

Visualizing a biological clockwork's cogs

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Recent structural studies of KaiA and KaiB, two bacterial biological clock proteins, mark the beginning of a new phase in the analysis of circadian clock mechanisms.

Have you ever looked inside an old-fashioned clock or wristwatch to see its oscillating gears and escapement in an attempt to infer how it keeps time? Biological clock researchers have the same goal when it comes to the self-sustained biochemical oscillators known as circadian clocks. These biological clocks regulate an enormous variety of processes, ranging from cell division to sleeping and waking. Their remarkable properties include temperature compensation, a time constant of ~24 hours and high precision, but these properties are difficult to explain by the currently known biochemical reactions. The ultimate explanation for the mechanism of these unusual oscillators will require characterizing the structures, functions and interactions of the molecular components of circadian clocks. In the past 20 years, many clock components have been identified in a variety of organisms, but the structures of these components have been unknown—that is, until now. Four recent reports, including that of Uzumaki *et al.*¹ in this issue of *Nature Structural & Molecular Biology*, reveal for the first time the three-dimensional structure of two essential circadian clock proteins, KaiA and KaiB¹⁻⁴.

The Kai proteins come from the simplest cells that are known to exhibit circadian phenomena, the prokaryotic cyanobacteria, where genetic and biochemical studies have been productive⁵. A mutational analysis from the cyanobacterium *Synechococcus elongatus* revealed that its circadian system is regulated by a cluster of three essential clock genes, *kaiA*, *kaiB* and *kaiC*⁶. A fourth gene encodes the histidine kinase SasA that is not essential for rhythmicity but significantly enhances the robustness of the oscillation⁷. We already know some things about the interactions and modifications of the proteins encoded by the *kai* genes (Fig. 1). KaiA, KaiB and KaiC interact with each other^{8,9} to form large complexes *in vivo*, with KaiC at the core¹⁰. KaiC can exist

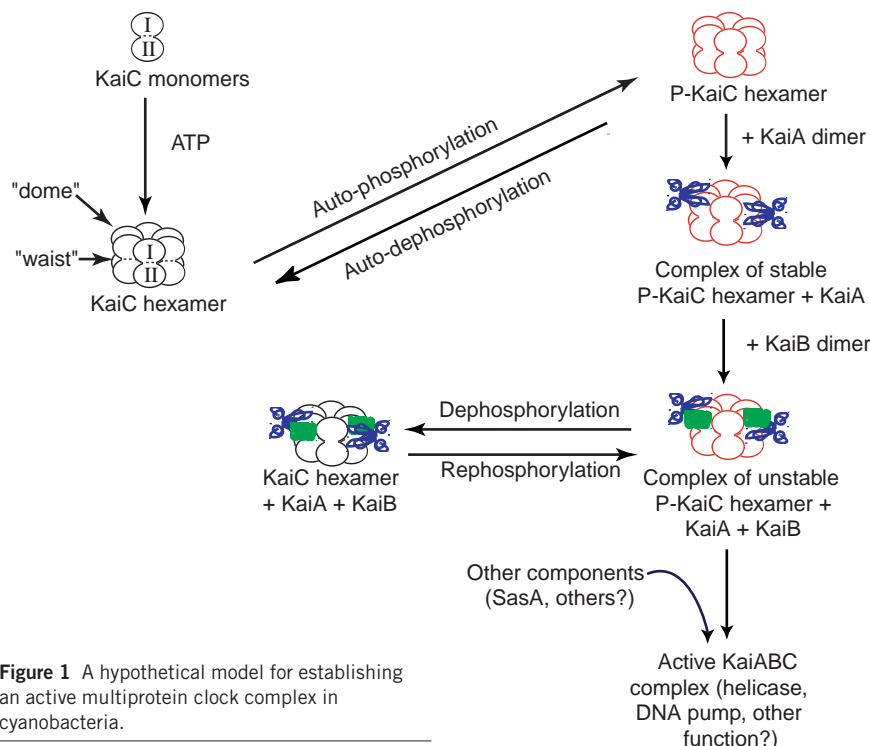


Figure 1 A hypothetical model for establishing an active multiprotein clock complex in cyanobacteria.

in both phosphorylated and non-phosphorylated forms¹¹⁻¹³, and its phosphorylation status is correlated with the period of the clock *in vivo*¹³. *In vitro*, KaiC can both auto-phosphorylate¹¹ and auto-dephosphorylate¹³. KaiA and KaiB modulate the phosphorylation status of KaiC *in vitro* and *in vivo*: KaiA enhances KaiC phosphorylation (and/or inhibits its dephosphorylation), whereas KaiB antagonizes the effects of KaiA¹²⁻¹⁵. Addition of ATP to purified KaiC *in vitro* initiates KaiC phosphorylation and also stimulates the formation of the hexameric KaiC ring complex^{16,17}. This last finding is consistent with the observation of KaiC in high-molecular-mass complexes *in vivo*¹⁰.

What have the structural studies added to this picture? Let's begin with KaiA. KaiA has two major domains^{1,3,14}. The N-terminal domain of KaiA shares structural, but not sequence, similarity with the receiver domain of bacterial two-component response regulators¹⁴. Nonetheless, the N-terminal domain of

KaiA is very unlikely to act as a true receiver domain, because it lacks the highly conserved aspartyl residues of this family¹⁴. Therefore, the N-terminal domain of KaiA has been labeled a 'pseudo-receiver' domain that suggests an alteration of function^{3,14}. Little is known about the exact function of the KaiA N-terminal domain, as it does not promote KaiC autophosphorylation *in vitro*^{1,14}. Furthermore, KaiA from another cyanobacterium (*Anabaena* sp. PCC 7120) doesn't even have the N-terminal part of KaiA¹⁴, suggesting that it might be dispensable. However, mutations in the N-terminal domain of KaiA from *Synechococcus elongatus* lengthen the circadian period, suggesting that it does play an important role¹⁸. Indeed, Williams and co-workers¹⁴ propose that this pseudo-receiver domain transduces input signals to the clock complex.

