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Intricate Protein-Protein Interactions in the Cyanobacterial Circadian Clock*

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Martin Egli[1]

From the Department of Biochemistry and Center for Structural Biology, Vanderbilt University, School of Medicine, Nashville, Tennessee 37232-0146

The cyanobacterial circadian clock consists of a post-translational oscillator (PTO) and a PTO-dependent transcription-translation feedback loop (TTFL). The PTO can be reconstituted *in vitro* with the KaiA, KaiB, and KaiC proteins, enabling detailed biochemical and biophysical investigations. Both the CI and the CII halves of the KaiC hexamer harbor ATPases, but only the C-terminal CII ring exhibits kinase and phospho-transferase activities. KaiA stimulates the kinase and KaiB associates with KaiC during the dephosphorylation phase and sequesters KaiA. Recent research has led to conflicting models of the KaiB-KaiC interaction, precluding a clear understanding of KaiB function and KaiABC clock mechanism.

The circadian clock in the cyanobacteria *Synechococcus elongatus* and *Thermosynechococcus elongatus* consists of a three-protein biochemical oscillator that is coupled to a transcription-translation feedback loop (TTFL)[2] (1–4). The KaiA, KaiB, and KaiC proteins in the presence of ATP reconstitute a temperature-compensated timer *in vitro* (5–7) and function as a post-translational oscillator (PTO) *in vivo*, whereby the latter constitutes the master clock (8). The cyanobacterial clock contradicts the widely held belief from studies of eukaryotic cells that the core mechanism of the circadian clock is based on a TTFL (9, 10).

KaiC is the only enzyme among the three proteins and exhibits rhythmic phosphorylation and dephosphorylation with an ~24-h period (5–7). Of the two phosphorylation sites observed in KaiC (Thr-432 and Ser-431 (11, 12)), Thr-432 becomes phosphorylated first followed by Ser-431, and dephosphorylation then proceeds in the same order: TS → pTS → pTpS → TpS → TS (13, 14). KaiC autophosphorylates and autodephosphorylates, but the other two proteins are needed to confer rhythmicity; early research demonstrated that KaiA stimulates KaiC phosphorylation (15, 16) and KaiB antagonizes KaiA action (17). Dephosphorylation is also accompanied by exchange of KaiC subunits among hexamers (18–20), a process that is believed to be important for synchronization of the clock (1). However, swapping of KaiC subunits also proceeds in the absence of either KaiA or KaiB (20), and sequestration of KaiA by the KaiBC complex may be more important for properly synchronizing the clock (21–24).

Crystal structures of Kai proteins revealed a domain-swapped dimer for KaiA (25) (Fig. 1*A*), a dimer of dimers for KaiB (26–30) (Fig. 1*B*), and a homohexamer for KaiC (31, 32) (Fig. 1*C*) (reviewed in Ref. 33). The quaternary structures in solution as established by small angle x-ray scattering (SAXS) remain the same as in the solid state (34). The 360-kDa KaiC hexamer resembles two stacked doughnuts with overall dimensions of ~100 × 100 × 100 Å (Fig. 1*D*). Twelve ATP molecules are lodged between subunits, six each in the N-terminal CI and C-terminal CII rings (Fig. 1*C*). The N-terminal region of CII forms a linker that tethers together the CI and CII domains of individual subunits (Fig. 1*C*). The C-terminal ends of CII domains include an S-shaped loop (31), termed A-loop (35), and the last 20 residues protrude from the dome-shaped surface of the upper ring (Fig. 1*C*).

KaiA binds to the C-terminal KaiC peptide and unravels the A-loop (36, 37). Molecular dynamics simulations provided evidence that unfolding of an A-loop increases the mobility of the P-loop region, ATP, and the loop region harboring Thr-432 and Ser-431 (38). Removal of an A-loop destabilizes adjacent loops and points to a concerted allosteric mechanism of the stimulation of KaiC phosphorylation by KaiA (38). This is also supported by the observation that a single KaiA dimer can push KaiC to the hyperphosphorylated state (39). The order of phosphorylation (Thr-432 first) is kinetically controlled as Thr-432 lies closer to the ATP γ-phosphate than Ser-431 (11, 40). Overall, the KaiA-KaiC interaction is better understood at this time than the KaiB-KaiC interaction or the interactions underlying the ternary KaiABC complex. This is because the binding site of KaiA on KaiC can be established without ambiguity and a plausible mechanism of the increase in KaiC phosphorylation in the presence of KaiA has been formulated.

Open Questions Regarding the Structure and Function of KaiB in the PTO

KaiB does not bind to the C-terminal KaiC tentacle, and the possibility of KaiB simply acting as a competitive inhibitor and displacing KaiA from KaiC can therefore be ruled out (29), but what triggers KaiB binding in the first place? Does KaiB bind to the same ring, CII, and therefore in the vicinity of KaiA (29), or at the opposite end, to CI (41, 42), and does KaiB bind as a tetramer (43), as a dimer (29), or as recent evidence suggests, in the monomeric form (30, 44)? The challenges that the KaiB-KaiC interaction has had in store for structural biology are matched by those at the functional level. Does KaiB sense the phosphorylation level of KaiC (18, 34) or the kinase state of KaiC, *i.e.* by preferential binding to a particular conformational or dynamic state of the latter (45), does it actively stimulate dephosphorylation (15, 17), or is its function in the PTO limited to sequestration of KaiA? The CI ATPase activity is required for KaiB binding (42, 46), but it is unclear whether KaiB binds there

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[1] To whom correspondence should be addressed. Tel.: 615-343-8070; E-mail: martin.egli@vanderbilt.edu.

[2] The abbreviations used are: TTFL, transcription translation feedback loop; PTO, post-translational oscillator; ESP, electrostatic surface potential; HDX, hydrogen-deuterium exchange; SAXS, small angle x-ray scattering; p, phosphorylated form.

