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Biochemical and Biophysical Research Communications 346 (2006) 95–101
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Serine 714 might be implicated in the regulation of the phosphorylation in other areas of mPer1 protein

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Received 27 April 2006
Available online 24 May 2006

Abstract

The phosphorylation of mPer proteins may play important roles in the mechanism of the circadian clock via changes in subcellular localization and degradation. However, the mechanism has remained unclear. Previously, we identified three putative casein kinase (CK)1ε phosphorylation motif clusters in mPer1. In this work, we examined the role of the phosphorylation of serine residue, Ser(S)714, in mPer1. mPer1 S[714–726]A mutant, in which potential phosphorylation serine residues replaced by alanine residues, is rapidly phosphorylated compared with wild-type mPer1 by CK1ε. Coexpression with S[714]G mutant of mPer1 advanced phase of circadian expression of mPer2-luc expression, which was monitored by in vitro bioluminescence system. This result showed that the mPER1 S[714]G mutation affects circadian core oscillator. Considering these, it seems that Ser 714 might be involved in the regulation of the phosphorylation of other sites in mPer1 by CK1ε.

Keywords: Circadian clock; Casein kinase 1ε (CK1ε); Period 1 (per1); Phosphorylation; Pulse-chase; Kinase assay

In mammals Per proteins are suggested to be implicated in the mechanism of molecular circadian oscillator as essential components. The circadian clock mechanism has been shown to be a highly conserved autoregulatory transcriptional-translational feedback loop of clock genes [1–4]. In the system a heterodimer of the transcriptional factors containing basic helix–loop–helix (bHLH) and PAS (Per-Arnt-Sim) domains activates the transcriptions of inhibitory factors. The inhibitory factors, synthesized in the cytoplasm, form complex, enter the nucleus, and inhibit the transcriptions of the clock genes. The circadian oscillator also exists in most peripheral cells and even in cultured cells [5,6].

Posttranslational regulations, such as phosphorylation, translocation, and degradation, are thought to be important in the molecular mechanism of circadian clock. The inhibitory factors, such as Frq, dPer, dTim, and mPers, detected by respective antibodies, are reported to show the phosphorylation-dependent mobility shifts and degradation in time-dependent manners [7–10]. Casein kinase 1ε (CK1ε) is one of such key phosphorylating enzymes. In *Drosophila*, Double-time (Dbt), which is a *Drosophila* homologue of mammalian CK1ε, was suggested to be implicated in the circadian mechanism [11]. The mutants of *Dbt* have been identified as alleles showing shortened, lengthened or abolished circadian locomotive rhythms. Moreover, changes in the phosphorylation status are thought to be related with the stability of Per proteins [12]. In mammals, *tau* mutant in Syrian hamster, which shows short free-running period, is caused by a point mutation of CK1ε [13]. The kinase activity of the mutant protein is much lower than that of wild-type CK1ε [13]. Furthermore, *hPer2* is a candidate gene for the cause of familial advanced sleep-phase syndrome (FASPS), which is an autosomal dominant circadian rhythm disorder, and produces 4 h phase advance of the daily sleep-wake rhythm [14]. This mutation causes a serine-to-glycine amino acid substitution at amino acid (aa) 662 of hPer2 which locates

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0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.05.082

within the CK1ε binding site and phosphorylation region, and the hPer2 mutant is less effectively phosphorylated by CK1ε in vitro. Recently, it was reported that another kinases, such as glycogen synthase kinase 3 (GSK3), and mitogen-activated protein kinase (MAPK), might be related with circadian molecular mechanism [15,16].

We previously reported that CK1ε interacted with mPer1, mPer2, and mPer3, and caused nuclear entries of mPer1 and mPer3 but not that of mPer2 in a phosphorylation-dependent manner in COS-7 cells [17]. CK1ε and δ are also known to bind, phosphorylate, and degrade mPer proteins [7,9,10]. And Serine 661 and Serine 663 of mPer1 are involved in the control of the subcellular localization of mPer1 [18]. Although these data suggest that CK1ε are important in generating the circadian rhythm in mammals, many unsolved problems, including precise molecular mechanisms how CK1ε affect mPer proteins, remain unclear. In this work, we report Serine 714 of the putative CK1ε-phosphorylation sites in mPer1. Our results suggested that Serine 714 might be implicated in the regulation of phosphorylation at other sites of mPer1.

