Biochemical analysis of three putative KaiC clock proteins from Synechocystis sp. PCC 6803 suggests their functional divergence

Anika Wiegard,1† Anja K. Dörrich,2† Hans-Tobias Deinzer,2 Christian Beck,1 Annegret Wilde,2‡ Julia Holtzendorff2 and Ilka M. Axmann1

1Institute for Theoretical Biology, Charité-Universitätsmedizin Berlin, Invalidenstrasse 43, D-10115 Berlin, Germany
2Institute for Microbiology and Molecular Biology, Justus-Liebig-University Giessen, Heinrich-Buff-Ring 26, D-35392 Giessen, Germany

Correspondence
Annegret Wilde
Annegret.Wilde@biologie.uni-freiburg.de

Received 5 December 2012
Accepted 23 February 2013

Cyanobacteria have been shown to have a circadian clock system that consists mainly of three protein components: KaiA, KaiB and KaiC. This system is well understood in the cyanobacterium Synechococcus elongatus PCC 7942, for which robust circadian oscillations have been shown. Like many other cyanobacteria, the chromosome of the model cyanobacterium Synechocystis sp. PCC 6803 contains additional kaiC and kaiB gene copies besides the standard kaiABC gene cluster. The respective gene products differ significantly in their amino acid sequences, especially in their C-terminal regions, suggesting different functional characteristics. Here, phosphorylation assays of the three Synechocystis sp. PCC 6803 KaiC proteins revealed that KaiC1 phosphorylation depends on KaiA, as is well documented for the Synechococcus elongatus PCC 7942 KaiC protein, whereas KaiC2 and KaiC3 autophosphorylate independently of KaiA. This was confirmed by in vivo protein–protein interaction studies, which demonstrate that only KaiC1 interacts with KaiA. Furthermore, we demonstrate that the three different Kai proteins form only homomeric complexes in vivo. As only KaiC1 phosphorylation depends on KaiA, a prerequisite for robust oscillations, we suggest that the kaiAB1C1 gene cluster in Synechocystis sp. PCC 6803 controls circadian timing in a manner similar to the clock described in Synechococcus elongatus PCC 7942.

INTRODUCTION

Most organisms are exposed to day and night cycles and have adapted their biological activities to the associated environmental changes. In particular, eukaryotic organisms evolved endogenous timing mechanisms that predict those day–night cycles and orchestrate gene expression and other biological activities in an approximate 24 h rhythm.

Although redox-based rhythms in Archaea have been reported very recently (Edgar et al., 2012), cyanobacteria are still the only prokaryotes for which a circadian oscillator has been biochemically identified. The inner clock system is well documented for the model organism Synechococcus elongatus PCC 7942 (hereafter Synechococcus), where it consists of just three proteins encoded by a cluster of the tandemly located genes kaiA, kaiB and kaiC (Ishiura et al., 1998). KaiC as the crucial component of the unique three-protein oscillator undergoes rhythmic autophosphorylation and dephosphorylation, inversely modulated by KaiA and KaiB (Iwasaki et al., 2002; Kitayama et al., 2003; Nishiwaki et al., 2004; Xu et al., 2003). The resulting phosphorylation cycles display a 24 h period, are temperature-compensated (Tomita et al., 2005), independent of transcription and translational processes (Tomita et al., 2005) and can even be reconstituted in vitro (Nakajima et al., 2005).

KaiC monomers contain two domains (CI and CII) and assemble into a homohexameric double-doughnut-shaped ring structure in an ATP-dependent manner (Hayashi et al., 2003; Iwasaki et al., 1999; Mori et al., 2002; Pattanayek et al., 2004). Intrinsic phosphorylation and dephosphorylation occur in the CII-half at residues T432 and S431 in an ordered pattern (Nishiwaki et al., 2004, 2007; Rust et al.,...
Kim et al. (2008) demonstrated that the rates of *Synechococcus* KaiC’s intrinsic phosphorylation and dephosphorylation activities are dependent on the state of a ten-residue segment (residues 488–497), termed the A-loop. If the A-loops are in a buried state, dephosphorylation dominates, whereas in the exposed state the rate of phosphorylation is enhanced. KaiA binds to the C terminus of KaiC (Akiyama et al., 2008; Pattanaeyk et al., 2006; Vakonakis & LiWang, 2004), stabilizes the A-loops in the exposed state and thereby stimulates autokinase activity of KaiC (Kim et al., 2008). KaiB attenuates the stimulatory effect of KaiA and thereby promotes autodephosphorylation of KaiC (Kitayama et al., 2003; Xu et al., 2003).

