Rapid activation of CLOCK by Ca^{2+} -dependent protein kinase C mediates resetting of the mammalian circadian clock

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In mammals, immediate-early transcription of the *Period 1* (*Per1*) gene is crucial for resetting the mammalian circadian clock. Here, we show that CLOCK is a real signalling molecule that mediates the serum-evoked rapid induction of *Per1* in fibroblasts through the Ca^{2+} -dependent protein kinase C (PKC) pathway. Stimulation with serum rapidly induced nuclear translocation, heterodimerization and Ser/Thr phosphorylation of CLOCK just before the surge of *Per1* transcription. Serum-induced CLOCK phosphorylation was abolished by treatment with PKC inhibitors but not by other kinase inhibitors. Consistently, the interaction between CLOCK and PKC was markedly increased shortly after serum shock, and the Ca^{2+} -dependent PKC isoforms PKC α and PKC γ phosphorylated CLOCK *in vitro*. Furthermore, phorbol myristic acetate treatment triggered immediate-early transcription of *Per1* and also CLOCK phosphorylation, which were blocked by a Ca^{2+} -dependent PKC inhibitor. These findings indicate that CLOCK activation through the Ca^{2+} -dependent PKC pathway might have a substantial role in phase resetting of the circadian clock.

Keywords: circadian rhythm; CLOCK; phase shift; phosphorylation; PKC

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INTRODUCTION

The circadian clock is an intrinsic timing device that typically runs with a period of about 24 h. Its main function is to optimize metabolic and physiologic behaviour in anticipation of cyclic changes in environmental conditions. The phase of the day and night cycle changes depending on the position of the Earth in its orbit around the sun; therefore, periodic resetting of the biological clock is required not only to maintain accurate timekeeping, but also to synchronize circadian rhythms with the solar day (Reppert & Weaver, 2002; Schibler & Sassone-Corsi, 2002). Although the phase resetting of the master clock in the suprachiasmatic nucleus (SCN) and the peripheral clocks throughout the body is regulated by different input signals, their downstream pathways are known to converge on the same, or similar, intracellular signalling cascades (Balsalobre *et al*, 2000; Yagita *et al*, 2001). The most prominent molecular event that leads to clock resetting is a rapid surge of *Period 1* (*Per1*) transcription in both the SCN and peripheral tissues. Furthermore, in cultured cell lines, diverse extracellular stimuli, including serum shock, can trigger immediate-early transcription of *Per1*, and this in turn seems to elicit a robust circadian oscillation of clock gene expression (Akashi & Nishida, 2000; Balsalobre *et al*, 2000; Yagita *et al*, 2001).

Intensive genetic studies on *Clock* mutants have provided an insight into the role of CLOCK in phase resetting. The homozygous *Clock* mutation significantly reduces the amplitude of the photic induction of *Per* genes in the SCN and abolishes the light-induced phase shift of behavioural rhythm (Vitaterna *et al*, 1994; Shearman & Weaver, 1999; Low-Zeddies & Takahashi, 2001). Although heterozygous *Clock* mutants maintained circadian rhythmicity under constant darkness, they were defective in phase shifting in response to both photic and non-photoc stimuli (Challet *et al*, 2000; Low-Zeddies & Takahashi, 2001). In addition, we have recently shown that overexpression of CLOCK Δ 19, a dominant-negative form of CLOCK, reduces the serum-evoked surge of *Per1* transcription without any significant effect on other immediate-early genes (Jung *et al*, 2003). Thus, CLOCK seems to be

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important in resetting the circadian clock and also in maintaining circadian gene oscillation. In this report, we provide the first evidence for the direct involvement of the core clock component CLOCK in the signalling pathways responsible for resetting the circadian rhythm.

