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Two KaiA-binding domains of cyanobacterial circadian clock protein KaiC

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Abstract ***kaiABC*, a gene cluster, encodes KaiA, KaiB and KaiC proteins that are essential to circadian rhythms in the unicellular cyanobacterium *Synechococcus* sp. strain PCC 7942. Kai proteins can interact with each other in all possible combinations. This study identified two KaiA-binding domains (C_{KABD1} and C_{KABD2}) in KaiC at corresponding regions of its duplicated structure. Clock mutations on the two domains and *kaiA* altered the strength of C_{KABD}–KaiA interactions assayed by the yeast two-hybrid system. Thus, interaction between KaiA and KaiC through C_{KABD1} and C_{KABD2} is likely important for circadian timing in the cyanobacterium.**

Key words: Circadian rhythm; *kai* gene; Protein–protein interaction; Cyanobacterium; *Synechococcus*

1. Introduction

Circadian rhythms, biological oscillations with a period of about 24 h, have been found in organisms ranging from cyanobacteria to green plants and mammals. These rhythms regulate various metabolic and behavioral activities and have evolved as an adaptation to daily changes in environmental conditions: such as light, temperature and humidity [1,2]. The circadian clock, an endogenous mechanism that generates the ca. 24 h oscillation, has been postulated as a basis for those rhythms.

Cyanobacteria are the simplest organisms that exhibit circadian rhythms [4,5]. We introduced a bioluminescence reporter gene into the unicellular cyanobacterium *Synechococcus* sp. strain PCC 7942 to monitor circadian gene expression [6], isolated various clock mutants [7] and identified the clock gene cluster *kaiABC* composed of three genes, *kaiA*, *kaiB* and *kaiC*, that was cloned [8]. While no similarity was found between Kai proteins and clock proteins from other organisms, disruption of any one of the *kai* genes completely abolished circadian rhythm. Disruption and overexpression experiments on the *kai* genes suggested that KaiC represses its own (*kaiBC*) expression, while KaiA enhances it. Thus, KaiC and KaiA have been proposed as negative and positive elements, respectively, of the molecular feedback loop of *kaiBC* expression [8]. 19 distinct mutants exhibiting a wide range of period length and arhythmia mapped onto the *kaiC* gene. It is therefore likely that the biochemistry of KaiC protein is a key to understanding circadian characteristics. ATP-binding and autophosphorylation activities of KaiC [9] have been demonstrated as important functional processes in the circadian timing mechanism. More recently, SasA, a regulatory system consisting of a two-component sensory kinase, has been found to amplify the *kai*-based oscillation by interacting with KaiC [10].

Feedback loops for molecular circadian cycling have also been proposed for *Neurospora*, *Drosophila* and mammals [3]. These models involve interactions among clock proteins as crucial step for the feedback loop within the circadian period [3]. A previous study [11] has revealed that KaiA (a positive regulator) and KaiC (a negative regulator) interact with each other in the yeast two-hybrid system, both in vitro and in *Synechococcus*. Each of homologous two half units of KaiC (the first (CI) and second (CII) half domains) interacts with KaiA. In the present study, we identify two KaiA-binding domains (C_{KABD1} and C_{KABD2}) in KaiC by using the yeast two-hybrid system and an in vitro interaction assay. C_{KABD1} and C_{KABD2} were found on corresponding C-terminal regions of CI and CII, respectively. We demonstrated that many of the known clock mutations on C_{KABD1} and C_{KABD2} and on *kaiA* [8] altered C_{KABD}–KaiA interactions, suggesting that a direct interaction between KaiA and KaiC through C_{KABD1} and C_{KABD2} is crucial for circadian timing in the cyanobacterium. Moreover, molecular modeling of KaiC based on similarity of KaiC with many ATP-binding proteins suggested that two domains are configured in the native form of KaiC so as to be accessed by other proteins.

