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Elevated ATPase Activity of KaiC Applies a Circadian Checkpoint on Cell Division in *Synechococcus elongatus*

Guogang Dong,[1,2] Qiong Yang,[3] Qiang Wang,[5] Yong-Ick Kim,[2] Thammajun L. Wood,[1] Katherine W. Osteryoung,[5] Alexander van Oudenaarden,[3,4] and Susan S. Golden[1,2,*]
[1]Center for Biological Clocks Research, Department of Biology, Texas A&M University, College Station, TX 77843-3258, USA
[2]Center for Chronobiology, Division of Biological Sciences, University of California San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0116, USA
[3]Department of Physics
[4]Department of Biology
Massachusetts Institute of Technology, Cambridge, MA 02139, USA
[5]Department of Plant Biology, Michigan State University, East Lansing, MI 48824, USA
*Correspondence: sgolden@ucsd.edu
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SUMMARY

A circadian clock coordinates physiology and behavior in diverse groups of living organisms. Another major cyclic cellular event, the cell cycle, is regulated by the circadian clock in the few cases where linkage of these cycles has been studied. In the cyanobacterium *Synechococcus elongatus*, the circadian clock gates cell division by an unknown mechanism. Using timelapse microscopy, we confirm the gating of cell division in the wild-type and demonstrate the regulation of cytokinesis by key clock components. Specifically, a state of the oscillator protein KaiC that is associated with elevated ATPase activity closes the gate by acting through a known clock output pathway to inhibit FtsZ ring formation at the division site. An activity that stimulates KaiC phosphorylation independently of the KaiA protein was also uncovered. We propose a model that separates the functions of KaiC ATPase and phosphorylation in cell division gating and other circadian behaviors.

INTRODUCTION

In organisms from microscopic cyanobacteria and fungi to plants and animals, genetically programmed daily cycles, known as circadian rhythms, pervade various aspects of physiology and behavior (Bell-Pedersen et al., 2005). In the cyanobacterium *Synechococcus elongatus*, the timing of cell division (Mori et al., 1996), global patterns of gene expression (Liu et al., 1995), and compaction of the chromosome (Smith and Williams, 2006) are all controlled by a circadian clock that exhibits the same properties as in eukaryotic organisms. However, the *S. elongatus* clock is distinct in components, mechanism, and evolutionary history from eukaryotic clock systems (Dong and Golden, 2008; Mackey and Golden, 2007).

In *S. elongatus*, three neighboring genes, *kaiA*, *kaiB*, and *kaiC*, encode proteins of the central oscillator. Inactivation of any of them abolishes the clock, as does overexpression of KaiA or KaiC (Ishiura et al., 1998). KaiC is an autokinase, autophosphatase, and ATPase; in complex with KaiA and KaiB, KaiC displays a daily rhythm of phosphorylation at residues Ser431 and Thr432 (Nishiwaki et al., 2004; Xu et al., 2004) both in vivo and in vitro (Nakajima et al., 2005; Tomita et al., 2005). KaiA stimulates KaiC autophosphorylation and KaiB opposes KaiA's stimulatory activity (Iwasaki et al., 2002; Kim et al., 2008; Rust et al., 2007; Williams et al., 2002). The oscillation of KaiC phosphorylation in a mixture of the three Kai proteins and ATP in vitro (Nakajima et al., 2005) suggests that the phosphorylation cycle is the fundamental timekeeping mechanism in cyanobacteria. However, the ATPase activity of KaiC also oscillates in a circadian manner, is intrinsically temperature compensated, and determines circadian period length, suggestive of a timekeeping role that may be separable from the phosphorylation cycle (Terauchi et al., 2007). A gene expression rhythm persists in the absence of a KaiC phosphorylation rhythm (Kitayama et al., 2008); thus, other aspects of KaiC, such as the ATPase activity, may underlie the basic timing mechanism instead of, or in addition to, KaiC phosphorylation.

Temporal information from the cyanobacterial oscillator is broadcast to downstream genes via the histidine protein kinase SasA, whose autophosphorylation is stimulated by interaction with KaiC (Iwasaki et al., 2000; Smith and Williams, 2006). SasA then transfers the phosphoryl group to RpaA, a response regulator with a DNA-binding domain. Disruption of either *sasA* or *rpaA* results in severely damped rhythms or arrhythmia, depending on growth conditions (Iwasaki et al., 2000; Takai et al., 2006b).

An input pathway that includes CikA, LdpA, and Pex relays environmental information to the oscillator for synchronization (Dong and Golden, 2008). Both CikA and LdpA sense light indirectly through cofactors that perceive changes in the cellular

redox state, which varies with photosynthetic activity (Ivleva et al., 2005, 2006). CikA is found in a complex with LdpA, KaiA, KaiC, and SasA in vivo, but no direct biochemical interaction has been detected between CikA and the oscillator. A *cikA* null mutant exhibits short-period, low-amplitude gene expression rhythms and fails to reset the phases of rhythms after an environmental cue (Schmitz et al., 2000); additionally, it is defective in cell division, resulting in elongated cells (Miyagishima et al., 2005).