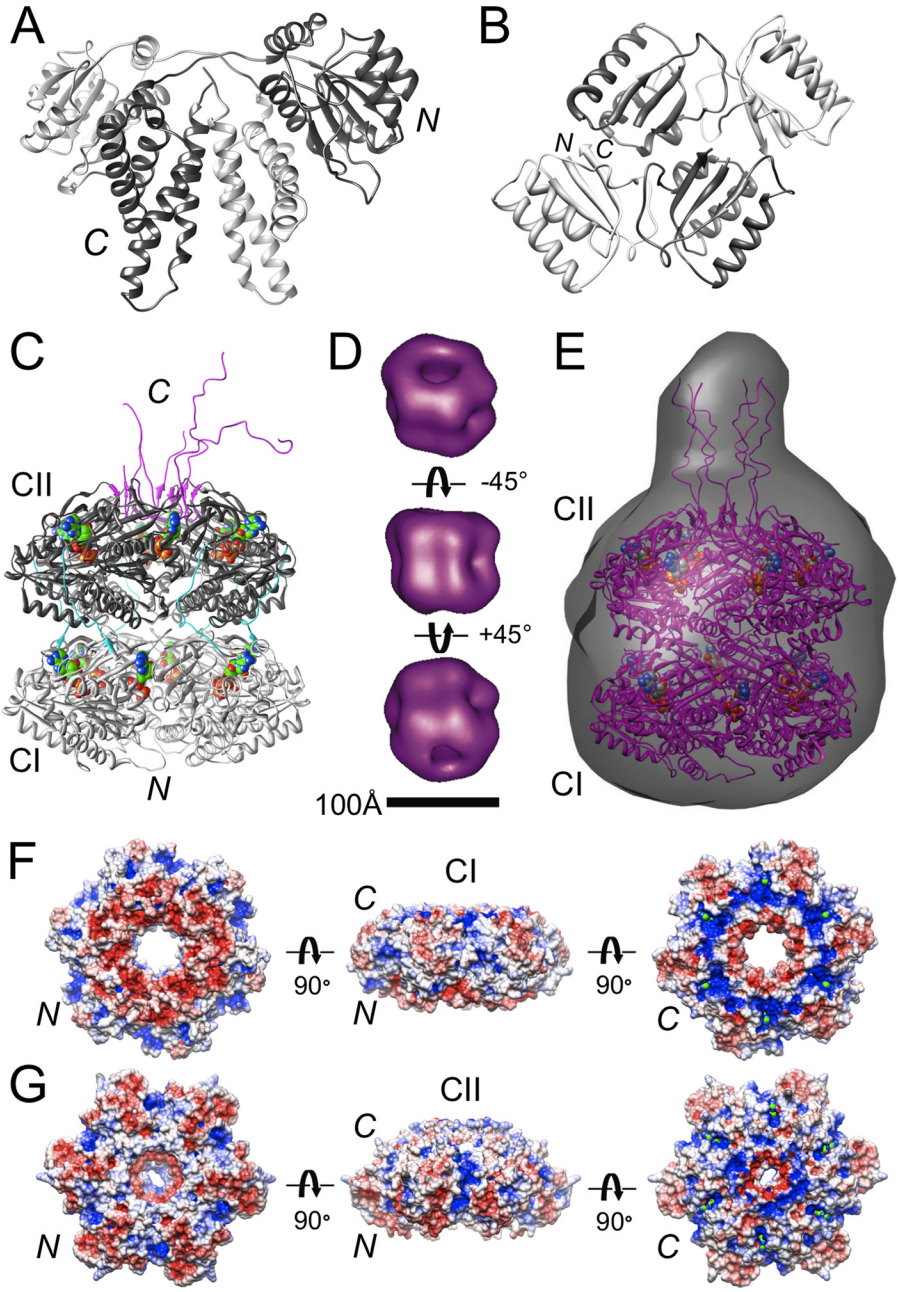


FIGURE 1. **Architecture of *S. elongatus* Kai proteins.** *A*, crystal structure of domain-swapped KaiA dimer with subunits colored in *light* and *dark gray*, respectively (61). *B*, crystal structure of KaiB tetramer (30). Structures of KaiC at high and low resolution are shown. *C*, crystal structure of *S. elongatus* KaiC with the N-terminal CI and C-terminal CII domains colored in *light* and *dark gray*, respectively (31, 37). Note the C-terminal tentacles (colored in *magenta*) constituting the KaiA binding site that are resolved to different degrees at 2.8 Å resolution. ATP molecules are drawn in space-filling mode with carbon atoms highlighted in *green*. In all depictions of KaiC included here, the original orientation has been retained, *i.e.* the N-terminal CI ring is at the *bottom* and the C-terminal CII ring is at the *top*. *D*, negative stain EM structure of KaiC viewed from the side (*center*) and rotated by +45° (*bottom*) and −45° (*top*) around the horizontal axis (37). *E*, SAXS structure of KaiC (34). The protrusion distinguishes the CII from the CI end. ESPs of the CI (*F*) and CII (*G*) rings in KaiC hexamer are shown. The minimum and maximum values of the electrostatic potential are −5 (*red*) and +5 *kt*/*e* (*blue*), respectively. KaiC rings are viewed from the N terminus (*left*), the side (*center*), and the C terminus (*right*). ATP molecules (*green*) can be seen inside positively polarized clefts in the views from the C-terminal side.

or whether the activity is sensed allosterically, *e.g.* at some distance from the CI ring?

Simplifying somewhat, one could attribute the difficulties to track down function and KaiC interactions of KaiB to the complexity of KaiC activities and architecture. The former are composed of kinase, phospho-transferase (ATP synthase (47, 48)), and ATPases in CII and CI. KaiB may modulate phosphorylation, dephosphorylation, or ATP hydrolysis, or perhaps influence the ratio between bound ADP and ATP (49). Because of the rotational symmetry of the KaiC particle (Fig. 1*D*), in the absence of high-resolution structural information, it is difficult to determine which ring is which and therefore the location of KaiB on KaiC. Moreover, the intrinsically dynamic nature of the KaiABC clock poses hurdles for structural studies of protein-protein interactions. Rather than moving in lockstep, individual Kai proteins coexist with binary and ternary complexes such that the concentrations of individual species in the PTO oscillate over the daily cycle both *in vitro* and *in vivo* (20).