In addition to the activities located in the CII-half, KaiC displays a very weak, temperature-compensated ATPase activity in the CI-half that is also inversely modulated by KaiA and KaiB (Terauchi et al., 2007). ATP hydrolysis in the CI domains is interlocked with KaiC phosphorylation by conformational transitions in the KaiC hexamer and acts as a basic timing system defining the 24 h period of the clock (Murayama et al., 2011; Terauchi et al., 2007).

Besides two other studies (Qin et al., 2010; Rust et al., 2007), we have demonstrated using a quantitative, highly non-linear feedback model that oscillations of the KaiABC system are a consequence of KaiA sequestration by serine-phosphorylated KaiBC complexes (Brettschneider et al., 2010; Clodong et al., 2007). Thus, our model suggested that most KaiA is inactive throughout the circadian cycle, which could be supported by native mass spectrometry revealing the existence of three KaiC binding sites for constant and phosphorylation-dependent sequestration of KaiA (Brettschneider et al., 2010).

Interestingly, the number of kai genes as well as the composition of the clockwork is not conserved among cyanobacterial species. For instance, Cyanobacteria belonging to the *Prochlorococcus* group harbour kaiB and kaiC, but have lost the kaiA gene by genome reduction during evolution (Axmann et al., 2009; Holtzendorff et al., 2008). Here, KaiC autophosphorylates independently of KaiA (Axmann et al., 2009). Other species contain multiple copies of kai genes, whose evolution involved gene duplication and/or lateral gene transfer (Dvornyk et al., 2003). Interestingly, we have never found more than one copy of a kaiA gene in a genome.

One of the species containing a high copy number of kai genes is *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*), a model organism for photosynthesis and industrial applications. Besides the orthologue of the well-known kaiABC cluster of *Synechococcus*, it harbours two more variants of both kaiB and kaiC (Aoki & Onai, 2009; Dvornyk et al., 2003). A phylogenetic analysis of the KaiBC amino acid sequences from different Cyanobacteria and other prokaryotic taxa demonstrated that the KaiC proteins of *Synechocystis* diverged into different subclasses (Dvornyk & Knudsen, 2005). In *in vivo* rhythm analysis of kai deletion mutants a clock role for the kaiAB1C1 cluster as well as the kaiB3 and kaiC3 genes has been demonstrated, but this has not been demonstrated for the kaiC2B2 cluster (Aoki & Onai, 2009). A putative involvement in fine-tuning of period length and phase of the circadian rhythm driven by a KaiAB1C1 oscillator has been discussed for kaiB3 and kaiC3 (Aoki & Onai, 2009).

Compared with *Synechococcus*, where at least 30% of all genes oscillate under constant light (Ito et al., 2009; Vijayan et al., 2009), in *Synechocystis* only 2–9% circadian genes have been detected by microarray experiments (Kucho et al., 2005). In *Synechococcus*, accumulation of kaiBC transcripts and its gene products exhibit circadian cycles allowing for a negative feedback control of kaiC expression by the KaiC protein that generates a circadian oscillation (Imai et al., 2004; Ishiura et al., 1998; Iwasaki & Kondo, 2004; Xu et al., 2000). Using a modelling approach, we have suggested recently that threonine and double-phosphorylated KaiC activate- and unphosphorylated KaiC hexamers suppress kaiBC transcription, connecting the KaiABC protein clock with a transcription–translation feedback on kaiBC comprehensively (Hertel et al., 2013).

Thus, the control of the kaiBC gene cluster governing its circadian expression is important for regulating the cyanobacterial clockwork in *Synechococcus*. As the number and amplitude of oscillating genes have been shown to be much lower, and most of the *Synechococcus* high-amplitude genes, including kaiB and kaiC, were not found to oscillate significantly in *Synechocystis* (Ito et al., 2009), the question arises of whether the multiple KaiC proteins present might have an influence on precision or robustness of the clockwork.

From *Synechocystis* we know that the robust oscillations are a consequence of defined stoichiometry of the three Kai proteins (Nakajima et al., 2010). At the beginning of our work it was unclear whether the related kai gene products from *Synechocystis* perform similar physical interactions and display similar biochemical activities.

