Two Antagonistic Clock-Regulated Histidine Kinases Time the Activation of Circadian Gene Expression

Andrian Gutu1,2 and Erin K. O’Shea1,2,3,*
1Howard Hughes Medical Institute
2Department of Molecular and Cellular Biology
3Department of Chemistry and Chemical Biology
Harvard University Faculty of Arts and Sciences Center for Systems Biology, Northwest Labs, 52 Oxford Street, Cambridge, MA 02138, USA
*Correspondence: erin_oshea@harvard.edu
http://dx.doi.org/10.1016/j.molcel.2013.02.022

SUMMARY

The cyanobacterial circadian pacemaker consists of a three-protein clock—KaiA, KaiB, and KaiC—that generates oscillations in the phosphorylation state of KaiC. Here we investigate how temporal information encoded in KaiC phosphorylation is transduced to RpaA, a transcription factor required for circadian gene expression. We show that phosphorylation of RpaA is regulated by two antagonistic histidine kinases, SasA and CikA, which are sequentially activated at distinct times by the Kai clock complex. SasA acts as a kinase toward RpaA, whereas CikA, previously implicated in clock input, acts as a phosphatase that dephosphorylates RpaA. SasA is bound and stimulated by KaiC (Iwasaki et al., 2000; Takai et al., 2006). Furthermore, SasA acts as a kinase toward RpaA, whereas CikA, previously implicated in clock input, acts as a phosphatase that dephosphorylates RpaA. SasA and CikA cooperate to generate an oscillation of RpaA activity that is distinct from that generated by either enzyme alone and offset from the rhythm of KaiC phosphorylation. Our observations reveal how circadian clocks can precisely control the timing of output pathways via the concerted action of two oppositely acting enzymes.

INTRODUCTION

Circadian clocks are endogenous oscillators found in a wide range of organisms that coordinate physiology and behavior with the diurnal cycle of the environment (Dunlap et al., 2004). Despite the lack of conservation of their underlying components, circadian clocks share a common set of fundamental properties—the ability to keep time with ~24 hr periodicity even in a constant environment (free run), the ability to adjust the phase to match that of environmental variation (entrainment), and a relative insensitivity of the period to temperature (temperature compensation) (Bell-Pedersen et al., 2005; Rosbash, 2009). In one of the simplest model systems known to possess a circadian clock, the cyanobacterium *Synechococcus elongatus* PCC 7942, the core pacemaker is made of the KaiA, KaiB, and KaiC proteins that interact to generate circadian oscillations in the phosphorylation state of KaiC (Nishiwaki et al., 2007; Qin et al., 2010; Rust et al., 2007). This phosphorylation cycle, which can be recapitulated in vitro, proceeds through sequential KaiA-promoted autophosphorylation of KaiC at two residues (threonine 432, T-KaiC; and serine 431, S-KaiC). As more S-KaiC accumulates, KaiB binds KaiC and inhibits KaiA, switching KaiC to autodephosphorylation mode, and brings the system to unphosphorylated state (Rust et al., 2007). This pacemaker receives input from the environment, tuning it to the external day/night cycle, and also orchestrates output, controlling physiological processes such as gene expression (Ito et al., 2009), chromosome compaction (Smith and Williams, 2006), and the onset of cell division (Dong et al., 2010). The majority of genes in cyanobacteria are under circadian control (Ito et al., 2009; Liu et al., 1995; Vijayan et al., 2009). This control extends to regulation of *kaiBC* operon, forming a transcription-translation feedback loop (Ishiura et al., 1998; Taniguchi et al., 2007, 2010).

The histidine kinase SasA and the DNA-binding domain containing response regulator RpaA were shown to be essential for clock-controlled gene expression (including *kaiBC*) (Iwasaki et al., 2000; Takai et al., 2006; Taniguchi et al., 2010). SasA is capable of phosphorylating RpaA in vitro at a conserved aspartate residue (a process referred to as phosphotransfer), and thus these proteins were proposed to form a cognate histidine kinase-response regulator pair (Takai et al., 2006). Furthermore, SasA is bound and stimulated by KaiC (Iwasaki et al., 2000; Smith and Williams, 2006), thereby coupling the phase of the oscillator with gene expression. CikA, another clock-associated histidine kinase (Ileva et al., 2006), also regulates *kaiBC* expression, and is thus proposed to form another clock output pathway (Taniguchi et al., 2010). However, the cognate response regulator controlled by CikA is not known. Intriguingly, CikA also has a role in clock input, as *cikA* mutants fail to entrain the phase of their clock in response to a 5 hr dark pulse (Schmitz et al., 2000).

