Nucleotide binding and autophosphorylation of the clock protein KaiC as a circadian timing process of cyanobacteria

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A negative feedback control of kaiC expression by KaiC protein has been proposed to generate a basic oscillation of the circadian clock in the cyanobacterium Synechococcus sp. PCC 7942. KaiC has two P loops or Walker’s motif As, that are potential ATP-/GTP-binding motifs and DXXG motifs conserved in various GTP-binding proteins. Herein, we demonstrate that in vitro KaiC binds ATP and, with lower affinity, GTP. Point mutation by site-directed mutagenesis of P loop 1 completely nullified the circadian rhythm of KaiC expression and markedly reduced ATP-binding activity. Moreover, KaiC can be autophosphorylated in vitro. These results suggest that the nucleotide-binding activity of KaiC plays important roles in the generation of circadian oscillation in cyanobacteria.

Circadian rhythms, biological oscillations with 24-h periodicity, are observed ubiquitously among eukaryotes and cyanobacteria. An endogenous oscillator named the circadian clock temporally regulates various biological activities to match them with daily environmental alterations (1). To elucidate the molecular mechanism of the circadian clock, several clock genes and clock-related genes have been cloned and analyzed in cyanobacteria, Neurospora, Drosophila, Arabidopsis, and mammals (2).

Cyanobacteria are the simplest organisms known to have the circadian clock. An essential clock gene cluster kaiABC was cloned from the cyanobacterium Synechococcus sp. strain PCC 7942 (3). Various circadian phenotypes can arise from mutations in any of the kai genes. In particular, 14 distinct clock mutations including those for period length and arrhythmia were mapped to kaiC. Experiments on the promoters of kai genes suggest that KaiC suppresses its own (kaiBC) expression in a basic negative feedback loop, and KaiA enhances kaiC expression to make the system oscillate (3). Moreover, the KaiA, KaiB, and KaiC proteins directly associate in all possible combinations in the yeast two-hybrid system, in vitro, and in cyanobacterial cells, and a long-period allele, kaiA1, markedly enhances KaiA–KaiB interaction (4). These observations suggest that physical interactions among the Kai proteins are crucial to the circadian timing mechanism.

Although the biochemical functions of the Kai proteins remain unknown, the amino acid sequence of KaiC contains two ATP-/GTP-binding motifs (P loops or Walker’s motif As) whose consensus is GXXXXGKT (X represents any amino acid) in the tandem duplicated domains of KaiC (CI and CII domains) (Fig. 1A; refs. 3–6). In addition, two DXXG motifs that are highly conserved in the GTPase superfamily (7, 8) are found in the CI domain. These motifs could provide clues to the biochemical function of KaiC in circadian rhythm generation. In this report, we show that KaiC binds ATP and, to a lesser extent, GTP in vitro, and disruption of the P loop in the CI domain causes arrhythmia and a marked reduction in ATP-binding activity. Moreover, we have identified autophosphorylation activity of KaiC in vitro. These observations strongly suggest that the ATP-binding activity of KaiC is important for the generation of circadian oscillation in Synechococcus.

Materials and Methods

Bacterial Strains, Media, Cultures, and Manipulation of DNA. Because the expression of the kaiBC operon was assumed to be the key process of the cyanobacteria oscillator (3), we used a reporter strain of Synechococcus, NUC39 (4), that carried a bacterial luciferase gene set luxAB fused to a promoter of kaiBC operon (PkaiBC) at a specific site of the chromosome (neutral site I) as the control strain of this study. The Synechococcus cells were grown at 30°C in BG-11 liquid medium or solid medium that contained 1.5% (wt/vol) Bacto Agar (Difco) under continuous light (LL) conditions of 46 μmol m−2 s−1 from white fluorescent lamps. Plasmids were introduced into Escherichia coli DH10B by electroporation. Synechococcus cells were transformed with plasmid DNA by natural transformation and selected with 40 μg/ml of spectinomycin sulfate (9).

Site-Directed Mutagenesis of the kai Locus. pCkaiABC targeting vector (3) containing the kaiABC cluster and a spectinomycin-resistant gene was mutagenized with the overlap extension method by PCR (10). All of the resulting mutations were confirmed by sequencing with a Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems). The mutant plasmids were introduced into kaiABC-deficient δkaiC reporter cells (NUC38) as described (4).

In Vitro Translation of KaiC and Its Derivatives. Expression vectors for in vitro transcription/translation were generated by inserting a wild-type or mutagenized PCR fragment into the SalI–BamHI site of pSP64-poly(A) vector (Promega). The TNT rabbit reticulocyte lysate system (Promega) was used for in vitro production and labeling of each KaiC-derived protein as described (4). Briefly, 25 μl of reaction mixture containing 1 μg each of the pSP64-poly(A) derivatives, SP6 RNA polymerase, and 12.5 μl of rabbit reticulocyte was incubated for 2 h at 30°C in the presence of 10 μCi of [35S]methionine (>1,000 Ci/mmol; 1 Ci = 37 GBq). Labeled proteins were confirmed by SDS/10% PAGE and analyzed by autoradiography with a BAS2000 Image Analyzer (Fuji).