In this study, we demonstrate the deduced amino acid sequence of the additional kaiC2B2 cluster in *Synechocystis* to be very similar to the sequence of gene products found in Proteobacteria and Archaea, as suggested previously by Dvornyk and colleagues (Dvornyk et al., 2003), whereas the orphan kaiB3 and kaiC3 gene products show amino acid sequence similarity to proteins encoded in *Cyanothece, Microcystis* and *Chloroflexus* genomes. Our biochemical analyses revealed that all KaiC proteins of *Synechocystis* display an *in vitro* autokinase activity, but no protein–protein interaction among the diverged KaiC proteins could be observed. Furthermore, only KaiC1 seems to interact with, and to be affected by, KaiA.

---

2007; Xu et al., 2004). Very recently, it has been shown that both enzymic activities occur at the same active site (Egli et al., 2012). During dephosphorylation, ATP is synthesized and subsequently hydrolysed by an ATPase activity located in the CII-half of KaiC (Egli et al., 2012; Nishiwaki & Kondo, 2012).
METHODS

Bacterial strains. The strain of Synechocystis used in this study was originally obtained from S. Sheshkakov (Moscow State University, Russia). Recombinant Kai proteins from Synechococcus were purified from *Escherichia coli* BL21 strains kindly provided by T. Kondo (Nagoya University, Japan).

Cloning and heterologous expression of recombinant Kai proteins. Genes encoding KaiA, KaiC1 and KaiC3 from *Synechocystis* (according to the names in Cyanobase) were amplified by PCR using specific primers and genomic wild-type DNA as template. The ORFs and the primers used were: *kaiA* (slr0756) (F 5'-tgtagcggtacccagctctccctc, R 5'-accagctggccgcttgattacgtc), *kaiC1* (slr0758) (F 5'-ctagggatccactccattgtaaagc, R 5'-gaagattggcgccgcttccaacgcttcctc) and *kaiC3* (slr1942) (F 5'-gaagatgatatggactgac, R 5'-gttacagcgcgccgcttgattacgtc). Amplified DNA fragments were cloned into the expression vector (a self-replicating expression vector designed for pUR (kaiC1)). Both vectors contain the copper repressible petI promoter. For the kaiC2 and kaiC3 vector constructs, a 3 x FLAG sequence (DYKDDDDKDQKDDDDKDDDDKDDDDKDDDDKDDDD) encoded by the annealed oligonucleotides FLAG-fwd (5'-gaaagtgcggccgctttgtagc), kaiC1 and kaiC3. The promoter region of petI together with a 3 x FLAG-tag from vector pSKFLAG (Peter et al., 2009) was amplified using the 5'-gtgcatgactccgcttgagc and 5'-gaagattgttgctcctcctcctc and kaiC3 (slr1942) (F 5'-gaagatgatatggactgac, R 5'-gttacagcgcgccgcttgattacgtc). Amplified DNA fragments were digested with BamH1 and NotI and inserted into the respective restriction sites of the vector pGEX-6P-1 (GE Healthcare) for expression with an N-terminal GST-tag. *E. coli* BL21 cells were transformed with the resulting plasmids (pGEX-kaiA, pGEX-kaiC1 and pGEX-kaiC3, respectively). For overexpression of Kai proteins from *Synechococcus* and *Synechocystis*, 1 L TB medium (Tarlow & Hobbs, 1987) containing 100 ug ampicillin ml⁻¹ was inoculated with 100 ml of the respective overnight culture and incubated at 37 °C and 200 r.p.m. Expression of recombinant GST-KaiA was induced by adding 1 mM IPTG after 3 h of incubation. Cells were further grown overnight. Expression of recombinant *Synechococcus* GST-KaiC and *Synechocystis* GST-KaiC1 and GST-KaiC3 proteins was carried out for 72 h without induction by IPTG. For affinity purification of recombinant GST-KaiA, cells were harvested and resuspended in 15 ml ice-cold extraction buffer (50 mM Tris/HCl (pH 8), 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT). Cells were lysed by 30 min incubation with lysozyme and Benzonase Nuclease (Novagen) followed by sonication. Proteins were bound to a glutathione resin (Protno Glutathione Agarose 4B, Macherey-Nagel or Glutathione Sepharose 4B, GE Healthcare) for 1 h at ambient temperature. Resins were thoroughly washed with ice-cold extraction buffer and recombinant proteins were cleaved off by incubation with PreScission protease (GE Healthcare) in cleavage buffer (50 mM Tris/HCl (pH 8), 150 mM NaCl, 1 mM EDTA, 1 mM DTT) at 4 °C overnight. Purification of recombinant KaiC proteins was performed as described above, except that MgCl₂ and ATP were added to extraction buffer (5 mM MgCl₂, 1 mM ATP) and cleavage buffer (5 mM MgCl₂, 0.5 mM ATP). Purity was determined by separating the proteins via SDS-PAGE. If it was not sufficient, the proteins were further purified by ion-exchange chromatography using a MonoQ 5/50 GL column (GE Healthcare). Affinity purification of KaiC1 always resulted in co-purification of an E. coli protein. Approaches to remove this protein by anion-exchange chromatography led to loss of KaiC1. Therefore, the inhomogeneous KaiC1 preparation was used for further analyses. Protein concentrations were determined either according to the method of Lowry et al. (1951) including precipitation with sodium deoxycholate and trichloroacetic acid following the method described by Bensadoun & Weinstein (1976) or by using the Bradford method (Bradford, 1976).