2. Materials and methods

2.1. Bacterial and yeast strains

Escherichia coli strains DH5α and DH10B were used as hosts for both plasmid construction and bacterial expression of GST fusion proteins. *Saccharomyces cerevisiae* strain L40 was used for two-hybrid analysis [12]. The *Synechococcus* arhythmic mutant ALAb, which carries the *kaiA4* mutation (T. Kondo and M. Ishiura, unpublished data), was isolated from the chemically mutagenized P_{psbAI} reporter strain AMC149 [7]. Wild-type and mutant *Synechococcus* strains listed

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Table 1
Cyanobacterial strains used in this study

Strains	Clock phenotypes	*kai* mutations[a]	References
AMC149[b]	wild-type (period: 24 h)	none	[6]
ALAb	arhythmia	*kaiA4* (Y166→C)	this study
A30a	long period (30 h)	*kaiA2* (R249→H)	[8]
C16a	short period (16 h)	*kaiC3* (R215→C)	[8]
CLAd	low amplitude	*kaiC6* (P248→L)	[8]
C44a	long period (44 h)	*kaiC11* (G421→R)	[8]
C60a	long period (60 h)	*kaiC12* (Y442→H)	[8]
CLAc	arhythmia	*kaiC14* (T495→A)	[8]

[a]Name of mutations and amino acid substitutions are listed.
[b]AMC149 is a derivative of wild-type *Synechococcus* that carries a *psbAI* reporter construct, P*psbAI::luxAB*. All mutant strains listed below were originally isolated from chemically mutagenized AMC149.

in Table 1 were each used for their respective genomic DNA preparations.

2.2. Plasmid construction

To construct expression vectors for KaiC deletion fragments (fragments 1–6) fused to LexA (pLexA-f1, -f2, -C_{KABD1}, -f4, -f5 and -C_{KABD2}), PCR was performed (primers listed in Table 2) on a wild-type *Synechococcus* genomic DNA template. For construction of point-mutated C_{KABD1} and C_{KABD2} fusions, PCR was performed similarly using a mutant *Synechococcus* genomic DNA (listed in Table 1) template. PCR products were cloned into the *Bam*HI site of pBTM116. Expression vectors for full-length KaiA, KaiB and KaiC protein fused to VP16 were designated as pLexA-KaiA, -KaiB and -KaiC [12]. For construction of point-mutated LexA-KaiA fusions, PCR was performed using *kaiA*-specific primers (listed in Table 2; [11]) and a mutant *Synechococcus* genomic DNA template (listed in Table 1). Each PCR product was cloned into the *Bam*HI site of pVP16 [12]. Plasmids expressing GST fused to the N-terminus of C_{KABD1} or C_{KABD2} (pGST-C_{KABD1} or -C_{KABD2}) were constructed by insertion of the smaller *Bam*HI fragment of pLexA-C_{KABD1} or -C_{KABD2} into the unique *Bam*HI site of pGEX-3X.

2.3. Yeast two-hybrid assay

The β-galactosidase filter assay was performed as described previously [11,13]. A quantitative liquid culture assay using *o*-nitrophenyl β-D-galactopyranoside (ONPG) as a substrate for β-galactosidase was performed as described [13].

2.4. Production of GST fusion proteins

GST, GST-C_{KABD1}, and GST-C_{KABD2} proteins were prepared and immobilized to glutathione Sepharose 4B (Pharmacia) as described previously [11], except that cells were induced with isopropyl-β-D-thiogalactopyranoside (IPTG) for 6 h prior to cell harvesting.

2.5. Production of ^{35}S-labeled KaiA

[^{35}S]Methionine-labeled KaiA was produced using the rabbit reticulocyte system as described previously [11].

2.6. In vitro assay for protein–protein interaction

An in vitro binding assay was performed as described previously [11].

2.7. Assay for bioluminescence rhythms

Bioluminescence rhythms were monitored as described previously [7].

2.8. Molecular modeling of KaiC

A three-dimensional (3D) structure for KaiC was modeled as follows. The amino acid sequence of KaiC was analyzed by a custom program developed to align its Walker motifs A and B, and its catalytic Glu to be configured in a fashion similar to other ATP-binding proteins such as RAS, F1-ATPase, elongation factor Tu and RecA. RecA had the best sequence match with half domains C1 and C2 of KaiC. Then, we applied a homology modeling technique to each half domain of KaiC, using the 3D structure of RecA as a template. The atomic coordinates of regions having no counterpart in RecA were estimated from corresponding regions of F1-ATPase. The homology module from insightII (SGI) was used in modeling of the half domains of KaiC. Molecular dynamics on the C1 and C2 domains of KaiC were carried out for 100 ps at 300 K, after which energy minimization was conducted with insightII.

