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CK2 α phosphorylates BMAL1 to regulate the mammalian clock

Teruya Tamaru¹, Jun Hirayama^{2,3}, Yasushi Isojima⁴, Katsuya Nagai⁴, Shigemi Norioka⁵, Ken Takamatsu¹, and Paolo Sassone-Corsi^{2,*}

¹Department of Physiology, Toho University School of Medicine, 5-21-16 Ohmori-nishi Ohta-ku Tokyo 143-8540, Japan

²Department of Pharmacology, School of Medicine, University of California, Irvine, California 92697, USA

³Medical Top Track Program, Medical Research Institute, Tokyo Medical and Dental University, Yushima 1-5-45, Bunkyo-ku, Tokyo 113, Japan

⁴Division of Protein Metabolism, Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka 565-0871, Japan

⁵Division of Protein Chemistry, Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka 565-0871, Japan

Abstract

Clock proteins govern circadian physiology and their function is regulated by a variety of mechanisms. Here we demonstrate that CK2 α phosphorylates the core circadian regulator BMAL1. Gene silencing of CK2 α or mutation of the highly conserved CK2-phosphorylation site in BMAL, Ser90, result in impaired nuclear BMAL1 accumulation and disruption of clock function. Importantly, phosphorylation at Ser90 follows a rhythmic pattern. These findings reveal that CK2 is an essential regulator of the mammalian circadian system.

The circadian clock orchestrates intrinsic timing in most organisms and controls a large variety of physiological and metabolic programs¹. The molecular core of the circadian clock is constituted by multiple gene products that operate in transcriptional-translational feedback loops^{1, 2}. The BMAL1-CLOCK heterodimer is central to the clock mechanism as it drives and maintains circadian oscillations. Several studies indicate that clock proteins are important targets of post-translational modifications^{3–7}, and their rhythmic phosphorylation appears to be a critical step for regulated function^{2, 8, 9}. Indeed, BMAL1 phosphorylation is rhythmic in serum-shocked-synchronized fibroblasts¹⁰. Furthermore, drastic circadian phenotypes in the fly are caused by mutations in kinases, such as *Doubletime (Dbt)*, the fly ortholog of CKI ϵ , shaggy (*sgg*), the fly ortholog of Glycogen synthase kinase 3 (GSK3), and CK2. The *Drosophila* dCK2 specifically regulates the nuclear entry of dPER thereby controlling the fly clock^{11–13}. In mammals, the *tau* mutant, characterized by impaired circadian rhythmicity, is due to a mutation in the gene encoding the clock regulating kinase CKI ϵ ¹⁴.

*Corresponding author: psc@uci.edu.

Microsequencing of purified p45^{PFK}, a kinase previously implicated in circadian control in mammals¹⁵, demonstrates that it corresponds to CK2 α (Suppl. Fig.1). Interestingly, purified p45^{PFK} phosphorylates BMAL1 and CLOCK *in vitro*^{15, 16} (Suppl. Fig. 2). The CK2 holoenzyme is constituted by two copies of the catalytic (CK2 α) and regulatory (CK2 β) subunits (α 2 β 2). CK2 α monomer also exists as an active form *in vivo*. To assess the role of CK2 β in modulating CK2 α -mediated BMAL1 phosphorylation, we co-incubated the subunits in kinase assays. CK2 α alone phosphorylated GST-BMAL1, whereas CK2 β inhibited BMAL1 phosphorylation in a dose-dependent manner (Fig. 1). These results demonstrate that CK2 α monomer phosphorylates BMAL1.

To establish the physiological role of CK2 α -mediated BMAL1 phosphorylation, we used microRNAi (miR)-mediated silencing in dexamethasone (Dex)-synchronized NIH-3T3 fibroblasts. A miR-CK2 α silencing vector specifically reduced CK2 α levels at less than the half of the control ($P=0.002$) (Fig. 2A), resulting in drastically impaired circadian *mPer2* oscillation as monitored by real-time *mPer2* promoter-driven luciferase bioluminescence⁴ (Fig. 2B). In fact, after a first peak with significantly reduced amplitude, *mPer2* oscillation was almost abolished in miR-CK2 α transfected cells (Fig. 2B). These results were confirmed by using an alternative target sequence (Suppl. Fig. 3). Thus, CK2 α is essential for mammalian circadian rhythmicity.