Cloning, expression and purification of 3 x FLAG-KaiC proteins. The coding sequences of the three KaiC proteins from *Synechocystis* were amplified by PCR using the following primer pairs: kaiC1 (slr0758) (F 5'-catagcactctgtgtaagc, R 5'-agtacttattataacccaggaac), kaiC2 (slr11595) (F 5'-catctacgataactcagcagct, R 5'-agctttctctggttttgtatg), kaiC3 (slr1942) (F 5'-catctacgataactcagcagct, R 5'-agctttctctggttttgtatg). The resulting products were cloned into the NdeI and BglII sites of the pSK9 expression vector (kaiC2 and kaiC3) (Kuchmna et al., 2012) or the *NdeI* and *BamHI* sites of a self-replicating expression vector designed for pUR (kaiC1). Both vectors contain the copper repressible petI promoter. For the kaiC2 and kaiC3 vector constructs, a 3 x FLAG sequence (DYKDDDDKDQKDDDDKDDDDKDDDDKDDDDKDDDD) encoded by the annealed oligonucleotides FLAG-fwd 5'-tattgattataaaatgtcatgggtcatgattgatttagattaaag and FLAG-rev 5'-taattctactcatctcatctcatctcatctcatctcatctctctccta and inserted into the NdeI site at the 5' ends of the kaiC2 and kaiC3 genes resulting in the plasmids pSK-F-KaiC2 and pSK-F-KaiC3, respectively.

The pUR vector was constructed based on a modified pVZ321 vector (Zinchenko et al., 1999), where the chloramphenicol resistance cassette was exchanged with the *aadA* gene conferring streptomycin resistance. The promoter region of petI together with a 3 x FLAG-tag from vector pSKFLAG (Peter et al., 2009) was amplified using the 5'-gtgcatgactccgcttgagc and 5'-gaagattgttgctcctcctc and kaiC3 (slr1942) (F 5'-gaagatgatatggactgac, R 5'-gttacagcgcgccgcttgattacgtc) and the 3 x FLAG sequence. The 3 x FLAG sequence was reintroduced into the NdeI site using the above-mentioned FLAG-oligonucleotides resulting in the plasmid pUR-F-KaiC1.

*Synechocystis* wild-type cells were transformed with the resulting plasmids pUR-F-KaiC1, pSK9-F-KaiC2 and pSK9-F-KaiC3. For transfer of the pUR-F-KaiC1 vector conjugation was used (Zinchenko et al., 1999). Cell cultures of the overexpression strains were grown under medium light intensities (30 μmol photons m⁻² s⁻¹) in 800 ml copper-depleted BG11 medium (Rippka et al., 1979) supplemented with 10 mM TES pH 8 at 30 °C and provided with 5 % carbon dioxide in air. Cells were harvested in the stationary growth phase by centrifugation. The pellet was resuspended in cold FLAG buffer [50 mM HEPES/NaOH (pH 7.5), 5 mM MgCl₂, 25 mM CaCl₂, 10 % (v/v) glycerol, 150 mM NaCl, 5 μg 6-aminohexaonic acid ml⁻¹, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 4 mM p-amino benzenazidine, 1 mM ATP]. The cells were disrupted with a 1:1 mixture of glass beads (0.1–0.11 mm and 0.25-0.5 mm size) in a bead beater (Reetsch) at 4 °C. The chlorophyll content of the homogenate was measured in 80 % acetone as published by Mackinney, 1941. Membrane proteins were solubilized by adding n-dodecyl-β-maltoside (β-DM) at a detergent-to-chlorophyll ratio of 20:1 at 4 °C for 1 h. Unsolubilized debris was removed by centrifugation at 9000 g for 60 min at 4 °C. The supernatant was incubated overnight with 400 μl anti-FLAG M2 affinity gel (Sigma-Aldrich) at 4 °C and loaded onto a Poly-Prep chromatography column (Bio-Rad) to separate the affinity gel from the flow through. The resin material was thoroughly washed with FLAG buffer containing 0.03 % β-DM. Since sufficient native elution of 3 x FLAG-KaiC2 from the anti-FLAG M2 affinity gel was not possible, phosphorylation assays were performed using the resin-bound protein. For gel analysis and Western blotting of 3 x FLAG fusion proteins, the respective protein was eluted from the resin using a 1 % w/v SDS solution. Protein concentrations were determined according to Lowry et al. (1951). For detection of the 3 x FLAG-tagged proteins, a monoclonal anti-FLAG M2 alkaline phosphatase antibody (Sigma-Aldrich) was used.