Here we show that RpaA is the cognate response regulator of both SasA and CikA. However, these histidine kinases exert antagonistic effects on RpaA, with SasA acting as a kinase and CikA as a phosphatase. Furthermore, we demonstrate that the Kai oscillator stimulates the activity of CikA with timing distinct from its activation of SasA. This temporal separation of two oppositely acting enzymes that converge onto the same substrate creates an RpaA–P oscillation that is phase advanced relative to that of KaiC–P, a phenomenon that is likely important for the accurate onset of gene expression.
RESULTS AND DISCUSSION

RpaA Is the Cognate Response Regulator of Both SasA and CikA

To identify candidate cognate response regulators for CikA, we used phosphotransfer profiling (Laub and Goulian, 2007; Skerker et al., 2005) to determine which of the predicted response regulators encoded by the S. elongatus genome (see Table S1 online) is preferentially phosphorylated in vitro by CikA→P (phosphorylated CikA). This method relies on the ability of a given phosphorylated histidine kinase to transfer a radiolabeled phosphor-ylated histidine kinase to transfer a radiolabeled phosphoryl group most rapidly to its cognate response regulator(s) when incubated in vitro with each receiver domain-containing protein from a genome of interest (Skerker et al., 2005). We find that the only response regulator phosphorylated by CikA→P after a short incubation time is RpaA (Figure 1A). We applied the same approach to SasA and find that RpaA is the most preferred substrate of SasA→P phosphotransfer (Figure 1A). The response regulator synPCC7942_1860 is also phosphorylated, but ∼32-fold less efficiently than is RpaA (Figure S1A). At longer incubation times other, likely noncognate (Skerker et al., 2005), response regulators are phosphorylated (Figure S1B). These results suggest that RpaA is the cognate response regulator of both SasA and CikA, indicating that it may be a key output node that integrates different circadian inputs.

Kai Proteins Control SasA Kinase and CikA Phosphatase Activities

Bacterial histidine kinases can act either as kinases (through the combined processes of autophosphorylation, in which they transfer the γ-phosphoryl group of ATP to their conserved histi-
In this "clock-rescue" strain we observe circadian oscillations regulating KaiC phosphorylation (Figure 2 A and Figure S2 A). 

KaiC production to a level that is sufficient to restore circadian clock-rescue strain grown in constant light elevates KaiB and 
Phe. We then deleted cikA (Figure 2B) or sasA (Figure 2C) in this "clock-rescue" strain background, demonstrated that oscillations in KaiC phosphorylation are restored, and analyzed RpaA phosphorylation. In

the absence of CikA, RpaA-P levels are high (Figure 2B), and conversely, in the absence of SasA, RpaA-P is practically undetectable (Figure 2C). Thus, in agreement with our biochemical observations, CikA and SasA have opposing actions on RpaA-P in vivo, with SasA promoting RpaA phosphorylation and CikA promoting accumulation of unphosphorylated RpaA. This observation is consistent with the high and low overall bioluminescence of a circadian gene reporter in cikA- or sasA-deficient strains, respectively (Taniguchi et al., 2010; Zhang et al., 2006).

**ST-KaiC Activates SasA Kinase Activity, and S-KaiC/ KaiB Complex Stimulates CikA Phosphatase Activity**

To determine which phosphorylation state of the KaiC oscillator is most potent in activating SasA and CikA, we measured the effect of adding partial clock reactions containing subsets of the Kai proteins on SasA kinase or CikA phosphatase activity. In partial clock reactions, KaiC transits through four phosphorylation states: unphosphorylated KaiC (U-KaiC), KaiC phosphorylated only on threonine 432 (T-KaiC), KaiC phosphorylated on both serine 431 and threonine 432 (ST-KaiC), and KaiC phosphorylated only on serine 431 (S-KaiC) (Figure 3A) (Rust et al., 2007). When we mix a partial clock reaction in which KaiC is phosphorylating (KaiC mixed with KaiA) with SasA and RpaA, we find that SasA kinase activity (reflected as RpaA-P accumulation) mirrors the abundance of the ST-KaiC phosphoform (Figure 3A), suggesting that this state of KaiC is the most potent activator of SasA kinase activity. A similar result was obtained when autophosphorylation of SasA was measured (Figure S3A), indicating that autophosphorylation is the activity regulated by the ST-KaiC phosphoform. When we mix CikA and RpaA-P with aliquots from a partial clock reaction in which KaiC is dephosphorylating (phosphorylated KaiC mixed with KaiB), we find that CikA phosphatase activity (reflected as a reduction in RpaA-P) correlates with the abundance of S-KaiC (Figure 3B), and this correlation is dependent on the presence of KaiB (Figure S3B). S-KaiC and KaiB form a complex during the clock cycle, which plays a critical role in feedback that maintains clock synchrony (Kageyama et al., 2006; Nishiwaki et al., 2007; Rust et al., 2007) and in this case also functions as an activator of CikA phosphatase activity.

