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Simplicity and complexity in the cyanobacterial circadian clock mechanism

Guogang Dong[*], **Yong-Ick Kim**[*], and **Susan S. Golden**
Center for Chronobiology, University of California – San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0116

Summary

The circadian clock of the cyanobacterium *Synechococcus elongatus* PCC 7942 is built on a three-protein central oscillator that can be reconstituted *in vitro*, a redox-sensitive input for synchronization with the environment, and a bacterial two-component signal transduction pathway for global transcriptional regulation. This review covers the most recent progress in our understanding of the biological and biochemical mechanism of this bacterial clock, such as the discovery of a quinone-binding activity of the oscillator protein KaiA, the molecular mechanism of circadian control of cell division, and the global control of gene expression via modulation of DNA topology.

Introduction

Most living organisms on Earth ranging from bacteria to humans experience striking changes in their environment that accompany the daily alternations of day and night, and have evolved an internal timing system named the circadian clock to cope with this predictable cycle. *Synechococcus elongatus* PCC 7942, a freshwater unicellular cyanobacterium, is the simplest and the most ancient form of life demonstrated to possess a circadian clock to date. The core oscillator of the cyanobacterial clock is composed of three proteins: KaiA, KaiB and KaiC [1], whose structures have been solved and biochemical activities well characterized [2-4]. KaiC is an autokinase, autophosphatase, and ATPase. Both the abundance and the phosphorylation states of KaiC cycle *in vivo*. Remarkably, a simple mixture of the three Kai proteins with ATP reconstitutes the circadian oscillation of KaiC phosphorylation and ATP hydrolysis *in vitro*, establishing the first and only cell-free circadian oscillator [5,6]. The circadian clock stays synchronized with the environment through one or more input pathways that inform the oscillator of changes in the cellular redox state, which in this photosynthetic organism correlate with the presence of light [4]. An output pathway that includes a bacterial two-component signal transduction system relays the timing information from the central oscillator to many key processes downstream. For the past two years significant progress has been made in many aspects of the clock, including redox sensing by the oscillator, the correlation between DNA topology and global transcriptional activity, and the mechanism of circadian gating of cell division in *S. elongtus*.

Corresponding authors: Guogang Dong (guogang.dong@gmail.com), Yong-Ick Kim (yik002@ucsd.edu).
[*]These authors contributed equally

Circadian transcriptome

An early promoter trap experiment with a luciferase reporter found that all promoters tested are expressed rhythmically [7]. With the advent of a complete genome sequence and microarray technology, two groups independently examined mRNA profiles of almost all annotated genes in constant light (clock free-running) conditions, and found that transcripts from 30% to 64% of the genomic repertoire, depending on the study and the analysis method adopted, accumulate rhythmically [8,9]. This is a considerably larger fraction than was shown by similar studies conducted in eukaryotic systems [10]. Both studies found that the majority of rhythmic genes peak either at subjective dawn or subjective dusk, and that, with the exception of genes that are arranged in an operon, there is no correlation between the phase angle (relative timing of peaks) of rhythmic transcripts and the location of their genes on the chromosome.

Recent results strengthen the correlation between DNA topology and the phasing of circadian transcript peaks that was suggested previously [11-13]. Vijayan *et al.* surveyed both the transcriptome and DNA topology at the same time points in a circadian cycle, and found a good correlation between the global transcription profile at a given time and DNA topology as displayed by an endogenous plasmid [8]. When a gyrase inhibitor is applied at the chromosome's most supercoiled phase of the circadian cycle (subjective dusk) to quickly relax DNA superhelicity, 79% of the rhythmic ORFs change in the direction that is predicted from the established correlation; e.g., expression of ORFs that peak when DNA is most supercoiled goes down. Furthermore, the authors found a significant difference in the GC content in the promoter regions of genes that peak at different times. Collectively, these results suggest a causal relationship between DNA topology and transcription activity, and further support the oscilloid model, which states that the clock controls global gene expression by modifying chromosome topology [14].

Ito *et al.* surprisingly discovered at least two rhythmic transcripts in a *kaiABC* null background [9], which suggests the presence of *kai*-independent oscillations. However, given the important implications for clock mechanism in this organism, this result needs to be further validated by a different method.