In vitro phosphorylation assays. To investigate the phosphorylation of *Synechococcus* KaiC and *Synechocystis* KaiC1 and KaiC3, 12 μg of the respective recombinant protein preparations was incubated in the presence or absence of 6 μg recombinant *Synechocystis* KaiA in 60 μl reaction buffer [20 mM Tris/HCl...
RESULTS AND DISCUSSION

Multiple kai gene copies in Synechocystis sp. PCC 6803

Starting from genome analysis, it is puzzling that besides marine Synechococcus and Prochlorococcus strains most cyanobacterial genomes contain multiple kai gene copies. Synechocystis harbours one kaiA, three kaiB and three kaiC genes (Aoki & Onai, 2009; Dvornyk et al., 2003). Based on amino acid sequence similarity kaiAB1C1 of Synechocystis is proposed to be the orthologue of the ‘standard’ kaiABC cluster of Synechococcus. The orphan kaiB3 and kaiC3 genes of Synechocystis show a higher sequence similarity to this kaiAB1C1 cluster than do the kaiCB2 genes. Furthermore, our BLAST analysis based on the Synechocystis KaiC1 amino acid sequence (Fig. 1a) revealed a complex pattern of KaiC relationships in Cyanobacteria. We also found sequences similar to KaiC1, outside the cyanobacterial phylum, in Proteobacteria, Chloroflexi and Archaea, an observation that has been suggested previously by Dvornyk et al. (2003) as well as Aoki & Onai (2009). Fig. 1 illustrates that species of the genera Cyanothece and Microcystis often maintain two kaiCs: one is more closely related to Synechocystis kaiC1 (Fig. 1a, KaiC1 group) and the other to kaiC3 (Fig. 1a, KaiC3 group). Synechocystis KaiC2 shows highest amino acid sequence similarity to proteins from Proteobacteria and Archaea (Fig. 1a, KaiC2 group). Furthermore, we found also a Chloroflexus strain, Chloroflexus aggregans, that harbours three kaiC genes, each clustering with a different lineage. Fig. 1(b) provides an insight into the synteny of kai genes in Synechocystis in comparison with example species. Here, kaiC2 and kaiB2 lie in a cluster not only in several Cyanobacteria but also in some Proteobacteria and Chloroflexi (Fig. 1b). The orphan gene kaiB3 appears often downstream of a gene encoding a NarL family two-component response regulator. Another orphan kaiB gene found in Cyanothece, Microcystis and Anaabaena is located downstream of a pilT gene encoding the twitching motility protein PilT.

Synechocystis KaiC proteins diverge in important C-terminal residues

Interestingly, the three Synechocystis KaiC proteins show common sequence properties, but also large sequence diversity. KaiC1 has the highest overall amino acid identity (82 %) to the well-studied Synechococcus KaiC, followed by KaiC3 (55 %) and KaiC2 (37 %).