We then quantified the effects of the ST and S forms of KaiC on the kinase and phosphatase activities of SasA and CikA by adding aliquots from complete clock reactions (KaiA + KaiB + KaiC) collected when ST-KaiC and S-KaiC are at their peak levels (Rust et al., 2007). Addition of a clock reaction aliquot withdrawn when ST-KaiC is at its peak greatly enhances the initial rate of SasA kinase activity by addition of both serine 431 and threonine 432 (ST-KaiC), KaiC phosphorylated on only on threonine 432 (T-KaiC), KaiC phosphorylated on

**Clock-Controlled Regulation of SasA and CikA Determines the Timing of RpaA Phosphorylation**

The timing of accumulation and decay of RpaA-P in cells is likely determined by the concerted action of SasA and CikA. Our in vivo
measurements indicate that the peak of RpaA→P precedes the peak of KaiC→P by ~4 hr (Figure 2A, Figure 4A, and Figure S4A). Intriguingly, the phase of RpaA→P oscillation that would be directed by SasA kinase activity alone, as measured in vitro in the presence of time-resolved clock aliquots, is coincident with the phase of KaiC→P oscillation (that coincides with ST-KaiC accumulation) (Figure 4B and Figure S4B). On the other hand, the RpaA→P oscillation produced in vitro by the phosphatase action of CikA alone in the presence of clock aliquots peaks 8 hr prior to the peak of KaiC→P (Figure 4C), and its trough coincides with the increase in S-KaiC that occurs 6 hr after the KaiC→P peak (Figure S4C). The profiles of these separate activities suggest that their combined action has the potential to produce a novel phase of oscillation of RpaA→P that peaks prior to the KaiC→P peak (Figure 4D) and would mirror the in vivo observations (Figure 4A). A phase-advanced peak of RpaA→P accumulation relative to that of KaiC→P is consistent with the peak of gene expression as observed in bioluminescence and RNA abundance assays, which occurs ~4 hr prior to the peak of KaiC→P (Takai et al., 2006; Tomita et al., 2005). Such sequential convergence of two inputs that have opposite effects on a key regulator may also enhance the accuracy of transduction of temporal information in the face of cellular and environmental fluctuations.

Our biochemical observations provide insight into the functions attributed to CikA by genetic studies. Recent work implicated CikA in a clock output pathway—CikA acts as a negative regulator of kaiBC expression and was proposed to operate during late subjective night (Taniguchi et al., 2010), corresponding to the time when KaiC is dephosphorylating and the S-KaiC level is high. Our results enable mechanistic understanding of this role, as CikA inhibits RpaA activity (i.e., promotes its dephosphorylation), which leads to reduced kaiBC expression. CikA was originally identified as a component of the clock input pathway, required for entrainment of the cyanobacterial clock to changes in light availability (Schmitz et al., 2000). It is possible that CikA has a role in input unrelated to regulation of RpaA activity. Alternatively, the entrainment defect in the cikA mutant may be a consequence of misregulation of RpaA activity that disrupts transcriptional feedback of kaiBC expression. In addition to a defect in entrainment, the cikA mutant also displays a cell elongation phenotype, due to its inability to properly gate the timing of cell division (Dong et al., 2010). We reason that this clock-dependent cell-elongation phenotype displayed by cikA and certain kai mutants (Dong et al., 2010) may be due to RpaA→P misregulation. For example, our observations predict that the cell elongation phenotypes of cikA and kaiB are due to an increased level of RpaA→P, in cikA caused by the absence of CikA phosphatase activity and in kaiB caused by lack of CikA activation.