3. Results and discussion

3.1. Identification of two KaiA-binding domains on KaiC

To identify KaiA-binding regions in KaiC by the yeast two-hybrid system, we first generated six deletion fragments (60–110 amino acid residues) of KaiC by exploiting an appropriate combination of restriction sites (Fig. 1A), then fused them to LexA, a DNA-binding protein. Each of the six deletion fusions was co-expressed in yeast cells with KaiA fused to a transcriptional activation domain of VP16 [11]. A filter assay

Table 2
Oligonucleotide primers used for amplification of full-length *kaiA* and deleted *kaiC* open reading frames

Fragment 1	5′-CCCTCTT**GGATCC**TGACTTCCGCT-3′ (−12 to +12)
	5′-AGCATCAA**GGATCC**ATAGTTTGCC-3′ (+324 to +301)
Fragment 2	5′-GTCAAG**GGATCC**TTGGCGGCTTCGATCTC-3′ (+341 to +369)
	5′-CGTAGCT**GGATCC**TTAGAGGGTGCGGCG-3′ (+671 to +649)
Fragment 3	5′-AACGTCG**GGATCC**TCCGCAACGTT-3′ (+610 to +630)
[C_{KABD1}]	5′-TCGGAC**GGATCC**AGATTAAACACGCAC-3′ (+807 to +781)
Fragment 4	5′-GTGGGG**GGATCC**TTAAGGACTCAATCATT-3′ (+824 to +852)
	5′-TTGGCG**GGATCC**TTAGTTGCTAACGCCCCG-3′ (+1182 to +1153)
Fragment 5	5′-CTCTCTG**GGATCC**CGCGGGGCGTTAGCA-3′ (+1138 to +1165)
	5′-CATTTC**GGATCC**AATCTAGACGTATTGGAG-3′ (+1347 to +1318)
Fragment 6	5′-AATACCA**GGATCC**AATTTATGGGA-3′ (+1240 to +1263)
[C_{KABD2}]	5′-AAGCG**GGATCC**CTGGCTAGCTCTC-3′ (+1575 to +1552)
Full-length KaiA	5′-AGGAGCG**AGATCT**TGCTCTCGCAA-3′ (−12 to +12)
	5′-AGCCGAGC**AGATCT**CCTCCTTTAC-3′ (+884 to +860)

As shown in Fig. 1, fragments 1–6 are partial sequences of KaiC. Bold letters indicate *Bam*HI and *Bgl*II sites. Stop codons are underlined. Numbers in parentheses indicate nucleotide position within the *kaiC* or *kaiA* gene; the first nucleotide, A (*kaiC*) or G (*kaiA*), of the translation initiation codon of each gene is numbered +1.