RESULTS AND DISCUSSION

Serum shock induces nuclear translocation of CLOCK

Most transcription factors that belong to the basic helix–loop–helix (bHLH)/PER-AHR/ARNT-SIM (PAS) family are crucial components of the signal-transduction pathways that allow organisms to adapt to environmental changes (Kewley *et al*, 2004). The CLOCK/brain and muscle Arnt-like protein-1 (BMAL1) heterodimer is a bHLH/PAS family transcription factor complex that regulates clock gene expression in response to the light and dark cycle, but its role in the signal-transduction pathway has not been described. Phylogenetic and structural analyses indicate that CLOCK is a Class I sensing factor and its partner BMAL1 is a Class II factor (Gu *et al*, 2000). The heterodimer that they form specifically recognizes the E-box located in the upstream regulatory regions of target genes, such as *Per1*, leading to transcriptional initiation. It is, therefore, reasonable to postulate that CLOCK is involved in the detection of, and adaptation to, changes in environmental conditions. To test this hypothesis, we examined the effect of serum shock (50% horse serum) on the subcellular distributions of CLOCK and BMAL1, and their interaction in NIH3T3 cells. Subcellular fractionation and Western blot analysis showed that CLOCK molecules rapidly translocated to the nucleus without significant alteration of the distribution of BMAL1 (Fig 1A; supplementary Fig 1 online). These observations were confirmed by immunocytochemistry (Fig 1B). The diffuse signal of CLOCK throughout the cells was concentrated in the nucleus shortly after serum shock (30 min), whereas BMAL1 showed a predominantly nuclear distribution regardless of serum treatment. We recently showed that BMAL1 has a functional nuclear localization signal (NLS) at its amino terminus, whereas CLOCK has no such signal (Kwon *et al*, 2006). Thus, heterodimerization with BMAL1 seems to be required for the rapid nuclear accumulation of CLOCK in response to serum. In favour of this idea, serum treatment markedly increased the amount of BMAL1 co-precipitated with CLOCK antibody and vice versa (Fig 1C; supplementary Fig 1 online). These results indicate that serum shock induces rapid nuclear translocation of CLOCK through heterodimerization with BMAL1.

PKC mediates the serum-evoked CLOCK phosphorylation

If CLOCK is a real signalling molecule modulating the circadian clock, it ought to be activated by post-translational modification in response to the resetting stimuli. Therefore, we conducted prolonged gel electrophoresis to examine the post-translational modification of CLOCK, and found transient but marked changes in its electrophoretic mobility (Fig 2A). The slowly migrating bands peaked at 30–60 min after serum shock and then gradually decreased. When the CLOCK immunoprecipitates were treated with λ protein phosphatase, the high-molecular-weight forms of CLOCK were reduced by more than 90%, and the addition of vanadate completely blocked this effect (Fig 2B, upper panel). In the same blot, the phospho-Ser antibody showed strong signals overlapping with the slow-migrating bands (Fig 2B, lower panel);

Fig 1 | Serum shock induces nuclear translocation and heterodimerization of CLOCK. (A) NIH3T3 cells were divided into cytoplasmic and nuclear fractions at the indicated time points after serum shock (SS) and subjected to immunoblotting by using specific antibodies for the indicated proteins. (B) Representative fluorescence images showing the nuclear accumulation of CLOCK. Upper panels show cellular distribution of CLOCK (green) and BMAL1 (red) in the cells before and after serum shock (30 min). DAPI staining (lower panels) is shown to define the nuclei. Scale bar, 25 μ m. CTL, control. (C) The protein–protein interaction between CLOCK and BMAL1 is increased after serum shock in the cells. Duplicate whole-cell lysates were immunoprecipitated (IP) with anti-CLOCK and anti-BMAL1, respectively and immunoblotted with both antibodies. BMAL1, brain and muscle Arnt-like protein 1; DAPI, 4,6-diamidino-2-phenylindole.

the phospho-Thr antibody gave similar results (data not shown). These observations show that serum shock rapidly induces Ser/Thr phosphorylation of CLOCK in cultured fibroblasts.

To identify the Ser/Thr kinase(s) responsible for the CLOCK phosphorylation, we used various kinase inhibitors and activators. Pretreatment of cells with GF109203X, a PKC inhibitor, completely prevented serum-induced CLOCK phosphorylation, whereas other kinase inhibitors including the PKA inhibitor KT5720 and

Fig 2 | Protein kinase C-dependent phosphorylation of CLOCK. (A) Time-course changes of the electrophoretic mobility of CLOCK. CLOCK immunoprecipitates (IP) were prepared at the indicated time points after serum shock (SS), separated by long SDS-PAGE on 6% gels and visualized by immunoblotting with anti-CLOCK. Arrowheads indicate the slow-migrating band (black) and the fast-migrating band (white). (B) CLOCK immunoprecipitates from the cells before and after serum shock (30 min) were incubated with λ protein phosphatase (λ PP) in the absence or presence of vanadate (Na_3VO_4) and were assessed by immunoblotting with the indicated antibodies. (C) The effect of various kinase inhibitors on CLOCK phosphorylation induced by serum shock. Cells were treated with $0.5 \mu\text{M}$ KT5720, $5 \mu\text{M}$ GF109203X (GF) or $1 \mu\text{M}$ KT5823 1 h before serum shock. Arrowheads indicate the phosphorylated form (black) and the naked form (white). (D) The effect of diverse kinase activators on phosphorylation of CLOCK. Cells were treated with 1 mM db-cAMP, 1 mM 8Br-cGMP or $1 \mu\text{M}$ PMA for 30 min and whole-cell lysates were subjected to immunoblotting with anti-CLOCK. (E) Characterization of PMA-induced CLOCK phosphorylation. Cells were treated with $5 \mu\text{M}$ GF109203X (GF) or $5 \mu\text{M}$ Gö6976 1 h before incubation with PMA for 30 min and analysed as described in (D). CTL, control; PMA, phorbol myristic acetate; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