KaiC Architecture

The two fused hexameric rings of KaiC that display similar widths and heights (31, 37) are consistent with the earlier notion that the protein is the result of a gene duplication (50). In the crystal structures of KaiC, with resolutions of up to 2.8 Å, the symmetry is broken by the C termini that protrude from the CII ring (Fig. 1*C*). In three-dimensional reconstructions of KaiC based on negative stain or cryo-EM (at 16 Å resolution or less), these C termini are invisible (Fig. 1*D*). Averaging typically wipes out electron density that could be attributed to the tails (20, 51, 52), and any attempt to distinguish between the CI and CII ends in raw negative stain images or class sums is guesswork at best. However, the KaiA dimer in EM images of KaiA-KaiC binary complexes marks the CII end because this protein binds to a C-terminal KaiC tail (37). Also SAXS studies of KaiC alone allow a distinction of the CI and CII ends, although the resolutions achieved with this technique and negative stain EM are probably not too different. Thus, the CII tails appear as a large protrusion in molecular envelopes computed to simulate scattering curves (Fig. 1*E*) (34). A comparison of the shapes of the KaiC hexamer based on crystallography, EM, or SAXS offers no evidence of large scale conformational changes, consistent with SAXS studies that established variations in KaiC volume of no more than 4% over the daily cycle (43).

At the level of the CI and CII domain sequences and folding, there is some similarity to replicative helicases (*e.g.* DnaB) and recombinases (*e.g.* RecA) (50), but the KaiC hexameric rings show less likeness to helicases. In terms of both the hexameric ring conformation and the active site configuration, KaiC shows the closest resemblance to F_1-ATPase, although the similarity is not apparent at the sequence level (31, 47). The KaiC crystal structure readily helps rationalize the higher stability of the CI hexamer (31, 32) relative to CII that does not form hexamers when expressed as a separate domain with KaiC from both *S. elongatus* (29) and *T. elongatus* (53). Similar active site configurations in KaiC and F_1-ATPase and the aforementioned lack of large conformational changes between the kinase and "phosphatase" states of KaiC are also in line with the ATP synthase mechanism of dephosphorylation (47). Lastly, hexameric assembly and kinase, ATP synthase, and ATPase all mapping to the same active sites at CII subunit interfaces are consistent with allosteric control of KaiA-stimulated KaiC phosphorylation (38) and with communication and cooperative effects between KaiC protomers (54).

Although the shapes and dimensions of the CI and CII rings appear to be similar at first glance, comparison of separate surface models reveals some important differences. The C-terminal surface of CI is essentially flat (Fig. 1*F*, *center panel*), whereas the corresponding side of CII has a dome-like appearance (Fig. 1*G*, *center panel*). ATP molecules wedged between subunits are apparent in clefts that open to the upper C-terminal surfaces of both rings (Fig. 1, *F* and *G*, *right panels*). However, these clefts in the CI hexamer are obscured at the waist by the CII hexamer. The electrostatic surface potentials (ESPs) of the bottom (N-terminal) and top (C-terminal surfaces) of the two rings are very different (55). The former is negatively polarized, and the latter is quite positive. This means that the N-terminal side of full-length KaiC is essentially flat and negatively polarized and the C-terminal side is convex and positively polarized (31).

The KaiCII ATPase integrates input signals through the ATP/ADP ratio and fuels the kinase activity, whereas the KaiCI ATPase acts as an input-independent timer and is needed for KaiB-KaiC complex formation (42, 46). Mixing separate CI and CII hexamers abrogates rhythmicity (53), and covalent linkage of the CI and CII domains of subunits is therefore needed for proper function. The structural basis for cross-talk between the CI and CII rings remains to be elucidated. It was suggested that rhythmic stacking and unstacking of the two rings drives the oscillator forward (41), whereby subunit interactions in the CII ring rhythmically tighten and relax (45). It was previously established that phosphorylation across subunit interfaces creates additional salt bridges and H-bonds (11) and that the volume of the KaiC particle is smallest in the hyperphosphorylated state (43). Crystal structures of KaiC hexamers offer no evidence for changes in the vertical separation of the CI and CII rings. However, a comparison of the structure of *S. elongatus* KaiC (31) with the recently determined structure of *T. elongatus* KaiC (32) revealed a difference between the relative rotations of CI and CII rings around the axis along the central channel that amounts to ~7 degrees. Therefore, it is possible that the KaiC hexamer undergoes a twisting motion over the daily oscillation instead of an up and down movement.

KaiC Phosphorylation Patterns

The strict order of phosphorylation of Thr-432 and Ser-431 is a hallmark of the KaiABC clock (13, 14). Researchers have replaced threonine and/or serine with aspartic or glutamic acid (*e.g.* T432E/S431E) to mimic phospho-threonine and phospho-serine sites, or with alanine to prevent transfer of the phosphate and lock KaiC either in the hypophosphorylated state (T432A/S431A) or in the second stage of dephosphorylation (S431D or S431E) (8, 23, 35, 41, 42, 46, 54, 56–58). Because binding of KaiB to KaiC involves either a particular conformational state and/or electrostatic polarization of the latter, it is important to keep in mind the limitation of such mimicry. Neither shape nor charge (−1) of aspartate or glutamate is a good match for phospho-threonine or -serine (charge −2). Not surprisingly, the ESPs of the hypophosphorylated (*e.g.* T432A/S431A) and hyperphosphorylated (pTpS) KaiC forms as well as the T432E/S431E mimic of the hyperphosphorylated state show significant differences (34). By comparison, the difference in ESP between the T432A/S431A and the TS hypophosphorylated forms is likely smaller, but functional assays also uncovered differences between the T432A/S431A mutant and unphosphorylated KaiC (59). Crystal structures of KaiC Thr-432 and/or Ser-431 mutants displayed phosphorylation patterns that are not always consistent with the particular state of phosphorylation that such mutants were intended to mimic (Table 1). Thus, T432E/S431A KaiC (pTS mimic) featured Thr-426 phosphorylation in four subunits, S431D KaiC (TpS mimic) featured three phosphorylated Thr-432 residues (40), and T432E/S431E (pTpS mimic) exhibited a new phosphorylation site at Ser-320 in two of the six subunits (34). The structural data suggest that the mimicry with Asp or Glu residues of particular KaiC phosphor-