The C-terminal domain of KaiA is the 'business end' in relation to KaiC auto-phosphorylation. The isolated C-terminal domain is completely active in promoting

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KaiC auto-phosphorylation *in vitro*^{1,14}, and it is the part that binds to KaiC¹. The structure of the C-terminal domain has been solved by both NMR² and crystallography^{1,3,4}. The agreement among various KaiA structures determined by two different techniques and by the several groups (and from three different species) is excellent. The C-terminal domain adopts a four-helix bundle that dimerizes and can interact with KaiC. The full-length structure of KaiA shows that the N-terminal domains are 'swapped' with respect to the C-terminal domains in the KaiA dimer³. Although Uzumaki *et al.*¹ suggest that KaiA may have three domains¹, the crystallographic analysis of the complete KaiA favors the interpretation that there are two predominant domains connected by a linker whose absence has functional consequences³.

How does this C-terminal domain interact with the core KaiC hexamer? KaiC is an internally duplicated protein, with its two halves named KaiCI and KaiCII⁸. Taniguchi *et al.*⁹ found that there were two widely separated regions in the KaiC sequence—one in each half—that interacted with KaiA. On the basis of this observation and their assumption that KaiA and KaiC will interact at a single site, these authors proposed that the two interaction regions wrapped back on themselves in the KaiC monomer so that they were in approximately the same relative spatial position. Vakonakis *et al.*² amalgamated the suggestion of Taniguchi *et al.*⁹ into a model using electron micrographs of the KaiC hexamer and the NMR structure of KaiA. In this model, the C-terminal domains of the KaiA dimer interact only with the 'waist' region of the KaiC hexamer that links the KaiCI and KaiCII domains (see dashed lines in KaiC monomer and hexamer in upper left quadrant, Fig. 1). On the basis of our own work on the KaiC structure, we now know the model of Vakonakis *et al.*² to be partially correct—KaiA-interacting regions are localized to the waist/linker region of KaiC. However, KaiA-interacting regions are also found along a spatially separated region of the KaiC hexamer—on the domes formed by the KaiCII domains (upper left quadrant, Fig. 1)—a result that was not predicted by the other groups (unpublished results)^{2,9}.

Therefore, there are two topologically distinct and separated regions of KaiC with which KaiA interacts. These interactions almost certainly underlie the influence of KaiA on the phosphorylation state of KaiC. Notably, only one KaiA dimer is enough to enhance the phosphorylation of one KaiC hexamer to an almost saturated level¹⁹. Considering that there is a six- to seven-fold excess of KaiC hexamers over KaiA dimers *in vivo*¹⁰, how can so few KaiA dimers maintain the phosphorylation status of the KaiC hexamers? Probably part of the answer is that not all the KaiC are phosphorylated *in vivo*¹².

Less is known about the structure and interactions of KaiB. The crystal structure of the cyanobacterium *Anabaena* KaiB⁴ now adds to our understanding. Like KaiA, KaiB from *Anabaena* purifies and crystallizes as a dimer. An important question to be answered from the structural studies is how KaiB antagonizes the effects of KaiA on KaiC phosphorylation. Garces *et al.*⁴ note that there are some common surface features between KaiA and KaiB from *Anabaena* and suggest that KaiA and KaiB may compete for a common binding site on KaiC. On the other hand, Ye *et al.*³ proposed on the basis of KaiA's structure that the action of KaiB might be to interact directly with KaiA. In particular, Ye *et al.*³ proposed that KaiB binds to a site in the C-terminal domain of KaiA that is either 'open' (available for KaiB binding) or 'closed' (masked by the N-terminal domain of KaiA). Therefore, the N-terminal pseudo-receiver domain was proposed to allosterically regulate the binding of KaiB to KaiA³. Time will tell which hypothesis—Garces *et al.*⁴ versus Ye *et al.*³, or even versus an as yet unformulated hypothesis—will prove to be correct for the action of KaiB. Cocrystallization studies of the possible combinations (A-C, B-C, A-B and A-B-C) may help to distinguish the hypotheses.

The structural information combined with what we know so far about the KaiABC system leads to a hypothetical sequence of assembling these components into an active complex (Fig. 1). Of note, the structural data on KaiA and KaiB come from three different species of cyanobacteria: the mesophilic *Synechococcus elongatus*, the thermophilic *Thermosynechococcus elongatus*, and the

filamentous *Anabaena*. Even though the KaiA and KaiB sequences for these species align well (with the exception of the N-terminal region of KaiA that varies), their biochemistry might not be directly comparable. That caveat aside, these structures allow us to refine our analyses of circadian mechanisms. As with all good science, however, the new results raise many new questions. What is the three-dimensional configuration of the entire KaiABC complex? Are other proteins besides SasA also members of this complex? What is the enzymatic activity of the KaiABC complex? The sequence similarity between KaiC and DNA helicases/recombinases suggested a hypothesis that the KaiABC complex mediates rhythmic changes in chromosomal topology that lead to global orchestration of gene expression⁵. The structural studies of the Kai proteins will undoubtedly provide useful tests to distinguish the various possible mechanisms of the circadian clockwork, including its precision, 24-hour time constant and temperature compensation.

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