Interestingly, silencing of CK2 α leads to substantial cytoplasmic BMAL1 retention at 24h post Dex treatment (Fig. 2C), a time when BMAL1 is normally mostly nuclear¹⁰. As the function of the CLOCK-BMAL1 complex is intimately dependent on the nuclear translocation of BMAL1¹⁷, we speculate that CK2 α exerts its control on the mammalian circadian clock by dictating BMAL1 intracellular localization. This is reminiscent of the role described for the *Drosophila* dCK2 in controlling dPER nuclear entry¹³.

We searched the BMAL1 primary sequence for CK2 consensus phosphoacceptor sites (S/T-X-X-D/E). The serine residue at position 90 is highly conserved among all vertebrate BMAL1s (Suppl. Fig. 4B), and a MALDI-Tof/MS analysis indicated that it is indeed a potential CK2-phosphorylation site (Suppl. Fig.4A). Interestingly, Ser90 is not conserved in dCYCLE, the *Drosophila* counterpart of BMAL1, consistent with the notion that dCK2 α is unable of phosphorylating dCYCLE¹³. These notions lead us to generate anti-phospho-S90 specific antibodies (see below) and to explore the *in vivo* relevance of Ser90 phosphorylation.

To establish the role of BMAL1 Ser90 phosphorylation in circadian function, we performed rescue experiments by expressing wild-type BMAL1 (BMAL1-WT), BMAL1-S90A mutant or GFP in mouse embryo fibroblasts (MEFs) derived from *Bmal1*-null mice which have a dysfunctional circadian clock^{4, 6, 7} (Fig. 2D). *mPer2* expression was monitored in Dex-synchronized MEFs by a real-time reporter assay. BMAL1-WT rescued circadian *mPer2* expression in Dex-synchronized MEFs (Suppl. Fig. 5), whereas the BMAL1-S90A mutant was unable to do so (Fig. 2F), despite its normal DNA-binding activity (not shown). These results indicate that BMAL1 phosphorylation at Ser90 is essential for circadian gene expression.

Ectopically expressed BMAL1-WT accumulated in the nucleus 24h post Dex treatment (Fig. 2F), paralleling the behavior of native BMAL1 in fibroblasts¹⁰. On the contrary, BMAL1-S90A remained mostly cytoplasmic (Fig. 2F). Consistent with previous reports indicating matching BMAL1-CLOCK localization pattern¹⁷, BMAL1-S90A suppressed nuclear CLOCK accumulation, possibly because of its decreased interaction potential with CLOCK (Suppl. Fig. 6). These data are consistent with the change in BMAL1 localization upon CK2 α silencing (Fig. 2C), and stress the notion that circadian function requires phosphorylation of BMAL1 and its nuclear accumulation.

To assess the temporal pattern of Ser90 phosphorylation *in vivo*, we raised an antibody specific for Ser90-phosphorylated BMAL1. Specificity was validated since this antibody (P-BMAL1-S90) detects Myc-BMAL1 but not the Myc-BMAL1-S90A mutant or Myc-GFP (Suppl. Fig. 7). Using the P-BMAL1-S90 antibody, we examined whether silencing of CK2 α indeed impaired BMAL1 phosphorylation at Ser-90. The P-BMAL1-S90 antibody detected a band corresponding to the highly phosphorylated protein (apparent migration pattern corresponding to ca. 85-90kDa) in the concentrated BMAL1-immunoprecipitated from the control- vector-transfected NIH-3T3 cells at 24h post Dex treatment (Fig. 3A). The targeting vectors for miR-CK2 α significantly decreased the P-BMAL1-S90 signal (Fig. 3A). Interestingly, knockdown of CK2 α strongly affects the total phosphorylation state of BMAL1, suggesting that CK2 α may influence/modulate BMAL1 multiple phosphorylations by other kinases, including ERKs and CKI ϵ ^{18, 19}.

Next, we examined whether P-BMAL1-S90 levels oscillated *in vivo*. In Dex-synchronized NIH-3T3 cells Ser90 phosphorylation showed a rhythmic pattern with peaks at 20-24 h and 44-48h, paralleling PER1 level oscillation (Fig. 3B). At peaking times, the P-BMAL1-S90 signal gradually shifted to higher molecular weight (~90kDa) corresponding to the hyper-phosphorylated forms. The timing of BMAL1-S90 phosphorylation fits with cyclic nuclear entry of BMAL1 in Dex-synchronized cells (not shown) and serum shock-synchronized cells¹⁰. The levels of CK2 subunits (α' is a subtype of CK2 catalytic subunit) are basically constant during the circadian cycle (Fig. 3B), thus they are unlikely to determine P-BMAL1-S90 oscillation. Finally, based on the BMAL1 phosphorylation pattern (Figs. 3A,B), it would seem that CK2 α could play a triggering role in multiple phosphorylation events.