TABLE 1
Phosphorylation patterns in WT and mutant KaiC crystal structures
Phosphorylation patterns for mutants are based on the *S. elongatus* KaiC sequence. Dashes indicate absence of phosphorylation.

KaiC protein	Number of phosphates in the CII half				Reference
	Thr-432[a]	Ser-431[a]	Thr-426[a]	Ser-320[a]	
Wild type[b]	6	4	—	—	31, 37
Wild type[c]	6	6	—	—	32
T432A	—	6	—	—	40
S431A	6	—	1	—	40
S431D	3	—	—	—	40
T426N	6	3	—	—	40
T432A/T426A	—	6	—	—	40
T432E/S431A	—	—	4	—	40
T432E/S431E	—	—	—	2	34
E318A	5	—	—	—	47
A422V	6	—	—	—	38

[a] Phosphorylation site.
[b] *S. elongatus*; quasi-hyperphosphorylated.
[c] *T. elongatus*; hyperphosphorylated.

ylation states along the daily cycle is not perfect, a caveat one should bear in mind when interpreting the outcome of experiments using the various KaiC single or double mutants discussed above.

Evidence That Supports Binding of KaiB to KaiCII

Looking back at the evolution of investigations directed at KaiB function and the KaiB-KaiC binding mode, it is no surprise that the first place to look for KaiB was the CII end. KaiA associates with CII-terminal tails, and because KaiB is thought to antagonize KaiA action, it is certainly possible that it does so by occupying a spot on KaiC in the vicinity of KaiA. Initial SAXS models of complexes featured both KaiA dimer and KaiB tetramer bound near the CII dome (43). Phosphorylation alters the ESP at the CII end but is not "felt" on the opposite side (34), a change that might be sensed by KaiB and trigger binding to CII (KaiB was reported to favor the phosphorylated form of KaiC (18)). However, no other evidence besides the assumption that antagonism could involve close association between KaiA and KaiB tied KaiB to KaiCII in the above SAXS study. Rather than surveying the evidence supporting a KaiCII binding mode by KaiB in a chronological order, let us start with the most recent data. An overview of structural and biophysical observations pertaining to the CII binding mode is given in Fig. 2.

Cryo-EM at 16 Å resolution on the one hand (30) and hydrogen-deuterium exchange (HDX) in combination with native MS on the other (44) independently provide support for six KaiB monomers interacting with the KaiC hexamer, *i.e.* a $KaiC_6B_6$ stoichiometry (Fig. 2, *A* and *E*, respectively). The resolution of the EM density alone is insufficient to unambiguously assign KaiB monomers to the CII side. However, regions of the strongest EM density normally associated with α-helical regions are consistent with a CII binding mode (Fig. 2*C*). This is because helical portions are concentrated in the N-terminal halves of the CI and CII rings (Fig. 1*C*). A negative stain EM investigation using KaiC with C-terminal His_6 tags and imaging the KaiBC complex with or without 1.8-nm nickel-nitrilotriacetic acid-Nanogold clearly showed gold, His tags, and KaiB congregating on the same side (CII; Fig. 2*B*) (55). The HDX data reveal protection in areas that include the subunit interface of one KaiC ring, and collisional cross-sections together with computational simulations favor the CII binding mode (Fig. 1*E*) (44). The cryo-EM results were also complemented by molecular dynamics simulations that indicated a clear advantage of the CII interface in terms of the buried surface when compared with CI (30).

The convergence of cryo-EM and HDX/MS in terms of the KaiBC model, the B-C stoichiometry, and the monomeric form of KaiB targeting the C hexamer is remarkable. The monomeric form of KaiB associating with KaiC subunits also eliminates a puzzle arising from a model of the complex formed by two KaiB dimers and a single KaiC hexamer (29), namely why subunits constituting the KaiB dimer would engage in deviating interactions with underlying KaiC subunits. In the $KaiC_6B_6$ complex, all six KaiB monomers are expected to occupy equivalent sites along the KaiCII ring and interact with KaiC in an identical fashion (Fig. 2*A*). What other evidence exists in support of the CII binding mode? The SAXS envelope computed for the KaiBC complex may seem inadequate to distinguish between KaiB binding at "the top" or at "the bottom" (Fig. 2*D*) (34). However, if we take into account the SAXS envelope for KaiC alone (Fig. 1*E*), it becomes evident that KaiBs are unlikely to be associated with the CI ring as the SAXS envelope of KaiBC should otherwise be composed of four layers (KaiB ring, CI ring, CII ring, CII tail protrusion).

It is important to stress that the conclusion that KaiB binds on the CII side was not reached by essentially trying to distinguish between the CI and CII rings at low resolution. Negative stain EM density does not allow one to assign the KaiC ring contacted by KaiB because of insufficient resolution and pseudo-symmetry of the KaiC hexamer (Fig. 1*D*). However, gold labeling gave a clear answer, and these experiments were carried out with full-length KaiB and KaiC proteins from *S. elongatus* (55) that constitute the *in vitro* PTO together with KaiA and ATP/Mg^{2+}. That KaiC His_6 tags may have introduced an artifact is unlikely because C-terminally His-tagged KaiC was used to reconstitute the PTO in the same fashion as WT KaiC (54). The earliest evidence for CII binding actually came from native PAGE experiments that used KaiB with full-length KaiC (Fig. 2*F*) or separate CI (Fig. 2*G*) or CII (Fig. 2*H*) (29). Although the CII domain does not form a stable hexamer in the absence of the CI ring (29, 53), mixing CII and B protein produced a band shift (Fig. 2*H*). Conversely, no complex formation was observed for CI hexamer alone and B protein (Fig. 2*G*).