A comparative alignment of their C-terminal sequences with those of Synechococcus and Thermosynechococcus elongatus BP-1 (Thermosynechococcus) KaiC (Fig. 2) demonstrates that all KaiC proteins possess two putative phosphorylation sites corresponding to those described for Synechococcus (S431 and T432). Furthermore, all KaiC proteins display the short-lived phosphorylation site aligning to T426 in Synechococcus, which has been proposed to be involved in dephosphorylation of KaiC.
Fig. 1. Reduced phylogenetic tree of Synechocystis KaiC1 homologues found in protein sequences at NCBI and synteny of Synechocystis kai genes compared with example species. (a) All BLAST hits for kaiC1 were clustered and for each group (size refers to the number of genes in each group) between one and six genes were selected to represent a cluster of the tree (see Methods for details). Species of the major taxa are indicated with different colours: green = Cyanobacteria, red = Archaea, blue = Proteobacteria and Chloroflexi. The cluster indicated by two asterisks consists of various genes with weak similarity to kaiC1. (b) Synteny of Synechocystis kaiA1B1C1 (CyanoBase accession nos slr0756, slr0757 and slr0758), orphan kaiB3 and kaiC3 genes (slr0486 and slr1942) and kaiC2B2 (slr1595 and slr1596) in comparison with similar genes of diverse cyanobacteria, Chloroflexus and Rhodopseudomonas.
However, Synechocystis KaiC2 contains two serine residues instead of S431 and T432 in Synechococcus and displays a prolonged C terminus. Furthermore, the A-loop as well as residues important for A-loop stabilization and KaiA-binding are conserved to different degrees in Synechocystis KaiC proteins. In KaiC1 the A-loop is completely identical to that of Synechococcus KaiC, whereas in KaiC2 and KaiC3 only half of the residues are identical or conservatively substituted. Strikingly, the last three residues of the A-loop are not conserved in the latter two KaiC homologues. For Synechococcus KaiC, Kim et al. (2008) demonstrated that these residues, especially I497, are important to stabilize the A-loop in the buried state. The observed low conservation of the A-loop and further residues that have been demonstrated to be involved in A-loop stabilization by Kim and colleagues (Fig. 2), suggests that A-loops of KaiC2 and KaiC3 predominate in the exposed state, making physical interaction with KaiA dispensable. In KaiC2 even an element that was described to couple A-loop state with phosphorylation (Kim et al., 2008) is likely to be absent. In Synechococcus KaiC interactions of amino acids 438–444 with the P-loop on the one hand and of E444 with the buried A-loop on the other hand disable phosphorylation, because due to these interactions the phosphoryl donor ATP, which is bound to the P-loop, cannot reach the phosphorylation sites (Kim et al., 2008). KaiC2 displays only low conservation of the 438–444 segment and lacks E444, whereas the segment is nearly conserved in KaiC1 and KaiC3, indicating that different regulatory mechanisms arose during KaiC evolution (Dvornyk et al., 2003). Vakonakis & LiWang (2004) crystallized the C-terminal domain of Thermosynechococcus KaiA in complex with a Thermosynechococcus KaiC-derived peptide. They identified residues involved in hydrophobic and electrostatic interactions as well as formation of hydrogen bonds between KaiA and KaiC. As stated previously by Dvornyk & Knudsen (2005), these residues are conserved to different degrees in cyanobacterial KaiC proteins. Most of these residues are identical or similar in Synechocystis KaiC1, but not conserved in KaiC2 and KaiC3 (Fig. 2). Therefore, a protein–protein interaction between Synechocystis KaiC1 and KaiA is very likely, whereas KaiC2 and KaiC3 might not bind KaiA.

**Synechocystis** KaiA can substitute its **Synechococcus** orthologue in promoting autophosphorylation of **Synechococcus** KaiC

Cyanobacterial KaiA proteins show great sequence divergence in their N-terminal domain, whereas the C-terminal KaiC-binding domain is highly conserved (Uzumaki et al.,...
KaiA shows only 41% identity to that of Synechocystis. Consistently, the overall protein sequence of agarose bound KaiC2 were incubated with \([\gamma^{32}\text{-P}]\) ATP revealed that Synechocystis KaiA significantly enhances Synechococcus KaiC’s autokinase activity (Fig. 3). Hence, the capability to stimulate kinase activity of KaiC is conserved in Synechocystis, implying that it might well sustain a circadian clock function by affecting KaiC phosphorylation. This goes along with findings of Iwase et al. (2005) who showed that Synechocystis KaiB1 can complement deletion of kaiB in Synechococcus.