Our findings demonstrate that CK2 α is a BMAL1 kinase that plays an essential role in the mammalian clock by regulating BMAL1 nuclear entry. The single Ser90 phosphorylation site is essential for circadian rhythmicity and its mutation causes a phenotype similar to that caused by depletion of CK2 α (compare Figs. 2BC & 2EF). These findings suggest that CK2 α plays a pivotal role in the mammalian circadian clock, by controlling BMAL1 intracellular distribution. While it is likely that CK2 influences circadian physiology at multiple levels because it phosphorylates a large array of cellular proteins²⁰, the remarkable specificity observed here on BMAL1-Ser90 provides a lead to further investigations. Finally, as BMAL1 has been shown to be SUMOylated and acetylated^{4, 7}, the regulatory pathway presented here may participate in the interplay between phosphorylation and other post-translational modifications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. CK2α phosphorylates BMAL1 *in vitro*

A) Mixtures of CK2α (50ng; visible in CBB staining) and CK2β (10ng; visible in autoradiography after phosphorylation) subunits were used in *in vitro* kinase assay with or without ATP using GST (lane 1) or GST-BMAL1 (lane 2) as substrates. **B)** CK2α (4 pmol) and CK2β (0-8 pmol) were used in kinase assays with GST-BMAL1 as substrate (500ng; optimal ratio to CK2α) kinase assay. GST-BMAL1 kinase activities (average values) is plotted at each CK2β/CK2α ratio, and normalized against activity in the absence of CK2β with error bars ($\pm$ SD values). Photographs are representative of duplicate experiments.

Figure 2. CK2α and BMAL1 Ser90 regulate nuclear accumulation and clock function (A-C) CK2α silencing/depletion affects BMAL1 nuclear localization and clock function. NIH-3T3 cells were transfected with microRNAi (miR) targeting vectors for mCK2α and the validated negative-control (control) vector, then Dex-synchronized. **A**) Immunoblot analysis for CK2α and Actin in cell lysates, with one representative experiment shown. Normalized average CK2α levels from triplicate experiments are shown below with error bars ($\pm$ SD values). **B**) Circadian *mPer2* expression was monitored by a real-time bioluminescence assay. Normalized average values from multiple experiments ($n=5$) are plotted with error bars ($\pm$ SD values of 1st/2nd peaks). **C**) Localization of BMAL1, visualized by immunofluorescence (red) at 24h post Dex treatment. Nuclei were stained with DAPI (blue) and miRNAi-transfected GFP-positive cells (green). Photographs are representative of triplicate experiments. Percentages of cells with BMAL1 predominantly nuclear among the GFP-positive cells from triplicate experiments are plotted as means $\pm$ SEM. (***; $P < 0.001$). (D-F) Mutation of BMAL1 Ser90 affects nuclear localization and clock function. **D**) Lysates from *Bmal1*^{-/-} MEFs stably expressing Myc-BMAL1-WT, Myc-BMAL1-S90A and Myc-GFP were analyzed with anti-Myc antibody. **E**) Circadian *mPer2* expression was not restored by BMAL1 S90A, as monitored as was monitored by a real-time bioluminescence assay. Normalized average values from multiple experiments ($n=5$) are plotted with error

bars ($\pm$ SD values of 1st/2nd peaks). **F)** MEFs at 24h post Dex treatment were stained with anti-BMAL1 (Green) antibody. Nuclei were stained with DAPI (Blue). Photographs are representative of multiple (n=4) experiments. Values of nuclear BMAL1-dominant cells (%) in MEFs from multiple (n=4) experiments are plotted as means $\pm$ SEM (***, $P < 0.001$).

Figure 3. Circadian phosphorylation of BMAL1-Ser90 by CK2α *in vivo*

A) NIH-3T3 cells expressing miR-CK2α and control vector at 24h post Dex treatment were subjected to BMAL1-immunoprecipitation (IP) followed by immunoblot analysis (WB) for BMAL1, P-BMAL1-S90, CK2α and Actin (Lysate). Arrows and bars designate BMAL1 and P-BMAL1-S90. **B)** BMAL1-immunoprecipitates and lysates of NIH-3T3 cells at each time point post Dex treatment were used in immunoblot analysis for P-BMAL1-S90; BMAL1, PER1, CK2 subunits and Actin. Arrows and bars designate BMAL1 and P-BMAL1-S90. Normalized average values for P-BMAL1-S90 and PER1 from triplicate experiments are plotted in the graph with error bars ($\pm$ SD values). All the photographs are representative of triplicate experiments.