Circadian oscillation of KaiC phosphorylation

A circadian oscillation in phosphorylation of KaiC occurs *in vivo* as well as *in vitro* [6,15, 16]. The mechanism of KaiC phosphorylation is comparatively well understood. Two residues in KaiC, serine at position 431 (S431) and threonine at position 432 (T432), can be phosphorylated and dephosphorylated with about a 24 hour period [17,18] (Fig. 1). T432 autophosphorylates (T-KaiC) upon stimulation of KaiC by KaiA, which binds to the C-terminal peptide of KaiC (A-Loop), and S431 subsequently autophosphorylates (ST-KaiC). ST-KaiC undergoes spontaneous dephosphorylation at T432 (S-KaiC). KaiB binds to S-KaiC, which then traps and inactivates KaiA, thus allowing further KaiC dephosphorylation. A recent review by Markson and O'Shea details the molecular events for this reaction, including time delay and synchronization, and the models that have been developed to explain the oscillation [19]. A third residue, T426, is important for KaiC function, as mutation to alanine abolishes circadian rhythm [20]; moreover, T426 can be be phosphorylated in mutants that carry substitutions at the known phosphorylation sites (S431A and S431A/T432E) [21]. The crystal structure of KaiC, which includes a mixture of phosphoforms, shows electron density around T426 suggestive of some level of phosphorylation [20]. KaiA is the primary stimulator of KaiC phosphorylation, and a mutant form of KaiA that lacks the last three residues has a stronger interaction with KaiC, resulting in changes in KaiC phosphorylation kinetics and a shorter circadian period *in vivo* [22].

KaiC is known to dephosphorylate in the absence of KaiA *in vitro* [23] and *in vivo* [24]. Surprisingly, we found partially phosphorylated KaiC *in vivo* in a *ΔcikAΔkaiA* mutant [25], suggestive of the presence of a KaiA-independent, but CikA-suppressed activity that stimulates KaiC phosphorylation. This finding implicates another condition or component that affects KaiC phosphorylation which would need to be added to allow the *in vitro* reaction to fully mimic *in vivo* conditions.

KaiC phosphorylation at the protomer level has been well described and many aspects of the interactions among KaiA, KaiB, and KaiC are also known [17-19,22,23,26-29]. However, KaiC functions as a hexamer at all phosphorylation states and in its interactions with other clock proteins [28,29]. The stoichiometry between the KaiA dimer and KaiC hexamer that supports oscillatory phosphorylation *in vitro* is 1:1; thus, it is of great interest to know how phosphorylation propagates within the KaiC hexamer when only one protomer is stimulated.

What is the role of the phosphorylation cycle? Phosphorylation of the adjacent amino acids likely causes conformational change [30] in the C-terminal domain of KaiC, which moves the activity or partner interface to the next stage in the cycle. Structural evidence of a progression of conformational change is not available for wild-type KaiC, but the published structure does provide some suggestive insights. Each protomer in the hexameric crystal structure has a different conformation, although the differences are small [31]. These variations may reflect sampling of the mixed phosphorylation states for the crystal. Because KaiC is autocatalytic, it is difficult to constrain the wild-type protein to yield a homogenous population of hexamers that have the same phosphorylation state. Recently, Pattnayek *et al.* solved crystal structures of KaiC mutants which have controlled phosphorylation states [32]. In these structures, KaiC mutants locked in different states clearly show conformational differences.

Gating of cell division and KaiC ATPase activity

In a cellular context, the circadian clock must coexist with the cell cycle, which operates with a different, and varying, period. In organisms for which the relationship between these two oscillators has been examined, the circadian clock gates cell division, allowing division at some phases while inhibiting it at others [33]. Similarly, the *S. elongatus* circadian clock gates cell division, whereas the cell cycle has no effect on the circadian clock, regardless of the speed at which the cell cycle runs [34,35]. Recent work from our lab unveiled a linear signal transduction pathway, involving all major components of the clock, that inhibits cytokinesis (Fig. 2) [36]. Specifically, mutants with elevated KaiC ATPase are associated with cell elongation. The absence of CikA, a major player of the input pathway, causes cell elongation in a KaiC-dependent manner, suggesting that it may suppress KaiC ATPase activity, directly or indirectly. Furthermore, clock output components SasA and RpaA act downstream of KaiC in cell division control, and formation of the septation ring by FtsZ, a bacterial tubulin homolog, is targeted by this clock pathway. Yang *et al.* tracked circadian rhythms and cell division events simultaneously in individual cyanobacterial cells and developed a mathematical model describing the coupling of the two oscillators, which could be extended to any coupled biological oscillations [37]; this work is discussed in more depth by Pando and Van Oudenaarden in this issue. It is still unclear, however, why there is a cell division gate. Given that daughter cells inherit the circadian clock with great precision from their mother cells [38], it is possible that the gate protects the clock from perturbations that would result from cell division at a vulnerable phase.