Binding of in Vitro Translated Proteins to ATP- or GTP-Agarose. An in vitro ATP-/GTP-binding assay was performed as described by Ismaa et al. (11) with minor modifications. A fraction of the radioactivity (3.0 × 105 cpm) of the in vitro translation reaction

This paper was submitted directly (Track II) to the PNAS office. Abbreviations: CI, tandem duplicated domain of KaiC (N-terminal half); CII, tandem duplicated domain of KaiC (C-terminal half); LL, continuous light; GST, glutathione S-transferase.

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UV Crosslink Assay. Glutathione S-transferase (GST) fused to KaiC, CI, and CII was prepared as described (4). To assess ATP crosslink to KaiC derivative proteins, 1.5 μg each of the fusion proteins was incubated with 0.1 mM [α-32P]ATP (10,000 Ci/mmol) or 0.2 mM [35S]ATP (1,000 Ci/mmol) in 20 μl of reaction buffer (100 mM Tris-HCl, pH 7.6/50 mM KCl/5 mM MgCl2/0.4 mM DTT) at 25°C for 30 min. Reactions were terminated by placing the mixture on ice. The mixture was exposed to UV light (Stratalinker 1800, Stratagene) for 40 min at 4°C, and 4 μl of 6× SDS/PAGE loading buffer was added to the mixture. After boiling for 5 min, samples were subjected to SDS/PAGE with 10% gels and autoradiography and then analyzed with the BAS2000 Image Analyzer.

Assay of Bioluminescence Rhythm. Synechococcus cells were inoculated onto BG-11 agar plates in a plastic dish (30 mm in diameter) and incubated under LL conditions (46 μmol m−2 s−1) to form 50–100 colonies. Then, cells were subjected to 12 h of darkness to synchronize the circadian clocks, and bioluminescence from the agar plates was automatically monitored under LL in the presence of decanal solution with a photomultiplier-tube-based bioluminescence-monitor system (3).

Protein Phosphorylation Assay. GST and GST fused to KaiA, KaiB, or KaiC were prepared as described (4). An autophosphorylation assay was performed as described by Aiba et al. (12) and Mc Cleary and Zusman (13) with some modifications. The protein sample (1.0 μg) was added to 15 μl of a reaction mixture (50 mM Tris-HCl, pH 7.5/100 mM KCl/5 mM MgCl2/0.5 mM EDTA/2 mM DTT/0.1 mM [γ-32P]ATP (10,000 cpm/mmol) or [α-32P]ATP (30,000 cpm/mmol)) at 25°C for 30 min. Reactions were terminated by the addition of 3 μl of 6× SDS/PAGE loading buffer. After heating at 65°C for 5 min, samples were subjected to SDS/PAGE with 10% gels and then blotted onto Immobilon-P membranes (Millipore). The radioactivities of phosphorylated proteins were analyzed with the BAS2000 Image Analyzer.

Chemical Stability Assay of Phospho-Linkage. A chemical stability assay was performed as described by McCleary and Zusman (13) with minor modifications. Equal amounts of GST-KaiC were phosphorylated and blotted onto an Immobilon-P membrane as described above. Individual blots were excised from the membrane and separately incubated in 50 mM Tris-HCl, pH 7.5/3 M NaOH/1 M HCl or 0.8 M hydroxylamine (pH 6.8) at 42°C. After incubation for 1 h, the membranes were washed with distilled water and dried in air, and then the radioactivities were analyzed with the BAS2000 Image Analyzer.

Results

KaC Binding to ATP and GTP. 35S-labeled KaiC was translated in vitro and incubated with ATP-agarose or GTP-agarose. As shown in Fig. 1B, radioactive KaiC was detected after incubation with ATP-agarose or GTP-agarose followed by SDS/PAGE (Fig. 1B, lanes 3 and 4). The binding of KaiC to ATP was much stronger than that to GTP. No activity was detected after incubation with control agarose resin (Fig. 1B, lane 2). In addition, [35S]KaiA associated with neither ATP- nor GTP-agarose (data not shown). These results indicate specific binding of KaiC to ATP and to GTP with lower affinity.