Circadian clocks enable organisms to time the regulation of physiological processes to exploit the predictable variation in the earth’s light/dark cycle. For example, cyanobacteria time the production of the photosynthetic apparatus to anticipate daylight (Stal and Krumbein, 1987; Vijayan et al., 2009) and also use the clock to gate the cell division (Dong et al., 2010; Mori et al., 1996). In some cases, the appropriate timing of regulation of physiological processes may not coincide with the phase of the core oscillator. We have shown how an organism can use differentially regulated and opposing enzymes converging on a single output protein to generate a phase of output distinct from that of the clock itself, a phenomenon that is likely to be important for all circadian clocks.

**EXPERIMENTAL PROCEDURES**

**Growth Conditions**

Wild-type, “clock-rescue,” and sasA and cikA derivative strains of S. elongatus were grown in standard BG11M medium, at 30 °C, in white light with O₂-enriched air bubbled through cultures that were repeatedly
Two Antagonistic Clock-Regulated Histidine Kinases Time the Activation of Circadian Gene Expression, Molecular Cell (2013), http://dx.doi.org/10.1016/j.molcel.2013.02.022

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A

B

C

D

Figure 4. Clock-Mediated Changes in SasA and CikA Activities Coordinate to Generate a Distinct Oscillation of RpA

(A) Circadian profile of KaiC-P and RpA-P in synchronized wild-type cells grown in continuous light, as measured by immunoblotting (see also Figure S4A).

(B) Relative kinase activity of SasA in the presence of clock aliquots obtained from an in vitro oscillating reaction. Activity (fold change) is expressed relative to the trough of RpA-P accumulation (14 hr clock time point) (see also Figure S4B).

(C) Effect of CikA on 32P-labeled-RpA-P in the presence of protein aliquots obtained from an oscillating clock reaction. Percent RpA-P is relative to the amount present when no clock reaction mix was added (see also Figure S4C).

(D) Model of the differential activation and convergence of SasA and CikA activities that create phase-advanced rhythms of RpA. SasA kinase activity is enhanced by ST-KaiC (doubly phosphorylated). As more S-KaiC accumulates, Kaib binds KaiC and inhibits the phosphorylation-promoting activity of diluted with fresh medium to maintain an OD 750 of ~0.3. Under these conditions a doubling time of 6.9 hr was recorded. For circadian experiments, the cultures grown in 6 μM IPTG were synchronized by two 12 hr dark periods, spaced by 12 hr in light. Sampling began 24 hr after release into constant light.

Western Blotting

Cells from 20 ml of culture were harvested by filtration on Whatman cellulose acetate filters and quickly frozen in liquid nitrogen. Lysates were obtained by bead-beating at 4°C in lysis buffer (8 M urea, 20 mM HEPEs-KOH [pH 8], 1 mM β-mercaptoethanol [β-ME]). Total protein content was determined by Bradford assay against a BSA standard curve. Kaic immunoblotting was performed as previously described (Rust et al., 2011). For detection of RpA-P, lysates were run at 4°C on 7% polyacrylamide gel (Hoefer SE 600 system) containing 50 μM of Phos-tag AAL-107 (Wako Chemicals) and 100 μM MnCl2 and then transferred to a nitrocellulose membrane. Custom-made rabbit anti-RpA polyclonal antibodies were used to detect phosphorylated and unphosphorylated RpA (see the Supplemental Information). Quantification of the western blot bands was performed using Alphalager EP software (Alpha Innotech). Within each lane, the lower and the upper (retarded) bands were delineated using two identically sized nonoverlapping boxes, which allowed the extraction of the sum of pixel intensities corresponding to each band. In addition, a similarly sized box positioned above each band set was used for background subtraction. The extent of phosphorylation at each time point (lane) was estimated by calculating the upper band signal as a fraction of the sum of the intensities of both bands (100*[upper band signal]/[total signal of both bands]).