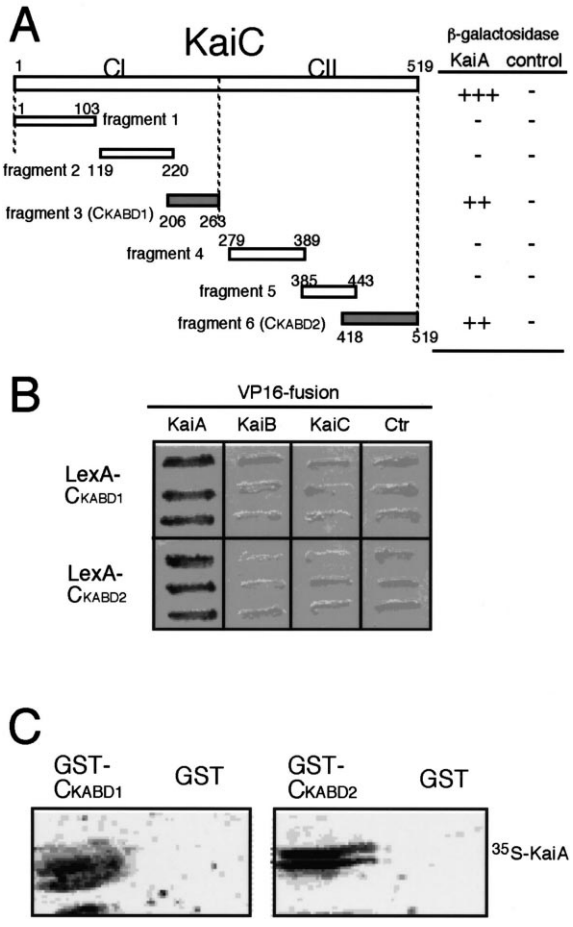

Fig. 1. Identification of two KaiA-binding domains in KaiC. A: Deletion analysis of KaiC for KaiA binding in the yeast two-hybrid system. Left: Open bars represent regions represented by expressed fusion proteins. Numeric labels refer to the first and last amino acid residues included. LexA domains, which were fused to the N-terminal end of each protein (the left of each bar), are not shown. Right: Results of two-hybrid tests. Two-hybrid interactions were estimated by the filter assay for β-galactosidase at 30°C for 3 h. +++, strongly positive interaction (cells were dark blue); ++, positive interaction (cells were blue); −, no interaction (cells remained white). Quantitative data for KaiA–fragment 3 (C_{KABD1}) and KaiA–fragment 6 (C_{KABD2}) interactions are shown in Table 2. B: C_{KABD1} and C_{KABD2} specifically interact with KaiA in yeast cells. β-Galactosidase was monitored by the filter assay as described in A. C: In vitro KaiA–C_{KABD} binding assay. GST, GST-C_{KABD1} or GST-C_{KABD2} were separately immobilized on glutathione Sepharose 4B and incubated with an equivalent volume of a reticulocyte reaction mixture containing ^{35}S-labeled KaiA. Proteins binding to the resin were eluted and then detected by SDS–PAGE on 10% acrylamide gels followed by autoradiography.

for β-galactosidase demonstrated that KaiA interacts with both a 58 amino acid fragment (fragment 3) and a 102 amino acid fragment (fragment 6) (Fig. 1B). In contrast, the other four fragments did not associate with KaiA (Fig. 1A). As shown in Fig. 1B, neither fragment 3 nor 6 interacted with KaiB or KaiC, suggesting that KaiC binds to KaiA through these two fragments exclusively. Therefore, fragments 3 and 6 were designated C_{KABD1} and C_{KABD2}, respectively. As expected from the repeat structure of KaiC, both C_{KABD1} and C_{KABD2} are located at equivalent C-terminal regions of the CI and CII domains, respectively. These observations support the general correlation of primary structure repeats and functional duplication in KaiC, but additional data also have uncovered some specific functional differences between CI and CII as well [8,13].

We confirmed the association between C_{KABD} and KaiA with an in vitro binding assay. Glutathione *S*-transferase (GST) fused to C_{KABD1} or C_{KABD2} and unfused control GST were separately expressed in *E. coli* and immobilized to an affinity resin. KaiA was labeled with ^{35}S using a rabbit reticulocyte system and incubated with each protein-bound resin. The amount of ^{35}S-labeled protein bound to, and subsequently eluted from, each resin was then analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by autoradiography. The autoradiogram demonstrated that both C_{KABD1} and C_{KABD2} bind KaiA in vitro (Fig. 1C). The doublet bands of in vitro translated KaiA may be due to alternative translation or degradation of KaiA in the rabbit reticulocyte system [11].