the PKG inhibitor KT5823 failed to do so (Fig 2C). Furthermore, treatment with the PKC activator phorbol myristic acetate (PMA) caused an electrophoretic mobility shift similar to serum shock, whereas neither the PKA activator db-cAMP nor the PKG activator 8-Br-cGMP had this effect (Fig 2D). Therefore, the PKC pathway seems to mediate the rapid phosphorylation of CLOCK induced by serum.

PKC comprises a family of isoenzymes that differ in Ca^{2+} requirement, substrate specificity and cell-tissue-specific expression. To identify the PKC pathway that regulates CLOCK phosphorylation, we tested the effects of GF109203X, a broad-specificity PKC inhibitor, and Gö6976, a specific inhibitor of the classic PKC that requires Ca^{2+} as co-activator. As shown in Fig 2E, both drugs eliminated PMA-induced CLOCK phosphorylation, indicating that CLOCK phosphorylation is mediated by the Ca^{2+} -dependent PKC pathway. Consistently, immunoprecipitation analysis using an antibody specific for the classic PKC together with CLOCK antibody showed a marked increase in the interaction between CLOCK and PKC after serum shock (Fig 3A). To determine whether CLOCK acts as a substrate for PKC and, if so, which PKC isoform is responsible, we carried out *in vitro* PKC assays. We first generated histidine-tagged CLOCK by using a cell-free protein synthesis system and incubated it with purified classic PKC in the presence and absence of Gö6976. The full-length CLOCK was readily phosphorylated by the PKC, which was completely blocked by the competitive PKC inhibitor (Fig 3B). Next, we carried out kinase assays for each isoform of the classic PKC family. Of the four isoforms both PKC α and PKC γ labelled CLOCK with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, but PKC γ was more effective than PKC α (Fig 3C). Importantly, these PKC isoforms were highly expressed in

the SCN (Van der Zee & Bult, 1995), and their messenger RNA levels peaked during early and/or late subjective night—that is, the permissive time period for phase resetting (Cagampang *et al*, 1998). Furthermore, inhibition of the PKC pathway abrogated the phase shift of SCN activity induced by melatonin and neuropeptide Y, whereas activation of the PKC pathway advanced circadian neuronal activity in the SCN (Biello *et al*, 1997; McArthur *et al*, 1997). Our results therefore provide an insight into the possible role of PKC in phase resetting of the mammalian central clock.

PKC mediates *Per1* activation and phase resetting

To investigate whether PKC-mediated CLOCK phosphorylation is important in the immediate-early response of *Per1*, we assessed the effects of PKC activation on the DNA-binding activity of CLOCK using chromatin immunoprecipitation (ChIP) assays (Fig 4A; supplementary Fig 2 online). PMA treatment enhanced the binding of both CLOCK and BMAL1 to E-box elements of the *Per1* promoter in NIH3T3 fibroblasts and these effects were blocked by pretreatment with Gö6976 and GF109203X. Furthermore, Re-ChIP assays with CLOCK and phospho-Ser antibodies showed that the PMA-induced DNA-binding activity of CLOCK was mediated by the PKC pathway. Next, we evaluated the effects of PMA on *Per1* gene expression in the cells (Fig 4B). *Per1* mRNA levels increased rapidly and reached a maximum as early as 1 h after the drug treatment; pretreatment with Gö6976 completely abrogated it. These data indicate that CLOCK is an effector in the PKC signalling cascade that leads to rapid induction of *Per1* mRNA.

To verify the role of PKC signalling in phase resetting, we first generated genetically modified NIH3T3 cells expressing *Per1*

Fig 3 | Phosphorylation of CLOCK by Ca^{2+} -dependent protein kinase C isoforms *in vitro*. (A) Serum-induced interaction between CLOCK and PKC. Cells were exposed to serum-rich medium for 30 min and then subjected to immunoprecipitation (IP) with CLOCK and PKC antibodies, followed by immunoblotting with both antibodies. (B) Histidine-tagged recombinant CLOCK was purified and subjected to *in vitro* PKC kinase assay in the absence or presence of 5 μM G66976. The reaction mixture was resolved by SDS-PAGE and transferred to a polyvinylidene fluoride membrane. The membrane was exposed to X-ray film for 24 h (upper panel). The same blot was then probed with CLOCK antibodies (lower panel). (C) Isoform specific kinase assays were carried out as described in (B). CTL, control; cPKC, classic PKC; PKC, protein kinase C; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SS, serum shock.