### All Synechocystis KaiC proteins show kinase activity in vitro

To determine whether the KaiC proteins exhibit autophosphorylation similar to the well-studied Synechococcus KaiC, we analysed the \([\gamma^{32}\text{-P}]\) phosphate uptake depending on Synechocystis KaiA. In the presence of KaiA, KaiC1 displayed strong autokinase activity resulting in formation of the phosphorylated KaiC1 protein (P-KaiC1). This could be observed by accumulation of radioactively labelled KaiC1 (Fig. 3) and a slower migrating band after separation via SDS-PAGE and Coomassie staining (Fig. S1a, available with the online version of this paper), representing most probably the phosphorylated form of KaiC1. In the absence of KaiA, only a single KaiC1 band (Fig. S1a) and nearly no incorporation of \([\gamma^{32}\text{-P}]\) phosphate (Fig. 3) could be detected.

In contrast, KaiC3 showed an intrinsic kinase activity that was independent of KaiA (Fig. 3). Interestingly, the ratio of the faster and slower migrating band did not change significantly during incubation (Fig. S1a). Control-treatment of KaiC3 with a protein phosphatase demonstrated the two bands to represent a phosphorylated (P-KaiC3) and non-phosphorylated (NP-KaiC3) form of KaiC3 (Fig. S1b). Accordingly, we have to assume that the ratio of phosphorylated to non-phosphorylated KaiC3 is rather constant under our experimental conditions.

As we were not able to express Synechocystis KaiC2 in E. coli cells in sufficient amounts and as a biochemically active form, a 3 × FLAG-tagged version of KaiC2 was produced in Synechocystis wild-type cells harbouring also the wild-type KaiC2 protein. The 3 × FLAG-tagged KaiC2 isolated from Synechocystis also showed autokinase activity that was independent of KaiA (Fig. 3). Western blot analysis using anti-FLAG and KaiC2 antibodies suggests that the two visible bands appearing after autoradiography as well as in SDS-PAGE correspond to the 3 × FLAG-tagged and untagged KaiC2, the latter of which was co-purified (Fig. S1d). Obviously, both forms built heterohexameric complexes in vivo. Wild-type as well as the tagged version of KaiC2 display autokinase activity in an equal fashion (Fig. 3).

In summary, our in vitro analyses clearly demonstrate, in consistency with our in silico analyses, that all KaiC proteins of Synechocystis exhibit autophosphorylation activity but only KaiC1’s kinase activity is positively affected by Synechocystis KaiA as has been observed for the Synechococcus KaiC protein. The KaiA-independent autophosphorylation activity obtained for KaiC2 and KaiC3 is similar to the in vitro activities of KaiC from Prochlorococcus marinus MED4. Marine Prochlorococcus species do not possess KaiA but possess functional KaiB and KaiC proteins (Axmann et al., 2009). The enhanced autophosphorylation activity we measured for Prochlorococcus KaiC might compensate for the absence of KaiA (Axmann et al., 2009). An hourglass-like mechanism has been suggested for Prochlorococcus cells (Axmann et al., 2009; Mullineaux & Stanewsky, 2009) that exhibit a biological diurnal rhythm, but no sustained rhythm under constant-light conditions (Holtzendorff et al., 2008). Thus, one might assume a possible KaiC2- or KaiC3-based timing mechanism for Synechocystis that is similar to the Prochlorococcus hourglass and runs in addition to the KaiABC clock. A putative fine-tuning of the KaiAB1C1 clock by KaiB3 and KaiC3 has been hypothesized previously (Aoki & Onai, 2009).
**KaiA only co-purifies with KaiC1. All Synechocystis KaiC proteins show homomeric subunit interactions**

In order to investigate direct protein–protein interactions between the three KaiC proteins and KaiA, N-terminal 3 × FLAG-KaiC1, -KaiC2 and -KaiC3 fusion proteins were separately expressed in *Synechocystis* cells. Cellular phenotypes of these mutant strains as determined by spectroscopy and microscopy remained unchanged upon 3 × FLAG-KaiC expression and were indistinguishable from the wild-type (data not shown). Total cell extracts and elution fractions of FLAG-agarose-bound proteins after immunoprecipitation were separated by SDS-PAGE, blotted and subjected to immunoblot analysis using different antibodies. As a control, the presence of the respective 3 × FLAG-KaiC proteins in the elution fractions was demonstrated by incubation of the blot membranes with alkaline-phosphatase-coupled z-FLAG antibody (Fig. 4a, lower panel). All 3 × FLAG-tagged KaiC proteins migrated in SDS-PAGE at their expected sizes, though 3 × FLAG-KaiC2 was hardly detectable in the crude cell extract, possibly due to its low expression level. Immunoblot analysis using a primary antibody raised against *Synechocystis* KaiA demonstrated that KaiA co-eluted only with 3 × FLAG-KaiC1 (Fig. 4a, upper panel). No immunolabelled bands were detected in the elution fractions of 3 × FLAG-KaiC2 and 3 × FLAG-KaiC3 samples. This result is consistent with our in vitro and in silico analyses, which suggest that no physical interaction takes place between KaiC2 or KaiC3 and KaiA. An antiserum detecting the AtpB subunit served as a control.