It is a surprise that elevated ATPase activity rather than a specific phosphorylation state of KaiC causes the closing of the gate. KaiC is an unorthodox ATPase, hydrolyzing an average of only 15 ATP molecules per KaiC monomer per day as measured *in vitro* [5,36]. However, this activity oscillates in a circadian manner, is correlated with circadian period, and is required

for the circadian clock [5]. Given the low level of this activity, it is unlikely for KaiC to affect the overall energy balance of the cell, or do any significant physical work. It is thus important to understand the role of KaiC ATPase, although its intimate coupling with KaiC phosphorylation complicates the analysis. Terauchi *et al.* concluded from KaiC phosphomimetics that KaiC phosphorylation suppresses ATPase activity. However, KaiC truncation mutants that are either constitutively phosphorylated or nonphosphorylated showed the opposite correlation [36]. The discrepancy lies in the nature of the mutants that were employed, as the phosphorylatable residues are replaced in the phosphomimetics, resulting in a KaiC that is unphosphorylated, but has elevated ATPase activity. In our study, the carboxyl terminus of KaiC that controls a switch between kinase and phosphatase activities is absent, but S431 and T432 are intact. It appears that KaiC ATPase activity is influenced more by its interaction with KaiA and KaiB than by a particular phosphorylation state.

The basic timing loop

In eukaryotes transcription translation feedback loops (TTFL) are essential for the fundamental oscillation of the circadian clock [39]. The Kondo group reported that the cyanobacterial oscillator can run without a TTFL [6,16], and that a gene expression rhythm remains when KaiC is arrested at a certain phosphorylation state [40]. These data suggest that the Kai protein oscillator and a TTFL exist independently, but that both are important for a robust circadian clock. However, Johnson and colleagues found that the gene expression rhythm quickly damps when KaiC phosphorylation is clamped, and concluded that the protein oscillator is the master pacemaker, whereas TTFL is a slave. Through mathematical modeling and experiments, they showed that the integration of TTFL with the protein oscillator improves the robustness of the circadian clock, and that a TTFL can provide an entraining signal to the post-translational Kai-based pacemaker [41].

Synchronization and resetting of circadian oscillations

Light is perceived directly through photoreceptors in eukaryotic clock systems [39]. However, evidence mounts that cyanobacteria synchronize the internal circadian oscillator by sampling cellular redox state rather than sensing light directly. Two major components of the input pathway, LdpA and CikA, are both redox sensitive. LdpA is an iron-sulfur protein [42,43], whereas the pseudo-receiver domain of CikA binds a quinone [44]. The N-terminus of KaiA is also a pseudo-receiver—a domain that shares the fold of phosphorylated signal transduction receiver domains, but lacks the conserved phosphoaccepting Asp residue [45]. We showed that KaiA binds the same quinone analog that binds to CikA, and aggregates upon such binding *in vitro*, which negatively affects KaiC phosphorylation [46]. More specifically, only the oxidized form of quinone binds to KaiA, whereas the reduced form has no effect on KaiA structure or activity. Therefore, external light cues that change the oxidation state of electron carriers in cyanobacteria may modulate the phosphorylation states of KaiC, and thereby synchronize the internal circadian clock to the external light/dark cycle.

Recently, CikA was implicated as an output pathway component, parallel to SasA and LabA, and downstream of KaiC, through a series of genetic analyses (Fig. 2) [47]. Although the three-component classification (input, central oscillator, and output) of the circadian clock research is somewhat arbitrary, there is no doubt that CikA acts in an input pathway based on evidence both old and new. CikA is the only known protein whose absence renders the clock blind to a dark pulse, which in the wild type causes a phase shift [48]. Analysis of clock mutants that affect cell length uncovered a specific role for CikA upstream of KaiC, which functions upstream of SasA and RpaA [25]. Although the mechanism remains elusive, CikA likely suppresses the kinase and ATPase activities of KaiC indirectly. The phenotypes of period and/or amplitude of circadian rhythms in *cikA* mutants can be explained by its role in modifying

KaiC's biochemical activity, *i.e.*, by its role in the input pathway. Nonetheless, involvement of this multifunctional protein at different points in the clock system is quite plausible.