Cell division is a cyclic event that is tightly regulated by and coordinated with other cellular activities. Few studies to date have focused on the interaction between the cell and circadian cycles, with even fewer molecular details. For example, in regenerating liver cells of mice, circadian clock proteins directly control the expression of Wee1, a kinase that inhibits the entry into mitosis (Matsuo et al., 2003). Cell division is also gated by the clock in mouse fibroblast cells cultured in vitro (Nagoshi et al., 2004) and in *S. elongatus* (Mori et al., 1996). The rate of DNA synthesis is constant in the cyanobacterium and not phase dependent, suggestive of regulation further downstream—such as cytokinesis (Mori et al., 1996). The mechanism of cell division gating in *S. elongatus* has remained unknown in the face of rich molecular details of the cyanobacterial circadian clock. Elucidation of this pathway would tie the oscillator to a key fitness component of cell physiology.

Here, we show that elevated ATPase activity of KaiC closes the cell division gate and demonstrate a linear signal transduction pathway from the input components to the central oscillator and to the output pathway in the regulation of cell division. We also show that localization of the bacterial tubulin homolog FtsZ is a target of clock control. This work revealed the surprising action of a KaiA-independent, but CikA-suppressed, activity that stimulates KaiC autophosphorylation. A model of the relationship of KaiC ATPase and phosphorylation activities, and how they are incorporated with the input and output pathways of the clock, emerges from this work.

RESULTS

Cell Division Is Gated in the Wild-Type and *cikA* Mutant

A previous report showed the gating of cell division in a population of *S. elongatus* cells measured over several circadian cycles (Mori et al., 1996). That work predated the identification of molecular components of the cyanobacterial clock and did not address the process in individual cells. CikA is the only clock component that has been reported to play a role in cell division (Miyagishima et al., 2005); therefore, we tested the requirement of *cikA* for the gating of cell division. Using timelapse microscopy, we directly monitored growing cells for 3 days, recording events of cell division and the circadian rhythm of *kaiBC* promoter activity as reported by a destabilized yellow fluorescent protein, YFP-SsrA(LVA) (Chabot et al., 2007).

Individual *cikA* mutant cells show rhythmic gene expression, with a period of 22.0 ± 1.1 hr, whereas the wild-type (WT) cells oscillate with a period of 24.9 ± 1.0 hr (Figure 1A), consistent with results from luciferase reporters (Schmitz et al., 2000). To address whether and how the circadian clock gates cell division, all division events were assigned to their corresponding circadian phases, normalized into one circadian period $0 \sim 2\pi$, and plotted as a histogram. To avoid sampling bias, we ensured that the initial circadian phases were evenly distributed; i.e., the cells examined are unsynchronized (data not shown). The occurrence of cell division in the WT is apparently suppressed around the peak of fluorescence (Figure 1B), indicating that cell division is gated. In the *cikA* mutant, a similar dip in the histogram was seen, although the overall occurrences of cell division during this window are higher and the duration of the inhibition is longer.

As a control we monitored cell division in an arrhythmic *kaiC* null mutant. Thus, the division events cannot be assigned to specific circadian phases. However, when we plotted the distribution of doubling time of all cells monitored (Figure 1C), the *kaiC* mutant had only a single peak in contrast to two peaks in the WT and *cikA* mutant. We reasoned that when gating is active, the doubling time of cells whose life spans cover the window of gating is prolonged compared to those whose life spans do not, resulting in two populations of cells with distinct doubling times. On the other hand, the doubling time of arrhythmic mutants follows a Gaussian distribution pattern in the absence of a gate. Both of these complementary types of analysis are able to detect gating of cell division. In conclusion, *cikA* is not essential to the gating of cell division, although its absence causes elongated cells and affects characteristics of the gate.

kaiC Is Epistatic to *cikA* in the Regulation of Cell Division

The phenotypic effects of a *cikA* null mutation on both circadian rhythms and cell length could be due to CikA's involvement in two pathways that separately govern circadian rhythms and cell division; alternatively, CikA may affect a clock pathway that regulates the gating of the cell cycle. If the second hypothesis is true, mutation of some clock genes would suppress the *cikA* null cell elongation phenotype. We made double mutants of *cikA* and central oscillator genes. Cell lengths of various mutants, grown under the same conditions simultaneously to minimize variations caused by cell density and/or light intensity (Figure S1 available online), were measured and compared. As expected, *cikA* mutant cells are significantly longer than the WT (Figures 1D and 1E). In the *ΔkaiCΔcikA* mutant, however, the absence of KaiC suppresses the cell division defect of *cikA*. The *ΔkaiAΔcikA* and *ΔkaiBΔcikA* mutants are still elongated. These data suggest that *cikA* regulates cell division through the clock, and that KaiC lies downstream of CikA in this pathway.

Loss of *kaiB* Leads to Increased Cell Length, Dependent on KaiC

To address the roles of other clock genes in cell-cycle control, single mutants were examined. Cells of a *kaiB* mutant average almost twice as long as WT, whereas mutants of *kaiA*, *kaiC*, or *ldpA* have normal cell lengths (Figures 1D and 1E and data not shown). The extensive evidence of KaiB-KaiC interaction suggested that inactivation of *kaiC* would also suppress the *kaiB* phenotype. Indeed, the cell length of a *kaiBC* double mutant is like WT (Figures 1D and 1E). We propose that the role of KaiB in determining cell length is the same as its function in modifying KaiC phosphorylation state.