For further protein–protein interaction analysis, epitope-directed antibodies specific for KaiC1, KaiC2 and KaiC3 were used. Specificity of these KaiC antibodies was achieved by choosing peptide sequences for antibody production that are discriminatory for the respective KaiC protein. KaiC1 antibody is raised against the N terminus of KaiC1 (NLPIVNERNRPDVPRKGVQ at position 2–20). KaiC2 and KaiC3 antibodies are targeted against peptide sequences in the diverged C terminus (EAIAKRQQALELSKRNFERKK at position 495–516 and MYTAQSEVERLSGLFDEKI at position 487–505, respectively). In order to reveal possible heteromeric interactions between the three *Synechocystis* KaiC proteins FLAG affinity purification of KaiC proteins was performed as described above and Western blot membranes were incubated with the epitope-directed antibodies raised against KaiC1, KaiC2 and KaiC3, respectively (Fig. 4b). For control, the blot membranes were afterwards incubated with alkaline-phosphatase-coupled z-FLAG antibodies (Fig. 4b, lower panel). These analyses revealed that, along with the tagged versions, also the respective wild-type KaiC proteins were co-purified, resulting in two bands in the respective lanes (Fig. 4b), but no heteromeric interactions between KaiC1, KaiC2 and KaiC3 were observed. Only a very faint immunolabelled band was detected in the 3 × FLAG-KaiC1 and 3 × FLAG-KaiC2 eluates after incubation with the KaiC3-specific antiserum. This could be due to protein–protein interaction but most probably reflects a weak affinity of the KaiC3 antibody to KaiC1 and KaiC2 or some contamination of KaiC3 in the eluate fraction, as was also detected for ATPase (Fig. 4a).

The wild-type proteins co-purified with their respective FLAG-tagged variants, indicating that all three Kai proteins form multimers (most probably hexamers as for the *Synechococcus* KaiC) and that the N-terminal 3 × FLAG-tag did not interfere with KaiC-multimerization. Therefore, we suggest that there exist three specific KaiC complexes in *Synechocystis* cells.
Conclusion

KaiC proteins are widespread not only in Cyanobacteria, but also in other Eubacteria and in Archaea. While the circadian clock system has been extensively researched in _Synechococcus_, studies on putative circadian clock systems in other Cyanobacteria as well as anoxygenic phototrophic and non-phototrophic prokaryotes are very rare. Our current analysis of the three different _Synechocystis_ KaiC proteins suggests that this cyanobacterium contains, along with a standard clock system, two other KaiC-related kinases, whose biological functions in circadian regulation or other processes have to be proven in future experiments. Notably, _Synechocystis_ does not show large-amplitude circadian oscillations in gene expression studies (Kucho et al., 2005). Although we could not find heteromeric interactions between the three different KaiC proteins in _Synechocystis_, we do not exclude the possibility that the divergent kaiC2 and kaiC3 gene products are involved in control of rhythmic behaviour of the cells, which would result in a more complex regulatory network in organisms containing multiple clock proteins. Taking into account that KaiC2 forms a group with sequences from other bacteria and represents an ancient type of KaiC proteins, _Synechocystis_ could be used as a model to study whether this specific KaiC type might have a clock-related function, similar to the hour-glass mechanism in _Prochlorococcus_, or possesses different roles. In recent years, post-translational oscillations in other organisms ranging from Archaea to humans have been discovered (Edgar et al., 2012; O’Neill & Reddy, 2011; O’Neill et al., 2011), which implies that protein rhythms are not unique to Cyanobacteria.

ACKNOWLEDGEMENTS

This work was supported by the Deutsche Forschungsgemeinschaft to A.W. and I.M.A., and by the German Ministry for Education and Research [Bundesministerium für Bildung und Forschung (BMBF)] through the Forschungseinheiten der Systembiologie (FORSYS) partner program (grant number 0315294) to I.M.A.

REFERENCES


Edited by: C.-C. Zhang