Point Mutations of the P Loops Affect Both ATP-/GTP-Binding of KaC and Circadian Rhythm. As illustrated in Fig. 1A, the first and second P loops (P loop 1 and P loop 2, respectively) were disrupted separately by substitution of the conserved lysine residues with histidine (K292H and K294H, respectively) to examine the effects of these mutations on the ATP-/GTP-binding activity of KaiC. Fig. 1C shows that the K292H substitution in P loop 1 markedly reduced the ATP-binding activity; however, the P loop 2 mutation (K294H) failed to alter the ATP-binding profile (Fig. 1D), and neither mutation altered the GTP-binding activities.
Next, we analyzed the effect of these mutations on the circadian oscillation of kaiBC expression. Initially, we constructed a DNA fragment that contained the entire kai gene cluster in which the kaiC ORF was modified to carry mutations in the nucleotide-binding motifs. Each mutant kai cluster was introduced into a kai−deficient strain at the original locus. Although the control strain that had regained the normal kai cluster had a robust circadian rhythm (Fig. 1B), the P loop 1-disrupted transformant (K52H) completely lost the rhythmicity (Fig. 1C), suggesting that the lysine residue in P loop 1 was indispensable to the generation of the circadian oscillation. The amino acid substitution at position 115 in Ras protein. The glutamine of Ras is one of the main sites of oncogenic transformation (14) and is conserved glycine residues with alanine (G71A and G114A). DXXG2 is followed by a glutamine residue (Q115) that is a cognate to the glutamine residue at position 61 in Ras protein. The glutamine of Ras is one of the main sites of oncogenic transformation (14) and is conserved glycine residues with alanine (G71A and G114A). DXXG1 lowered the amplitude and distorted the waveform (Fig. 3C). The G114A mutation in DXXG motif 2 extended the period to 27 h and caused a bimodal waveform (Fig. 3B). Thus, the circadian oscillation in kaiBC expression was modified considerably by these mutations, although periodicity remained in the circadian range. On the other hand, Q115R mutation abolished the circadian rhythm (Fig. 3C). None of these mutations affected the average level of bioluminescence (Fig. 1B–D) or the ATP-binding profile of KaiC (Fig. 2B). On the other hand, only a faint signal of ATP-binding was detected for either normal or mutated CI domains (Fig. 2 C and D). To exclude a possibility that CI is merely inaccessible to agarose-linked ATP, we also performed a UV crosslinking assay to assess the ATP-binding profile of KaiC, CI, and CII fusion proteins. GST fused to KaiC, CI, or CII was incubated with [α-32P]ATP or [35S]ATP·γ-S, and UV crosslinked ATP was analyzed by SDS/PAGE and autoradiography. The results confirmed that ATP bound to KaiC and CI but failed to associate with CII (Fig. 2E and data not shown). These results suggest that ATP-binding activity mainly resides on the CI domain and that the P loop 1 is responsible for this activity.

**Mutation in DXXG Motifs Affects the Clock.** Two DXXG motifs (DXXG1 and DXXG2) in the CI domain were disrupted individually by substitution of the conserved glycine residues with alanine (G71A and G114A). DXXG2 is followed by a glutamine residue (Q115) that is a cognate to the glutamine residue at position 61 in Ras protein. The glutamine of Ras is one of the main sites of oncogenic transformation (14) and is conserved glycine residues with alanine (G71A and G114A). DXXG1 lowered the amplitude and distorted the waveform (Fig. 3A). The G114A mutation in DXXG motif 2 extended the period to 27 h and caused a bimodal waveform (Fig. 3B). Thus, the circadian oscillation in kaiBC expression was modified considerably by these mutations, although periodicity remained in the circadian range. On the other hand, Q115R mutation abolished the circadian rhythm (Fig. 3C). None of these mutations affected the average level of bioluminescence (Fig. 1B–D) or the ATP-binding profile of KaiC (Fig. 2B). On the other hand, only a faint signal of ATP-binding was detected for either normal or mutated CI domains (Fig. 2 C and D). To exclude a possibility that CI is merely inaccessible to agarose-linked ATP, we also performed a UV crosslinking assay to assess the ATP-binding profile of KaiC, CI, and CII fusion proteins. GST fused to KaiC, CI, or CII was incubated with [α-32P]ATP or [35S]ATP·γ-S, and UV crosslinked ATP was analyzed by SDS/PAGE and autoradiography. The results confirmed that ATP bound to KaiC and CI but failed to associate with CII (Fig. 2E and data not shown). These results suggest that ATP-binding activity mainly resides on the CI domain and that the P loop 1 is responsible for this activity.

**Autophosphorylation of KaiC.** It is known that some bacterial proteins that contain the P loop and Walker’s motif B have autokinase activity (17, 18). To examine the autophosphorylation of KaiC, GST-KaiC (4) was produced in E. coli, purified with an affinity resin, and then incubated with [γ-32P]ATP or [α-32P]ATP in the presence of Mg2+. As shown in Fig. 4A, GST-KaiC was labeled with 32P after incubation with [γ-32P]ATP but not with [α-32P]ATP. No phosphorylation activity was detected in unfused GST and GST fused to KaiA or KaiB. Therefore, GST-KaiC was specifically autophosphorylated by γ-phosphate.