What are the implications of the KaiCII binding mode for KaiB function? Cryo-EM and HDX/MS in conjunction with modeling both place KaiB monomers at CII subunit interfaces, thereby covering ATP binding clefts (Figs. 1*G* and 2*A*) (30, 44). Because KaiBC association is closely linked to the phosphorylation of Ser-431 (13, 14), *i.e.* KaiB binding occurs when KaiC phosphorylation peaks and continues during dephosphorylation, KaiB could possibly flip the switch from kinase to ATP synthase by perturbing active sites at subunit interfaces. Binding by KaiB would initially be triggered by the change in ESP on CII as a result of phosphorylation (34), but perhaps KaiB does not actively promote KaiC dephosphorylation and instead antagonizes KaiA action only by sequestering KaiA? A recent EM model of the KaiABC ternary complex placed KaiA at the

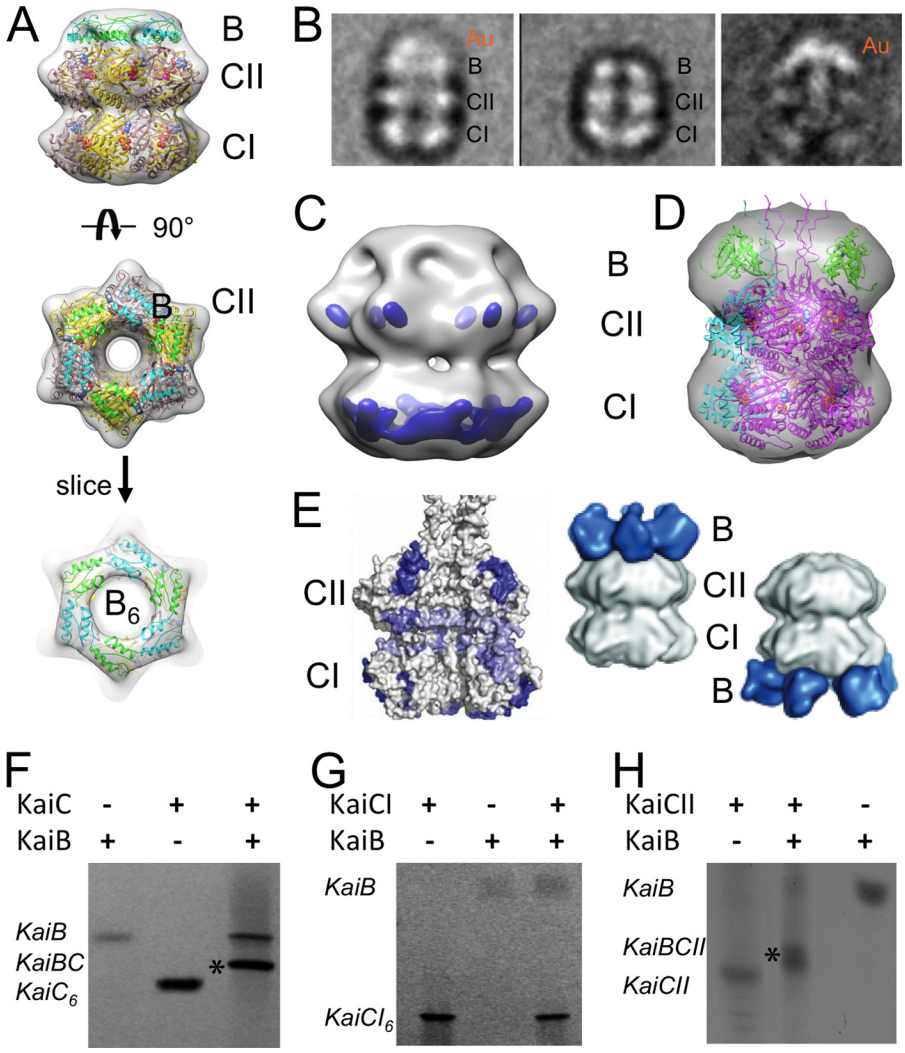
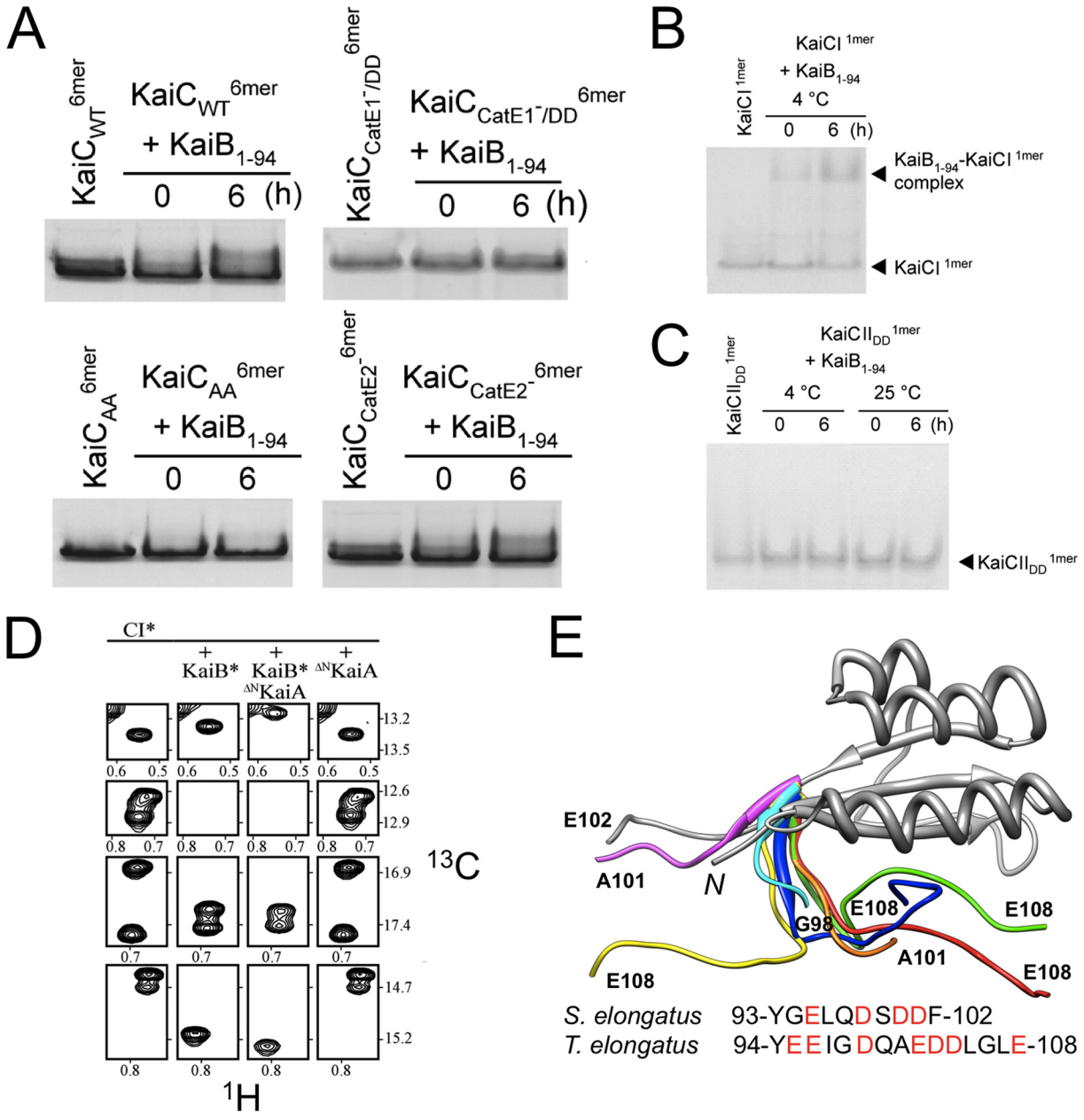