Materials and method

Cell cultures and transfection. Complementary DNAs of rat *CK1ε*, mouse *Per1*, and their mutants were transfected into COS-7 cells as described in the previous report [18], but the method is explained briefly below. The site-directed mutagenesis of *mper1* in which some serine residues changed to alanine or glycine residues was realized with a single step by PCR. The mutated DNA was picked up by *Dpn*I selection on hemimethylated DNA, and the sequences were verified with dideoxynucleotide chain-reaction method using BigDye sequencing kit and ABI Prism model 310 genetic analyzer (Perkin-Elmer-Applied Biosystems). Cultures of COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Cells were transfected with expression vectors carrying *rck1ε* and *mper* cDNAs with LipofectAmine 2000 (Invitrogen) according to the manufacturer's protocol.

Pulse-chase analysis. mPer1 was co-transfected with rCK1ε or empty pcDNA3 vector into COS-7 cells. Twenty-four hours after the transfection, transfected COS-7 cells were incubated in methionine-free DMEM supplemented with 14.3 mCi/ml [^{35}S]methionine/cysteine (Amersham) for 1 h. Then the medium was changed to fresh DMEM containing nonlabeled methionine and 50 mM MG-132, an inhibitor of the 26S proteasome. Incubating for indicated time, cells were lysed in 500 μl of the TNE buffer. The lysates were immunoprecipitated with an anti-HA antibody. The immunopurified material was separated by electrophoresis using a 6% or 10 % polyacrylamide gel and radioactivity of mPer1 was detected by BAS 2000 image analyzer.

In vitro kinase assay. For in vitro kinase assay, the cDNA of CK1ε was subcloned into pGEX4T-3 vector (Qiagen) to produce GST-fused CK1ε (GST-CK1ε). These expression vectors were transformed into the *Escherichia coli* expression system of BL21 (DE3) (Stratagene). GST-rCK1ε was purified with glutathione Sepharose 4B (Pharmacia) as per manufacturer's protocol. As substrates of kinase assay, GST-fused mPer fragment proteins and hemagglutinin-tagged full-length mPer1 (HA-mPer1) protein were used. GST-fused mPer fragment proteins were produced in a manner similar to the production of GST-rCK1ε as described above. HA-mPer1 were purified by immunoprecipitation using anti-HA monoclonal antibody (Invitrogen) from the lysate of COS-7 cells overexpressed HA-mPer1. The kinase reaction was performed in a kinase buffer containing 45 mM Tris–HCl, pH 7.4, 9 mM $MgCl_2$, 0.9 mM β-mercaptoethanol, and 40 μM ATP and 74 kBq [γ-^{32}P]ATP. Incubating for 10 min at 37 °C, the reaction was stopped by the addition of SDS–PAGE sample buffer and the proteins were electrophoresed in 6 % or 10 % polyacrylamide gels. The incorporation of radio-labeled phosphate into the substrates was detected by BAS 3000 image analyzer (Fuji Film). The samples were also analyzed by Coomassie brilliant blue staining to confirm molecular sizes of substrates.

Real-time monitoring of luciferase activity in cultured cells. mPer2-luc was co-transfected with *mPer1* or its mutants in NIH3T3 cultured cells and incubated. After 24 h, the cells were stimulated with DMEM supplemented with 50% newborn bovine serum. Two hours later, this medium was replaced with normal culture medium (DMEM, supplemented with 1% fetal bovine serum) and supplemented with 0.1 mM luciferin. Light emission was counted and integrated for 1 min at an interval of 15 min with a photomultiplier tube in photon detection unit (Hamamatsu Photonics) as described previously [19]. The measurements of phase and period rhythm were calculated as described in previous studies [20–23]. Data sets were detrended by subtracting the 24 h running average from the raw data. The maximum between the smoothed curves dor each cycle (the peak and the trough) were used to calculate the amplitude of each cycle.