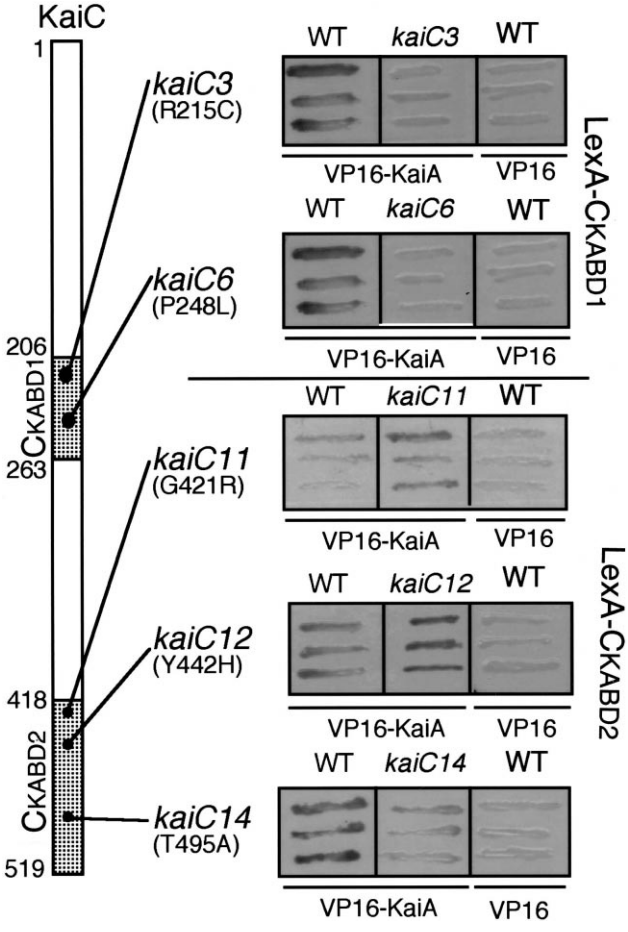

Fig. 2. *kaiC* clock mutations alter KaiA–C_{KABD} interactions. Left: Schematic representation of five clock mutations on either C_{KABD1} or C_{KABD2}. Filled bars indicate C_{KABD1} and C_{KABD2} segments. Numeric labels refer to the amino acid residue number. Black dots indicate locations of various mutations causing the indicated rhythm variations. Right: Effects of KaiC mutations on KaiA–C_{KABD} binding. LexA-C_{KABD1} or LexA-C_{KABD2} was used as bait in the two-hybrid system, while either VP16-KaiA or unfused VP16 was used as prey. β-Galactosidase filter assays were performed as described for Fig. 1A. Photographs show interaction profiles at different reaction times for clarity: incubation for 80 min (*kaiC12*), 120 min (*kaiC11*), 150 min (*kaiC14*), or 180 min (*kaiC3* and *kaiC6*). Note that introduction of each mutation did not alter the background signal of cells expressing C_{KABD} hybrid and unfused VP16 control (data not shown).

3.2. Clock mutations of KaiA-binding domains of kaiC alter C_{KABD}–KaiA interactions

More than half (64%) of the missense mutations in *kaiC* localize to 31% (160 amino acids of C_{KABD1} and C_{KABD2} per 519 amino acids of KaiC) of the *kaiC* gene [8]. Therefore, we examined the effects of these mutations on the association between C_{KABD} and KaiA by the yeast two-hybrid assay. As shown in Fig. 2 and Table 3, we found that five *kaiC* mutations altered the C_{KABD}–KaiA interaction in yeast cells. C_{KABD1}–KaiA interaction was greatly reduced by *kaiC3* (substitution at residue R215 to C) and *kaiC6* (P248 to L) mutations (Fig. 2). *kaiC3* shortens the period to 16 h with a reduced amplitude and *kaiC6* exhibits arhythmia [7,8]. In contrast, C_{KABD2}–KaiA interaction was enhanced by *kaiC11* (G421 to R) and *kaiC12* (Y442 to H) mutations, while it was reduced by *kaiC14* (T495 to A). *kaiC11* and *kaiC12* lengthen the period to 44 and 60 h, respectively, and *kaiC14* abolishes rhythmicity [7,8]. These observations suggest that both KaiA–C_{KABD1} and KaiA–C_{KABD2} interactions may play a role in the generation of circadian rhythms in *Synechococcus*. Binding of KaiC to KaiA is thought to enhance KaiA activity, promoting *kaiBC* expression. Arhythmicity or damping of the oscillation caused by *kaiC3*, *kaiC6* and *kaiC14* mutations could be ascribed to a change in KaiA activity by losing KaiC binding. In contrast, enhanced KaiA–C_{KABD2} binding by the two long-period mutations (*kaiC11* and *kaiC12*) suggests that the more strongly KaiA and C_{KABD} interact, the more slowly the clock proceeds. Elevation of KaiA–C_{KABD} binding would induce a higher level of KaiA activation, which would extend the period. Further studies of *kaiBC* expression profiles and Kai protein complex formation in *Synechococcus* are necessary to address these possibilities.

