylamine or acidic conditions. These results also support the expectation that the histidyl residue in the H-box as a typical phosphoacceptor residue (42).

One of the potential CikA partners identified in a yeast two-hybrid screen is a protein that carries a canonical GAF. Whether or not CikA naturally binds a co-factor that would serve as a chromophore, the GAF domain likely serves a regulatory role, as evidenced by the negative effect of GAF removal on HPK activity. We favor interaction with a partner as the regulatory role of the CikA GAF domain.

As standards for holoprotein formation, the PCB-producing cells were transformed with the gene for Cph1 of Synechocystis sp. Strain PCC 6803 (46) and the BV cells with the gene for BphP of Agrobacterium tumefaciens (47).

attachment at 25 °C 

Fig. 5. Co-expression of CikA and bilins in E. coli. Strains of E. coli engineered to produce PCB or BV were transformed with a plasmid that encodes CikA. As standards for holoprotein formation, the PCB-producing cells were transformed with the gene for Cph1 of Synechocystis sp. Strain PCC 6803 (46) and the BV cells with the gene for BphP of Agrobacterium tumefaciens (47).

CikA Activities

![Diagram of CikA Activities]

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Purified full-length CikA tagged by His6 or Trx and some of the Trx-tagged truncated variants that carry the HPK domain dramatically reduced the autophosphorylation activity as compared with CikA6HIS. Because Trx is a larger tag than His6, the physical structure of the N-terminal region of CikA may have been modified. This is consistent with the finding that a functional domain that influences kinase activity resides in this portion of the protein.

The C terminus of CikA contains a similar sequence to the receiver domains of response regulators involved in two-component signal transduction systems. Although this cryptic receiver domain conserves other key residues of the motif, the invariable aspartyl residue that is phosphorylated by a cognate HPK in bona fide receivers is absent from the sequence (27). From the negative results of various in vitro trans-phosphorelay experiments using the HPK domain and the C-terminal domain of CikA (such as in Fig. 9), we conclude that the latter is a pseudo-receiver and does not accept a phosphoryl group.

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J.-S. Choi, S. Canales, and S. S. Golden, unpublished data.
from the kinase domain of CikA. The N-terminal domain of the circadian clock protein KaiA has been identified structurally as a pseudo-receiver that is unlikely to be involved in phosphorylation (51). Sequence also predicts a pseudo-receiver, unrelated to phosphoryl transfer, in the A. thaliana putative clock component TOC1 and its APRR family members (35–38). We predict that each of these acts as a protein-protein interaction domain that induces conformational changes in another domain to modulate its activity. Unlike the N-terminal region and GAF domain, elimination of the pseudo-receiver domain increased autokinase activity 10-fold over full-length Trx-CikA. These results suggest that the receiver domain functions as an autophosphorylation suppressor of the CikA HPK transmitter but not as a phosphoryl receiver. This is consistent with a role for the pseudo-receiver similar to that in AmiR, an antiterminator whose RNA-binding domain is regulated by interaction of

![Fig. 7. Autophosphorylation by purified CikA variants.](image)

A, each recombinant protein (2 μg) loaded on SDS-PAGE was visualized by Coomassie Brilliant Blue to check the mobility, purity, and protein concentration. B, phosphorimaging visualization of autophosphorylation of each CikA by [γ-32P]ATP. The proteins (200 ng) were used for each standard reaction and applied to 8% SDS-PAGE. C, graphical representation of relative autophosphorylation activity. 100% is the average of the highest activity (n = 4, ΔRR), n.d., not detectable. D, summary figure of interactions among proposed functional domains. Phosphorylation at the H-box of the kinase domain is regulated positively by GAF and the N-terminal region and negatively by the pseudo-receiver.

![Fig. 8. Time course of autophosphorylation activity of purified CikA variants.](image)

A, phosphorimage of autophosphorylation time courses from CikA variants. The protein concentration was adjusted to achieve comparable signal intensity among variants (400 ng of Trx-CikA, 100 ng of CikA6HIS, and 40 ng of ΔRR). B, graphic representation of the time course from CikA6HIS. Autophosphorylation was quantified, and the average of the maximum activity from four separate assays was set as 100% (n = 4).
the pseudo-receiver with its ligand-binding partner AmiC (52).

Bacteriophytchrome two-component systems are present in other cyanobacterial species and have been characterized best from *Synechocystis* sp. PCC 6803. Cph1 carries a typical GAF domain that includes the critical cysteine residue for bilin attachment and has an HPK domain; Rcp1 is its response regulator (19). Like plant phytochromes, Cph1 exists in photoconvertible Pr and Pfr forms that are generated by absorption of red or far-red light, respectively. Cph1 autophosphorylation is attenuated by red light and increased by far-red light; the phosphorylated Pr form transfers a phosphoryl group to Rcp1 (19). In the case of plant phytochromes, light affects a Ser/Thr protein kinase activity (15, 16). CikA reconstituted with purified PCB had the same level of autophosphorylation activity as CikA apoprotein (data not shown) and showed no spectral activity. These differences between CikA and phytochromes/bacteriophytochromes suggest a very distinct mechanism for modulating signal transduction, despite an expected shared identity as CikA apoprotein (data not shown) and showed no spectral activity.

Whether multiple signals converge on CikA as an integrator that can sense various inputs directly remains to be determined. The basic function of the protein as a kinase has been established; further understanding its role depends on discovering the other molecules with which it directly interacts.

**Acknowledgments**—We are grateful to J. Clark Lagarias for support and for materials, advice, and insightful comments regarding the manuscript. Specifically, we thank him for providing *E. coli* cells that produce PhoB and PCB and for the cph1 control plasmid. We also thank B. Karniol and R. Vierstra, who generously provided plasmids used for co-expression of BV and AtEph1P in *E. coli*.

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Biochemical Properties of CikA, an Unusual Phytochrome-like Histidine Protein Kinase That Resets the Circadian Clock in *Synechococcus elongatus* PCC 7942

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*J. Biol. Chem.* 2003, 278:19102-19110.
doi: 10.1074/jbc.M213255200 originally published online March 7, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M213255200

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