Results and discussions

S[714]G showed hyperphosphorylation by Western blot

We previously reported that CK1ε phosphorylated all mPer proteins and affected their subcellular localizations [17]. Moreover, we identified the three phosphorylation clusters by CK1ε, Serine, Threonine (ST)653–663, Serine (S)714–726, and ST784–787. In particular, phosphorylation of S661 and S663 was involved in controlling subcellular localization of mPer1 [18]. Phosphorylation motif cluster of S714–726 is conserved in all mPer proteins (Per1–3) and this region is suggested to be related with their ubiquitinations [8]. But the role of S714–726 in mPer1 still remained unclear.

When COS-7 cells were transfected with vectors expressing HA-mPer1 or its mutants, along with rCK1ε or empty control vector, the phosphorylation-status of mPer1 or its mutant proteins was initially assessed by the shift of electrophoretic mobility on the SDS–PAGE gel detected by immunoblot method. Wild-type mPer1 showed the obvious mobility shift depending on the intact CK1ε activity (Fig. 1, lanes 1–3). Patterns of mobility shift of mPer1

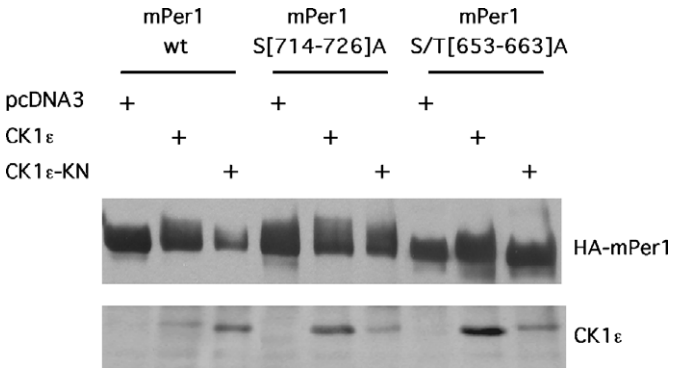


Fig. 1. Immunoblot analysis for the phosphorylation of mPer1 and its mutants by CK1ε. HA-tagged mPer1 and its mutants were coexpressed with the empty vector pcDNA3, rCK1ε or rCK1ε-KN (kinase negative) (K38R) in COS-7 cells and immunoprecipitated by anti-HA antibody. The phosphorylation status of HA-mPer1 was assessed by SDS–PAGE as a shift in electrophoretic mobility. Upper panel, blotted by anti-HA antibody. Lower panel, blotted by anti-CK1ε antibody.

Serine (S)[653–663]Alanine (A) mutant were similar to that of wild-type mPer1 (Fig. 1, lanes 7–9). However, mPer1 S[714–726]A mutant showed striking electrophoretic mobility shift and the shift probably due to mPer1 phosphorylation was observed even in the absence of rCK1ε protein (Fig. 1, lanes 4–6). Considering the fact that the replacement of serine to glycine at the N-terminal end of hPer2 (hPer2 S[662]G), which corresponds to mPer1 S[714]G, was reported to cause the shortening of circadian period of sleep-wakefulness (FASPS) [14], these results suggest that S[714–726] might regulate phosphorylation of other sites of mPer1.

S[714]G showed rapid phosphorylation in pulse-chase analysis

To clarify the function of S714 phosphorylation motif cluster in mPer1, the time course of phosphorylation of mPer1 or its mutants coexpressed with CK1ε in COS-7 cells was examined using a pulse chase method (Fig. 2). It was difficult to track the phosphorylation of mPer proteins by CK1ε, because the phosphorylation of mPer proteins caused their degradations (Fig. 2A). Thus, MG-132, an inhibitor of the 26S proteasome, was added to the medium to prevent their degradation in this experiment (Fig. 2B). As indicated in the previous reports [9,10,24] ubiquitin-proteasome pathway in the phosphorylation-dependent degradation of mPer proteins by CK1ε the inhibitor prevented the degradation of these proteins. In *Drosophila*, it was reported that Slimb, an F-box/WD40-repeat protein functioning in the ubiquitin-proteasome pathway, interacted preferentially with phosphorylated dPer protein and stimulated its degradation [25,26]. Recently, in mammals, $SCF^{\beta\text{-}TRCP}$ collaborates with CK1ε to promote turnover of Per1 and Per2 [27,28].