promoter-controlled destabilized luciferase reporter (*Per1*-dsLuc) and showed that serum shock induced robust oscillation of the bioluminescence with an approximate 24 h periodicity in these cells (supplementary Fig 3A online). Then, at various time points after serum shock, the effects of a 15 min PMA pulse on the phase shift and immediate-early response of *Per1* gene expression were determined in the cells (Fig 4C; supplementary Fig 3B online). Depending on the circadian time point at which PMA was applied, both phase shift and *Per1* mRNA induction varied. This indicates that activation of the PKC pathway can indeed act as a phase-resetting signal of the molecular clock and also as an inducer of *Per1* gene expression in cultured fibroblasts. Recently, CLOCK-deficient mice have been established and characterized (Debruyne et al, 2006). Surprisingly, but consistent with our present hypothesis, these animals showed abnormal responses to light stimuli but continued to show robust circadian rhythms of locomotor activity similar to wild-type animals. Together, these results further emphasize the role of CLOCK in the resetting of the circadian clock rather than in generating circadian rhythmicity.

CLOCK activation is independent of the CREB pathway

It is widely accepted that cAMP response element-binding protein (CREB) seems to mediate the rapid induction of *Per* gene expression, as it is rapidly phosphorylated by the light stimulus in the SCN (Ginty et al, 1993), and both *Per1* and *Per2* carry a canonical CRE in their promoter (Travnickova-Bendova et al, 2002). This prompted us to test the possibility that activation of the PKC pathway also affects CREB activation. Indeed, treatment with PMA stimulated phosphorylation of CREB and also CLOCK (Fig 5A). However, the Ca^{2+} -dependent PKC inhibitor G66976 did not prevent CREB phosphorylation, whereas PD98059, a MAPK kinase (MEK) inhibitor, completely eliminated it without significant effect on CLOCK phosphorylation and *Per1* expression (Fig 5A,B). Furthermore, PMA augmented CLOCK/BMAL1-induced *Per1* transcription in a PKC-dependent manner. As shown in Fig 5C, treatment with PMA significantly elevated CLOCK/BMAL1-induced *Per1* promoter activity, which was blocked by G66976 but not by PD98059. PMA alone also increased *Per1* promoter activity, presumably reflecting phosphorylation of endogenous CLOCK. Thus, the Ca^{2+} -dependent PKC pathway seems to be crucial in both CLOCK phosphorylation and *Per1* transcription.

Intuitively, rapid activation of CREB alone cannot account for the induction of the immediate-early response of the *Per* genes as CREB activation causes rapid activation of only a few genes, including *c-fos*, *Per1* and *Per2*, despite the existence of various genes containing functional CREs in their upstream regulatory regions. Furthermore, there are some kinetic differences in the expression profiles of *c-fos* and the *Per* genes. It is therefore likely that the induction of *Per* gene expression does not depend on CREB activation, alone. Indeed, the promoters of *Per1* and *Per2* contain the configuration of CRE and E-boxes appropriate for cooperative action as shown in other systems (Benjanirut et al, 2006), and combined treatment with forskolin and PMA synergistically enhanced *Per1* promoter activity (supplementary Fig 4 online). This synergism further explains the previous study showing that serum shock evokes selective transcription of *Per1* and *Per2* among CLOCK/BMAL1-controlled genes (Balsalobre et al, 1998). On the basis of these observations, we propose a schematic model for the rapid induction of *Per1* gene expression triggered by the resetting signals (Fig 5D).

In conclusion, we have provided the first evidence that CLOCK is phosphorylated by Ca^{2+} -dependent PKC isoforms immediately after the application of extracellular stimuli that lead to synchronization of individual cellular clocks. This phosphorylation-dependent CLOCK activation is essential for the rapid surge of *Per1* transcription and also for activation of circadian gene expression in cultured cells. Furthermore, our results strongly indicate that CLOCK is a real signalling molecule mediating the resetting of the circadian clock in a process distinct from its role in the positive limb of the core clock feedback loop. In the SCN, this type of clock-specific signalling mechanism would be applicable to the selective induction of *Per* gene expression among the diverse CREB target genes that can be triggered by Ca^{2+} influx.

METHODS

Experimental procedures are provided in detail in the supplementary information online.