FIGURE 2. **Evidence in support of a KaiCII binding mode by KaiB.** *A*, six KaiB monomers forming a third layer on top of the KaiCII ring, consistent with cryo-EM density (16-Å resolution) and a more extensive buried surface at the KaiCII end based on molecular dynamics flexible fitting (MDFF) simulations when compared with a KaiCI binding mode (30). *B*, negative stain EM study of KaiC with a C-terminal His_6 tail (identical to the construct used for the crystal structure determination; Fig. 1*C*), bound to KaiB and in the presence of 1.8-nm nickel-nitrilotriacetic acid Nanogold (*left panel*), bound to KaiB without gold (*central panel*), and applying a weighted Fourier difference electron density analysis (*right panel*) (55). The gold labeling approach supports binding of KaiB to KaiCII because of the co-localization of His tag, KaiB, and the *gold cloud* on top of the CII ring. *C*, cryo-EM density of the $KaiC_6B_6$ complex (30). The strongest density (*blue regions*) maps to the α-helical regions of KaiCI and KaiCII (Fig. 1*C*), which is supportive of KaiBs bound to the β-rich side of the KaiCII ring. *D*, SAXS investigation of the KaiBC complex with crystallographic models of KaiC hexamer (*magenta*, with one subunit highlighted in *cyan*) and KaiB dimers (*green*) modeled into the three-dimensional envelope (34). The observation that the protrusions produced by the C-terminal KaiCII tails (Fig. 1*C*) and by KaiB appear on the same side supports a KaiCII binding mode. *E*, side view of three KaiC subunits rendered in surface mode with *blue patches* indicating regions between subunits that are protected from HDX (*left panel*). HADDOCK-based models of the $KaiC_6B_6$ complex with KaiB monomers bound to either the CII (*central panel*) or the CI ring (*right panel*) (44). The former is favored based on MS-based ion mobility spectrometry-derived collisional cross-section data. *F–H*, native PAGE assays of the complex formations between full-length KaiC hexamer and KaiB (*F*), KaiCI hexamer and KaiB (*G*), and KaiCII and KaiB (*H*) (KaiCII alone does not form a stable hexamer) (29). KaiB binds to KaiC and to KaiCII but not to KaiCI (complex bands are marked by an *asterisk*).

KaiBCII interface, such that KaiAs protrude from the side of the third (KaiB) ring (34). In this fashion, each KaiB monomer can sequester a KaiA molecule, thus preventing them from contacting KaiC C-terminal tails and stimulating phosphorylation. It would appear that a CII binding mode by KaiB is consistent with the current (however incomplete) understanding of the function of KaiB. However, a celebratory attitude may be somewhat premature because a further requirement for KaiB binding to KaiC cannot be readily reconciled with the KaiCII binding mode. According to two recent studies, KaiBC complex formation is preceded by a cycle of ATP hydrolysis in the CI half (42, 46).