Rhythms in other cyanobacterial species

Bacterial circadian rhythms were first rigorously demonstrated in the cyanobacterium *Synechococcus* sp. RF-1 [49]. Since then, *S. elongatus* has emerged as the model for the prokaryotic circadian clock, largely due to the superior genetic tools available for this organism. To date, cyanobacteria remain the only prokaryotes shown to possess a true circadian clock. However, cyanobacteria are a very diverse group and differ greatly in their genome size, morphology, metabolism, and ecology; therefore, other cyanobacterial species can help us understand the evolutionary history and adaptive significance of the clock. In *Crocosphaera watsonii* WH 8501 staining of the chromosome, reflecting DNA topology, is rhythmic under constant light conditions [50], confirming the circadian chromosome compaction seen in *S. elongatus* [12]. Circadian rhythms have also been reported in *Synechocystis* PCC 6803 [51], *Cyanothece* sp. ATCC 51142 [52], and *Thermosynechococcus elongatus* BP-1 [53], which all have at least one set of the *kaiABC* genes. Of the 49 complete cyanobacterial genomes at GenBank, only *Gloeobacter violaceus* PCC 7421 has no apparent homolog of any of the *kai* genes. *Gloeobacter* is unique as it lacks thylakoid membranes and, phylogenetically, branches off from other cyanobacteria very early [54]. Interestingly, *Prochlorococcus* species have lost *kaiA* as a result of genome streamlining but retain *kaiBC* [55]. In *Prochlorococcus marinus* PCC 9511, rhythms of cell division and mRNA accumulation from several genes are apparent in light dark cycles, but are quickly lost when cells are released into constant light [55], failing a test of circadian clocks. Recombinant KaiC protein from *Prochlorococcus* sp. MED4 (ProKaiC) is phosphorylated at the neighboring Ser and Thr residues like *S. elongatus* KaiC, and displays a similarly weak ATPase activity. However, contrary to KaiC, ProKaiC is constitutively phosphorylated when incubated alone, and the addition of KaiA from *S. elongatus* or KaiB from either species has no effect on this activity [56]. Therefore, no oscillation of phosphorylation has been reconstituted under the tested conditions. Taken together, these data suggest that *Prochlorococcus* species may have lost the autonomous circadian clock, but retain a timing system that needs to be reset daily with environmental cues, analogous to an hourglass [57]. It remains to be seen whether the *kaiBC* genes are part of this timing system.

Conclusions

The seemingly simple and elegant *in vitro* oscillator has allowed scientists to make strides in understanding the core mechanism of the cyanobacterial clock and gain insights into how the clock synchronizes with the environment. Future studies will strive to incorporate the input and output aspects of the clock into the *in vitro* system. Meanwhile, the power of genetics continues to reveal how the circadian clock is integrated within the cell to coordinate physiology and behavior. Some key questions that remain are outlined in Box 1.

Box 1: Key questions

How does the conformation of KaiC change with changes in phosphorylation state?

What is the correlation between phosphorylation state and ATPase activity?

What is the role of monomer shuffling of KaiC?

How is KaiC phosphorylation propagated and synchronized within a hexamer and among the entire population?

How does the clock control DNA topology, and *vice versa*?

How do CikA and LdpA relay environmental information to the central oscillator?

How are clock components distributed in the cell?

What is the function of the circadian cell division gate?

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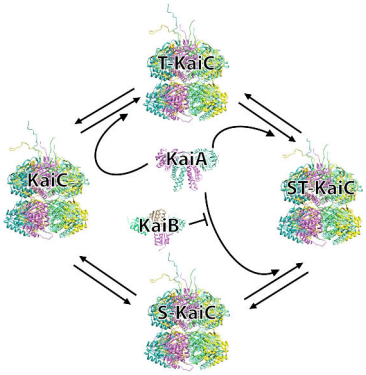

Figure 1. Model of the circadian cycle of KaiC phosphorylation

Two residues (S431 and T432) in KaiC undergo autophosphorylation and autodephosphorylation. KaiA shifts the equilibrium toward T-KaiC and ST-KaiC. T432 on ST-KaiC spontaneously dephosphorylates and forms S-KaiC. KaiB binds to S-KaiC and inactivates KaiA to shift the equilibrium towards unphosphorylated KaiC.

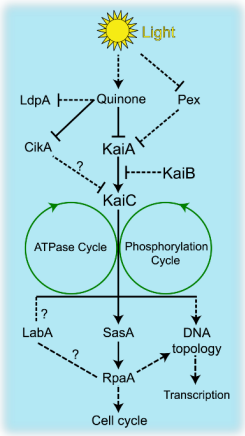

Figure 2. Molecular Pathways of the Circadian Clock in *S. elongatus*

The central oscillator of the clock is composed of KaiA, KaiB, and KaiC, which together generate circadian rhythms of KaiC phosphorylation (see Figure 1 for details) and ATPase activity that are intimately coupled. CikA, LdpA, and Pex are known components of the input pathway. Both CikA and LdpA are redox sensitive, as the addition of a quinone analog destabilizes them *in vivo*. CikA binds a quinone directly, and represses KaiC's phosphorylation/ATPase activity through a cryptic activity. LdpA is an iron-sulfur protein that is involved in light-dependent modulation of circadian period [43]. Its mechanism of action remains unknown. The *pex* gene is light suppressed, and encodes a transcription repressor of the *kaiA* gene [58]. Oxidized quinone binds and destabilizes KaiA, providing a direct input to the central oscillator by cellular redox. SasA is a histidine kinase that physically interacts with KaiC. RpaA is the cognate response regulator of SasA, and bears a DNA binding domain, whose target(s) has yet to be identified [59]. LabA is proposed to act negatively on RpaA [60]. Global transcription rhythms are regulated through DNA topology via an unknown mechanism. Timing of cell division is inhibited by elevated KaiC ATPase through the SasA-RpaA output pathway, and localization or polymerization of bacterial tubilin homolog FtsZ is targeted.