Cell culture and transient transfection. NIH3T3 and mouse embryonic fibroblast cells were cultivated in DMEM supplemented

Fig 4 | Involvement of protein kinase C-mediated CLOCK phosphorylation in the rapid activation of *Per1* and phase resetting of the molecular clock. (A) Chromatin was extracted from NIH3T3 cells that were treated with 1 μ M PMA for 1 h in the presence and absence of Gö6976 or GF109203X (GF). ChIP assays were carried out with specific antibodies for the indicated proteins. Re-ChIP experiments were carried out with a two-step immunoprecipitation with CLOCK and phospho-Ser antibodies (pSer). The association of each protein with the *Per1* promoter was analysed by PCR using a primer set flanking the proximal E-box. ChIP data of histone H3 are shown as a control. (B) Cells were treated with 1 μ M PMA for 2 h, and the medium was replaced with serum-free medium. A set of cells was incubated with Gö6976 for 1 h before treatment with PMA. *Per1* messenger RNA levels were determined by reverse transcription-PCR and normalized with those of *G3PDH*. Data are shown as means $\pm$ s.e.m. of three independent experiments. (C) Phase responses to a 15 min PMA pulse. NIH3T3 cells stably transfected with a plasmid containing the *Per1*-dsLuc were synchronized by serum shock and then treated with PMA for 15 min at various time points. The *Per1*-dsLuc activities were monitored continuously using a real-time measuring system and the resulting shift in the second peak time of the bioluminescence was expressed as phase shift of the molecular clock. BMAL1, brain and muscle Arnt-like protein 1; ChIP, chromatin immunoprecipitation; PMA, phorbol myristic acetate.

Fig 5 | Characterization of the signal-transduction pathways leading to the rapid induction of *Per1*. (A) Cells were stimulated with 1 μ M PMA for 30 min in the absence or presence of Gö6976 and PD98059, and subjected to immunoblotting with the indicated antibodies. (B) Under the same conditions, messenger RNA levels of *Per1* were determined as described in Fig 4B (* P < 0.05). (C) Cells were transiently transfected with *Per1*-dsLuc reporter construct with or without plasmids expressing CLOCK and BMAL1. One day after serum depletion, they were treated with PMA for 4 h. Inhibitors were added 1 h before the addition of PMA. The resulting luciferase activities were expressed as mean fold $\pm$ s.e.m. of the control group (* P < 0.01). (D) Schematic model for the rapid induction of *Per1* gene expression by resetting signals. The immediate-early response of the *Per1* gene is mediated by independent signalling pathways. The Ca^{2+} -dependent PKC pathway leads to CLOCK activation, whereas the PKA and MAPK pathways converge on CREB. BMAL1, brain and muscle Arnt-like protein 1; CRE, cAMP response element; CREB, cAMP response element-binding protein; MAPK, MAP kinase; PKC, protein kinase C; P, phosphate; PMA, phorbol myristic acetate.

with 10% fetal bovine serum (FBS). For drug application, confluent cells were serum-starved (1% FBS) for 1 day before treatment. DNA transfections were carried out using Lipofectamine plus reagents (Invitrogen, Carlsbad, CA, USA) and reporter gene activity was measured using the dual luciferase assay reagents (Promega, Madison, WI, USA).

Synthesis of CLOCK protein and protein kinase C kinase assay. Histidine-tagged CLOCK protein was prepared in a cell-free protein synthesis system and purified with a commercial histidine-Bind kit (Novagen, Darmstadt, Germany). *In vitro* PKC kinase assays were carried out at 30 °C for 15 min with [γ - 32 P]-ATP (10 μ Ci) and purified rat brain PKC (Promega)/recombinant PKC isozymes (Sigma, St Louis, MO, USA) in the presence or absence of a PKC inhibitor (Gö6976, 5 μ M).

Chromatin immunoprecipitation assay. ChIP assays were carried out using a commercial ChIP assay kit (Upstate Biotech, Chicago, IL, USA) according to the manufacturer's instructions. The primer sets used for ChIP assays are provided as supplementary information online.

Real-time measurement of bioluminescence. Cells were transfected with 0.5 μ g of the full-length *Per1* promoter (5.7 kb) fused with a destabilized firefly luciferase (dsLuc) and preincubated in serum-free DMEM for 24 h. The medium was then replaced with 3 ml of DMEM supplemented with 10% FBS, 10 mM HEPES (pH 7.4) and 0.1 mM luciferin (Ueda et al, 2002). GF109203X (5 μ M) was added 1 h before the serum treatment. Bioluminescence was measured with photomultiplier tube detector assemblies (AB-2500 Kronos, ATTO, Tokyo, Japan). Photon counts were integrated at 1 min intervals.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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