Evidence That Supports Binding of KaiB to KaiCI

Using the proteins from *S. elongatus*, Rust and co-workers (46) found that the amount of WT KaiB bound to a hexameric KaiC with the T432E/S431E double mutation in CII and catalytic glutamates replaced by glutamine in CI (abolishing the ATPase there) was strongly reduced relative to WT KaiC (to ~20%). Ishiura and co-workers (42) observed that the hexameric *T. elongatus* KaiC T432D/S431D mutant without the ATPase in CI ($KaiC_{CatE1^-/DD}$) and KaiB lacking the C-terminal tail (14 amino acids; $KaiB_{1-94}$) do not interact (Fig. 3*A*). The same KaiB mutant does bind to a KaiC mutant hexamer that lacks the ATPase in CII ($KaiC_{CatE2^-}$) and to WT KaiC, but not to


A
KaiC_WT 6mer
KaiC_WT 6mer + KaiB_1-94
0 6 (h)
KaiC_CatE1-/DD 6mer
KaiC_CatE1-/DD 6mer + KaiB_1-94
0 6 (h)
KaiC_AA 6mer
KaiC_AA 6mer + KaiB_1-94
0 6
KaiC_CatE2- 6mer
KaiC_CatE2- 6mer + KaiB_1-94
0 6
B
KaiCI 1mer
KaiCI 1mer + KaiB_1-94
4 °C
0 6 (h)
KaiB_1-94-KaiCI 1mer complex
KaiCI 1mer
C
KaiCII_DD 1mer
KaiCII_DD 1mer + KaiB_1-94
4 °C 25 °C
0 6 0 6 (h)
KaiCII_DD 1mer
D
CI*
+ KaiB*
+ KaiB* ΔNKaiA
+ ΔNKaiA
13C
1H
E
E102
A101
N
G98
E108
E108
A101
E108
E108
S. elongatus 93-YGELQDSDDF-102
T. elongatus 94-YEEIGDQAEDDLGLE-108
F
E108
Y94
Y94
G
G250
P5
B-loop

the KaiC T432A/S431A mutant hexamer that mimics the hypophosphorylated state (Fig. 3*A*). Moreover, the $KaiB_{1-94}$ deletion mutant binds to KaiC monomer and a KaiCI monomeric domain (Fig. 3*B*), but not to a KaiCII monomeric domain with the T432D/S431D double mutation (Fig. 3*C*) (42). Although these observations are intriguing, it is important to remember that WT KaiB does not form a complex with a KaiC monomer (42). NMR studies of a KaiB mutant lacking the N-terminal tail (seven residues) in addition to the above C-terminal deletion and additional Y8A/Y94A mutations (KaiB*) mixed with a FLAG-tagged monomeric CI domain (CI*) revealed formation of a specific complex (Fig. 3*D*) (60). No complex is observed when CI* and KaiA lacking the N-terminal domain ($^{\Delta N}$KaiA) are mixed alone (Fig. 3*D*). Conversely, shifts in NMR signals are consistent with formation of a ternary CI*-B*-$^{\Delta N}$A complex (Fig. 3*D*), indicating that the CI*B* complex can sequester KaiA.

Why use a KaiB deletion mutant in the first place? Removal of the C-terminal tail of KaiB abolished the *in vivo* rhythm of the KaiABC clock (28). A subsequent publication reported normal *in vitro* oscillations based on the $KaiB_{1-94}$ mutant, but attenuated amplitudes of oscillations and a lack of normal gene expression rhythms in cells expressing $KaiB_{1-94}$ (7). Both $KaiB_{1-94}$ and KaiB* appear to favor the dimeric over the tetrameric state (7, 42, 60). As demonstrated by native MS, the quaternary structure adopted by KaiB, tetramer, dimer, or monomer, is influenced by both protein concentration and temperature, with lower concentrations and reduced temperatures favoring the monomer (44). From KaiB crystal structures (no NMR solution structure of KaiB has been reported to date), it is not obvious why removing the C-terminal tail should favor the dimer. The tails adopt a wide range of conformations in the crystal and do not fold back on the core structure in the tetramer (Fig. 3*E*). However, removal of the tail reduces significantly the number of acidic residues (Fig. 3*E*) and inverts the ESP on one face of KaiB from strongly negative to weakly positive (Fig. 3*F*). KaiA and KaiB do not form a complex according to native PAGE and fluorescence assays (29). However, KaiA ($^{\Delta N}$KaiA) and KaiB* do form a complex as demonstrated by NMR (60). Clearly, this observation argues against the KaiB C-terminal tail contacting KaiA. Rather, the tail might be involved in the KaiBC interaction such that it is tucked away at the KaiBC interface and then allows sequestration of KaiA. In this context, it is crucial to remember that WT KaiB does not bind KaiC monomer and that for the KaiB C-terminal tail to properly engage, a KaiC hexamer (including subunit interfaces) appears necessary.

What are the implications of the KaiCI binding mode for KaiB function? The ATPase activity needed to promote KaiB binding there could go along with a conformational change in CI that exposes a new interface or expands an existing one (42). The C-terminal tail of KaiB might assist in prying open an already weakened subunit interaction in the CI ring as a result of the hydrolysis of ATP to ADP and thus assist in the formation of a stable interaction between KaiB and CI from individual subunits. It has been suggested that the binding site on CI involves the so-called B-loop (Fig. 3*G*) (61). This loop is highly acidic and contributes to the overall negative ESP at the CI end of the hexamer (Fig. 1*F*). The loop is more extended when compared with the corresponding CII region that is buried at the waist (32). It is somewhat implausible that KaiB with a negatively polarized ESP as a result of the C-terminal tail (Fig. 3*F*) would interact with a negatively polarized B-loop alone. For one, B-loops are available on the outer surface of the CI ring, and accessing them does not necessitate ATPase activity. Moreover, computational simulations of $KaiC_6B_6$ complexes with KaiB monomers located on either the CI or the CII end demonstrated a significantly enhanced buried surface for the latter interaction (30, 44) (Fig. 2*E*). In any case, KaiB binding to CI seems to involve individual C subunits, unlike the proposed interaction at the CII end, where KaiB monomers bridge adjacent KaiC subunits by covering ATP binding clefts (Fig. 2*A*). Because the CI ring is more stable, KaiB docking there could disrupt the structural platform critical for holding together the entire hexamer, which is more in line with subunit shuffling during the dephosphorylation phase (18–20) than a CII binding mode. However, initial experiments to probe the effects of the presence of either KaiA or KaiB on KaiC monomer exchange did not show an enhancement of the process by KaiB (20). Both the CI and the CII binding modes by KaiB in principle allow sequestration of KaiA at the KaiBC interface. A key difference lies in the initial trigger of KaiB association with KaiC. The KaiCII interaction is preceded by kinase activity and a concomitant change in the ESP that may be sensed by KaiB; the