Four other C_{KABD} mutations did not alter KaiA–C_{KABD} binding profiles (data not shown). Our assay might not be able to detect slight alterations in KaiA–C_{KABD} binding that could be generated by these mutations. Alternatively, these mutations could affect properties of KaiC other than KaiA binding, which are currently unknown but should be assessed. We have not yet found a *kaiC* mutation that affects the interaction between full-length KaiC and KaiA in yeast cells. The duplication of C_{KABD} in KaiC may explain these results, because loss of affinity of either C_{KABD1} or C_{KABD2} could be compensated by the other binding domain. However, even using CI- or CII-VP16 protein, we have not yet found clock mutations that alter the interaction of CI or CII with KaiA (H. Iwasaki, unpublished data), suggesting that the effects of these mutations on binding are too small to alter the configuration of entire domains.

Table 3
Quantitative liquid assay for yeast two-hybrid interactions between KaiA and C_{KABD}

Bait	Prey	β-Galactosidase activity, mean value ± S.D. (arbitrary units)
LexA-C_{KABD1} (wild-type)	VP16	<0.07
LexA-C_{KABD1} (wild-type)	VP16-KaiA	5.4 ± 0.74
LexA-C_{KABD1} (*kaiC3*)	VP16-KaiA	0.22 ± 0.06
LexA-C_{KABD1} (*kaiC6*)	VP16-KaiA	ND
LexA-C_{KABD2} (wild-type)	VP16	0.12 ± 0.03
LexA-C_{KABD2} (wild-type)	VP16-KaiA	5.5 ± 1.3
LexA-C_{KABD2} (*kaiC11*)	VP16-KaiA	8.0 ± 1.8
LexA-C_{KABD2} (*kaiC12*)	VP16-KaiA	15.0 ± 2.6
LexA-C_{KABD2} (*kaiC14*)	VP16-KaiA	ND

ND, not determined.

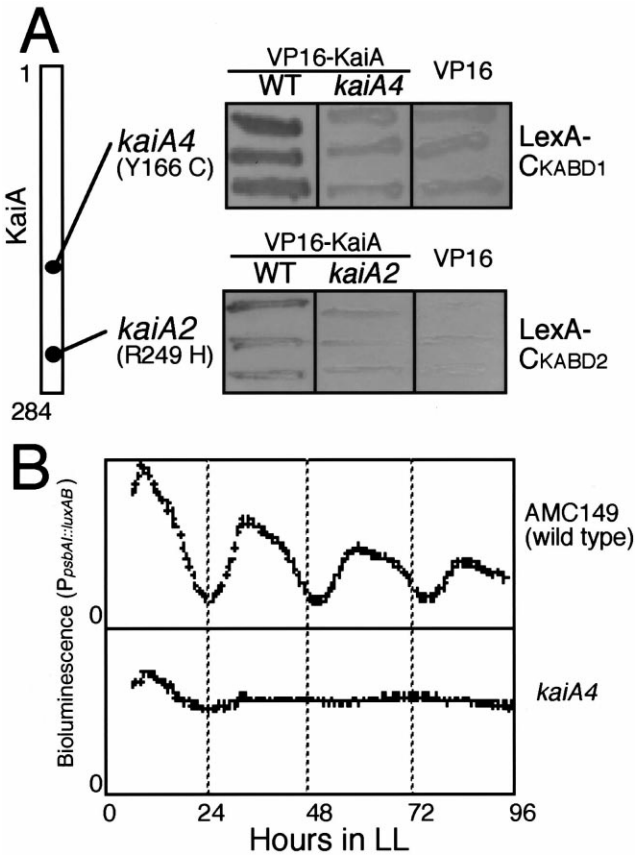

Fig. 3. *kaiA* clock mutations alter KaiA–C_{KABD} interactions. A: Left: Schematic representation of five *kaiA* clock mutations. Reaction conditions were as described in Fig. 2 (left). Black dots mark locations of the indicated mutations. Right: Effects of mutations on KaiA–C_{KABD} binding. Each KaiA mutation was introduced into the VP16-KaiA prey construct. Then, the yeast reporter strain was transformed with the resulting prey plasmid and either LexA-C_{KABD1} or LexA-C_{KABD2} as bait. β-Galactosidase filter assays were performed as described for Fig. 1A. B: Temporal pattern of expression of a clock-controlled gene (*psbAI*) monitored by a bioluminescence reporter in wild-type and ALAb (*kaiA4*) mutant strains.