Based on the stability of the phosphate bond in various...
conditions, phosphorylated GST-KaiC was assayed to determine the class of phosphorylated amino acid residue (19). As shown in Fig. 4B, 90% or more radioactivity was retained after treatments with HCl and hydroxylamine, whereas base (NaOH) treatment markedly reduced the level to 12% of the control. These results suggest that the phosphate link in phosphorylated GST-KaiC is an O-phosphate linkage. Among O-phosphate bonds, phosphotyrosine is base stable, whereas phosphoserine and phosphothreonine are base labile (19). Therefore, KaiC was likely to be autophosphorylated at serine or/threonine residue or residues.

Discussion

Ishii et al. (3) proposed a model of circadian oscillation of cyanobacteria in which negative feedback of the kaiBC expression by KaiC and positive regulation by KaiA play key roles. Thus, Kai proteins, especially KaiC, are evidently key components of the circadian oscillator of cyanobacteria (3). In this report, we have presented evidence of the biochemical activity of KaiC. The ATP-binding of KaiC could be essential to generate the circadian oscillation, because a marked decrease in ATP-binding activity of KaiC and positive regulation by KaiA play key roles. Thus, Kai proteins, especially KaiC, are evidently key components of the circadian oscillator.

In various clock models, phosphorylation of clock proteins is considered to be an important part of the circadian timing loop (2). In Drosophila, phosphorylation of Period (PER) controls the initial rate of PER accumulation and seems necessary for PER cycling. Double-time (Dbt), a Drosophila homolog of casein kinase Iε, was revealed to phosphorylate PER (27, 28). As is the case for PER, autophosphorylation of KaiC, revealed in this study, could alter the stability of KaiC that regulates negative feedback of kaiBC transcription. Alternatively, after autophosphorylation, KaiC might change its conformation to regulate as-yet unknown biochemical activities. Our previous analysis for ATP-binding of KaiC. This observation also suggests distinct functions of CI and CII domains. This possibility is pertinent to our previous findings that the KaiA–KaiB association was enhanced by the CI but not the CII domain (4). However, note also that substitution of the lysine residue of P loop 2 with histidine extremely extended the period of the rhythms (≈70 h) and of conserved threonine with alanine induced arrhythmicity. Thus, it is evident that this region of P loop 2 is also important for normal rhythmicity. Mutation of P loop 2 might affect some unknown function (e.g., maintaining a functional configuration of KaiC) and have a large effect on the circadian oscillator; however, it is still possible that P loop 2 is also a functional ATP-binding motif but that its ATP/GTP-binding affinity is below the detection limit of the current assay.

The GTP-binding activity detected in this study was much weaker than the ATP-binding activity and was not altered by point mutations in the P loops and DXXG motifs (Fig. 1 B–D Left; Fig. 2; data not shown). Mutations of invariant glycine to alanine in GXXG motifs 1 and 2 altered the circadian rhythm phenotype; however, rhythmicity still remained obvious, and the period length was in the circadian range (Fig. 3). Moreover, KaiC contained P loops and DXXG motifs but not NXXD and (C/S)AX motifs that are conserved in many GTP-binding proteins (7, 8). Thus, the GTP binding to KaiC seems not to be essential to the timing mechanism of the circadian oscillator. However, because a substitution of Q115, which is cognate to Q61 of Ras (7, 8), with arginine resulted in complete arrhythmia (Fig. 3C), it is still possible that GTP hydrolysis by this residue is responsible for the generation of circadian rhythm, as is the case for signal transduction by Ras (15, 16).

Yoshida and Amano (20) revealed a consistency in topologies of ATPases whose crystal structures had already been solved, such as F1-ATPase and RecA. These investigators identified catalytic carboxylate 24 ± 2 residues from the lysine residue of P loop and Walker’s motif B 50–130 residues from the C-terminal side of the P loop (20). In the case of KaiC, the distances between P loops and Walker’s motif Bs in CI and CII domains are 87 and 78 residues, respectively, and two continuous glutamate residues are located 25 and 24 residues from the lysine residues of P loops in CI and CII domains, respectively (Fig. 1A; ref. 3). Therefore, it is likely that the topology of the CI and CII domains of KaiC are similar to that of the ATPases.
revealed that KaiC interacted with both KaiA and KaiB (4). Autophosphorylated KaiC may alter affinities between the Kai proteins. Conversely, it is possible that the binding of KaiA and/or KaiB regulates the autophosphorylation activity of KaiC.

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