FIGURE 3. **Evidence in support of a KaiCI binding mode by KaiB.** *A*, a native PAGE assay indicates that KaiBC complex formation requires KaiCI ATPase activity (*T. elongatus* Kai proteins (42)). Clockwise from *top left*: WT KaiC forms a complex with a KaiB truncation mutant that lacks the 14 C-terminal residues (13% of full-length KaiB; $KaiB_{1-94}$). No complex is observed with a KaiC mutant that is unable to hydrolyze ATP in CI ($KaiC_{CatE1}$-) and contains the T432D/S431D double mutation in CII that mimics hyperphosphorylation (*DD*); conversely, complex formation occurs with a KaiC mutant that lacks kinase and ATPase activity in CII ($KaiC_{CatE2}$-); the T432A/S431A double mutation in CII that mimics hypophosphorylation (*AA*) abolishes complex formation. Please also see Ref. 46 for the requirement of CI ATPase activity to support KaiBC complex formation (*S. elongatus* Kai proteins). *B*, the monomeric KaiCI domain forms a complex with $KaiB_{1-94}$ (42) *C*, on the contrary, complex formation with $KaiB_{1-94}$ is not observed using the monomeric KaiCII domain that carries the T432D/S431D double mutation (*DD*) (42). *D*, methyl transverse relaxation optimized spectroscopy (TROSY) spectra of U-[^{15}N,^{2}H]Ile-δ1-^{13}C,^{1}H-labeled *T. elongatus* CI* alone (* indicates N- and C-terminal FLAG tags (*column 1*); in the presence of KaiB* (= Y8A/Y94A double mutation, 8–94 truncation form) (*column 2*); in the presence of KaiB* + $^{\Delta N}$KaiA (KaiA lacking the N-terminal domain) (*column 3*); and in the presence of $^{\Delta N}$KaiA alone (*column 4*) (60). These data support binding between KaiCI monomer and KaiB* dimer, whereby the dimer form is supported by combined SDS-PAGE/gel filtration assays (not shown). *E*, superimposition of eight *T. elongatus* KaiB monomers (from crystal structures of *T. elongatus* WT KaiB (29) and Thr-64 mutant tetramers (28)) with C-terminal tails (residues 94 up to 108) that were resolved to various degrees in electron density maps colored differently for individual monomers. The C-terminal sequences of *S. elongatus* KaiB (102 amino acids) and *T. elongatus* KaiB (108 amino acids) are included below the drawing for comparison. Note the different numberings (Tyr-94 in *T. elongatus* KaiB corresponds to Tyr-93 in *S. elongatus* KaiB) and highly acidic character of the tails (Glu and Asp are highlighted in *red*). *F*, electrostatic surface potentials (ESPs) of *T. elongatus* KaiB encompassing all 108 residues (*left*) and with the C-terminal residues 95–108 removed (right; $KaiA_{1-94}$, see *panels A* and *B*). The minimum and maximum values of the electrostatic potential are −5 (*red*) and +5 *kt*/*e* (*blue*), respectively. *G*, secondary structure graphic of a monomeric CI domain from the crystal structure of *T. elongatus* KaiC (32). ATP/Mg^{2+} and a negatively polarized loop region (now termed B-loop (60)) located at the N-terminal surface of the KaiC hexamer are highlighted in *ball-and-stick mode* and in *magenta*, respectively.

KaiCI interaction is preceded by ATPase activity and a concomitant conformational change that provides access for KaiB.

Perspective

Studies of the cyanobacterial KaiABC circadian clock have led to new insights into the mechanism of circadian time keeping. To a large degree this is due to the discovery that the KaiA, KaiB, and KaiC proteins in the presence of ATP constitute a PTO that can be studied independently from the TTFL. As far as KaiB function and its interactions with KaiC and KaiA are concerned, there are currently more questions than answers. Clearly, sequestration of KaiA by KaiB that is itself bound to KaiC is of central importance for maintaining a stable oscillator. Further, there is strong evidence that ATPase activity in the KaiCI half boosts KaiBC complex formation. KaiB tetramers dissociate, and it is the monomeric form of KaiB that interacts with KaiC. However, no consensus exists in the field as to where KaiB binds on the KaiC hexamer. There are observations that cannot be rationalized with a KaiCI binding mode and others that defy a binding mode that involves only KaiCII. KaiB monomers bound at the CII end are shared between adjacent subunits, but KaiB docking at the CI end involves only individual subunits (monomers).

A hybrid structural biology approach has been useful to characterize Kai protein-protein interactions at low resolution. A better understanding of KaiB function and its role in KaiA sequestration at the level of KaiC hexamer now requires crystal or high-resolution cryo-EM structures of KaiBC and KaiABC complexes. KaiB interacting with a KaiC monomer or a KaiCI domain opens the door to structure determination of complexes by solution NMR. However, it appears unrealistic to gain a complete understanding of a >400-kDa molecular machine by analyzing 40-kDa fragments in the absence of (i) ATP, (ii) KaiC phosphorylation, and (iii) cooperative effects among KaiC subunits, and with (iv) KaiB mutants that bind KaiC monomers although WT KaiB does not. Recent observations raise the possibility that KaiB can contact KaiC at both the CI and the CII ends. Experiments using tagged KaiC constructs for EM or mutants carrying spin labels for electron paramagnetic resonance could be used to address the hypothesis that KaiB binds both halves. A dual binding mode seems unnecessary just to sequester KaiA. Perhaps KaiB is not just a key component of the PTO but is also involved in the regulation of the clock output pathway. These roles may necessitate different KaiC binding modes and involve either KaiC hexamer or KaiC monomer. Breakthroughs in both the structural and the functional realms are still needed for a comprehensive mechanistic understanding of the cyanobacterial KaiABC PTO.

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