3.3. Clock mutations of kaiA alter C_{KABD}–KaiA interactions

Reciprocally, we also examined whether clock mutations at KaiA altered binding between KaiA and C_{KABD} in yeast cells (Fig. 3A). *kaiA4*, that causes arhythmicity (Fig. 3B), exhibited reduced KaiA–C_{KABD1} binding. *KaiA4* mapped to the middle of *kaiA* (A to G transition at nucleotide +497, where the first G of the initiator GTG codon is numbered +1) causing an expanded 30 h period [7,8] demonstrated reduced KaiA–C_{KABD2} binding. While reduction of KaiA–C_{KABD2} association by the long period *kaiA2* mutation deviates from the possibilities mentioned in Section 3.2, it is still likely that KaiA–C_{KABD} interaction is an important process to determine parameters of the circadian oscillator of *Synechococcus*, such as period or amplitude.

These observations also support the notion that the C_{KABD}–KaiA interaction plays a role in circadian rhythms in cyanobacteria, and in that period would be extended by alteration of C_{KABD}–KaiA interaction both to tighter or weaker.

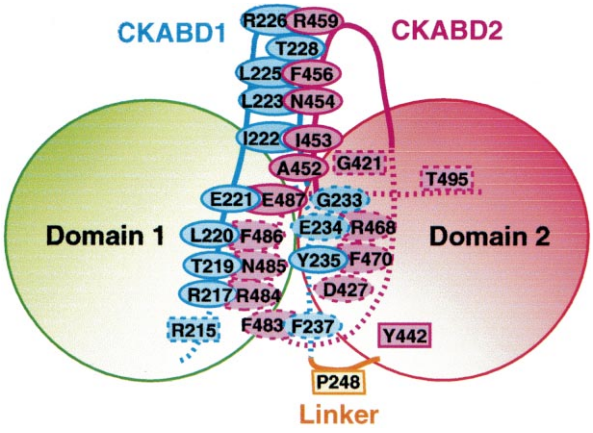

Fig. 4. Putative molecular structure of KaiA-binding domains of KaiC. A putative structure of the two KaiA-binding domains of KaiC is illustrated. Blue and red lines represent C_{KABD1} and C_{KABD2}, respectively. Solid lines depict peptide chains exposed to solvent, and dashed lines indicate buried regions. Each oval indicates a non-polar amino acid residue. Predicted interactions between amino acid side chains are illustrated. Boxes indicate KaiC mutations that modify C_{KABD}–KaiA binding. Amino acid residues near P248 form an interface between the CI and CII domains, and are colored with green and pink circles, respectively.

3.4. Structural modeling of the two KaiA-binding domains

To discuss the functions of the two KaiA-binding domains identified in this study, a 3D structure of KaiC would be extremely helpful. While no 3D structural information is available for KaiC, the similarity of KaiC sequence to those of several nucleotide-binding proteins, such as RAS, F1-ATPase, adenylate cyclase and RecA [14], might help to model the 3D structure. Based on the assumption that common functional motifs of these proteins (Walker's motif A, Walker's motif B, and catalytic Glu) would exhibit structural conservation, the 3D structure of KaiC was computed, based on homology of each half domain of KaiC (CI and CII) to RecA (Yamaguchi et al., in preparation). The predicted structure, as shown in Fig. 4, suggests that both C_{KABD1} and C_{KABD2} are located at the interface between the CI and CII domains, and are stabilized by forming hydrophobic contacts. Two domains seem to be exposed to the surface, which can be accessed by KaiA. This computational model is consistent with our observations that mutations in KaiC profoundly affect KaiA–KaiC association, resulting in abnormality in circadian timing processes.

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