In Vitro Assays

Purification of Kai proteins and clock reactions were largely performed as described previously (Rust et al., 2007), with the exception that the clock buffer contained an ATP-regeneration system (phosphoenoacylpyruvate and recombiant pyruvate kinase of Bacillus steaformorphus [Sigma]). Full-length recombinant SasA, CikA, and RpA lacking a tag were prepared as described in the Supplemental Information. Except for phosphotransfer profiling, which was performed as previously described (Skerker et al., 2005), the ratios and the concentrations of the recombinant proteins used in assays were based on quantitative western blotting (Figures S2C–S2G) and standard oscillating clock reactions (Rust et al., 2011): 3.5 μM KaiC, 2.5 μM KaiB, 1.5 μM KaiA, 2.5 μM RpA, 0.65 μM SasA, and 0.65 μM CikA. The kinase buffer used was 20 mM HEPEs-KOH (pH 8), 150 mM KCl, 10% glycerol, and 5 mM MgCl2. Unless indicated otherwise, kinase reactions were initiated with addition of ATP-containing Kai protein mixes or ATP-containing buffer (1 mM ATP final concentration) and incubated at 30°C. Aliquots from the reaction mix were quenched with the β-ME-based Laemmli buffer and run on 7% polyacrylamide gels made with the Phos-tag reagent (Wako Chemicals) as described in the western blotting section. For estimation of kinase rates, the linear slopes of percentage RpA-P accumulation over time was measured. The sampling for Figure 2B was done for all combinations of Kai proteins in parallel at 0.5, 2, and 10 min after addition of SasA or CikA (for CikA and Kaib combination, only 0.5 and 10 min time points were obtained). To capture SasA kinase activity, as shown in Figure 3A and Figure 4B the reactions were done in parallel for all the partial clock and complete clock aliquots with 2 min incubation with SasA and RpA. For Figure 3C, the times sampled were 0.5, 2, 5, 7, and 10 min after addition of ST-KaiC-enriched clock aliquot to SasA/RpA or CikA/RpA mixes. After electrophoresis, the gels were stained with SyproRuby (Invitrogen) and imaged with the Typhoon Trio System (GE Healthcare). Densitometry analysis was performed with ImageQuant TL7.0 (GE Healthcare).

For phosphatase assays, radiolabelled RpA-P was independently prepared before each experiment by utilizing a CikA-coupled resin (see the KaiA, switching KaiC in autodephosphorylation mode. The S-KaiC-KaiB complex enhances the phosphatase activity of CikA. The integration of SasA and CikA activities generate on oscillation of RpA-P that is phase-advanced relative to KaiC-P.
Supplemental Information. Reaction conditions were similar to the ones used for kinase assays. Aliquots from reactions were stopped with the addition of Laemmli loading dye and analyzed by SDS-PAGE and autoradiography. The time points sampled in Figure 1C and Figure 3D were 0, 15, 30, and 60 min following addition of Kai protein mixtures. Background-subtracted signal intensity of each band was normalized against the band signal at time 0 (which was actually ~30 s after addition of the Kai protein mixtures) in order to obtain exponential decay rates from the nonlinear fits of each time series (as exemplified in Figure S1C). As dephosphorylation of RpaA–P appears to proceed through reverse phosphotransfer (Figure S1C), we also included in the calculations the signal present in the histidine kinase bands, summing it with the signal of the corresponding RpaA–P band. All regressions were performed with Prism (GraphPad). The relative phosphatase activity represents the fold increase in the rate of dephosphorylation relative to the rate of RpaA–P dephosphorylation alone. In cases where the phosphatase activity was estimated from a single time point (15 min, Figure 3B and Figure S3B; 30 min, Figure 4C and Figure S4C), we reported only the fraction of RpaA–P remaining (sum of CikA–P and RpaA–P signal) relative to the signal present in the control lane (B, buffer added only). Each assay was independently performed at least twice, with similar results.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, one table, Supplemental Experimental Procedures, and Supplemental References and can be found with this article at http://dx.doi.org/10.1016/j.molcel.2013.02.022.

ACKNOWLEDGMENTS

We thank J.S. Markson for assistance with protein purification, for providing rpaA- and “clock-rescue” strains, and for insightful discussions. We thank M. Laub for providing MBP- and TRX-based destination vectors and for input on the project and manuscript, and we thank V. Vijayan, J. Piechura, C. Chidley, E. Czeko, R. Alvey, and V. Denic for valuable comments on the paper. This work was supported by the Howard Hughes Medical Institute.

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Please cite this article in press as: Gutu and O’Shea, Two Antagonistic Clock-Regulated Histidine Kinases Time the Activation of Circadian Gene Expression, Molecular Cell (2013), http://dx.doi.org/10.1016/j.molcel.2013.02.022.


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