Apparent mobility shift of mPer1 protein was observed proportionally to the time after the start of the pulse labeling when coexpressed with CK1ε (Fig. 2B, lanes

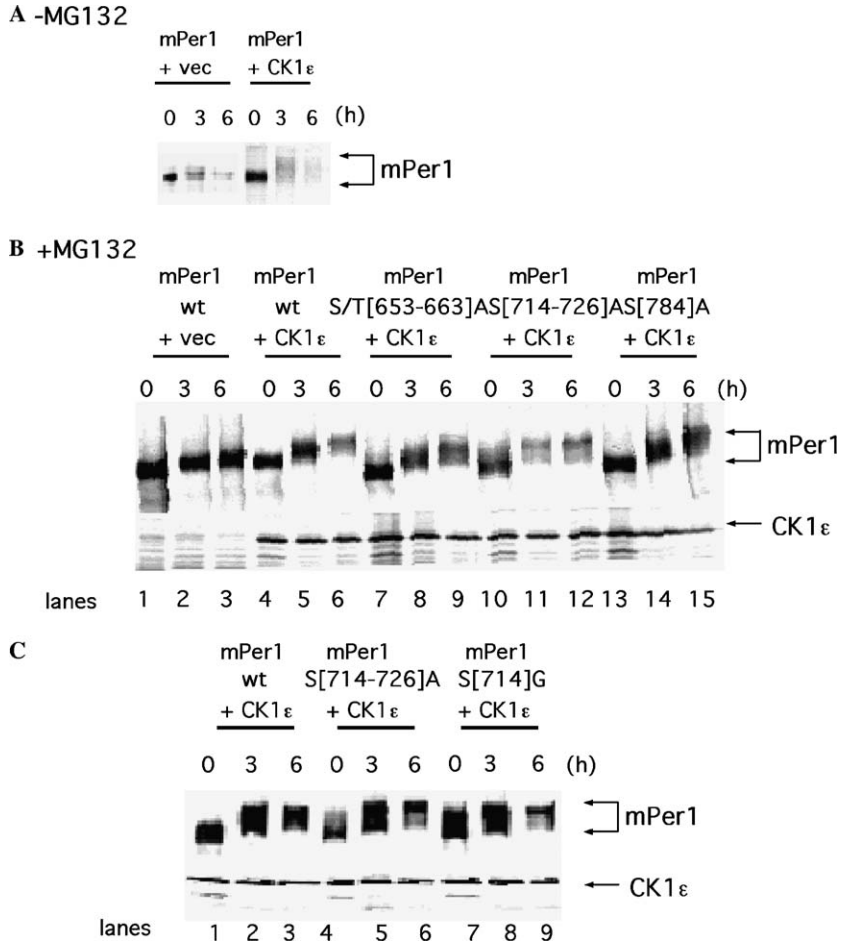


Fig. 2. Pulse-chase analyses of phosphorylation in wild-type mPer1 and its mutants coexpressed with CK1ε in COS-7 cells. (A) COS-7 cells cotransfected with *mper1* and *ck1ε* or empty pcDNA3 vector were lysed and analyzed by SDS–PAGE at indicated time after the pulse label by [^{35}S]methionine. mPer1 or its mutants were immunoprecipitated with anti-HA antibody. (B) COS-7 cells cotransfected with *mper1*, S/T[653–663]A, S[714–726]A, or S[784]A mutants and *ck1ε* or empty pcDNA3 vector were lysed and analyzed by SDS–PAGE at indicated times after the pulse label by [^{35}S]methionine. mPer1 or its mutants were immunoprecipitated with anti-HA antibody. (C) COS-7 cells cotransfected with *mper1*, S[714]G or S[714–726]A mutants and *ck1ε* or empty pcDNA3 vector were lysed and analyzed by SDS–PAGE at indicated times after the pulse label by [^{35}S]methionine. MG-132, an inhibitor of the 26S proteasome, was used in (B,C) not (A). The mobility shifts of immunoprecipitated mPer1 or its mutants were examined by autoradiography. rCK1ε bound to mPer1 or its mutants were confirmed by the immunoblot analysis using anti-rCK1ε antibody (lower panel).

4–18), and only a slight shift was seen when cotransfected with empty vector and this level remained constant throughout the time course (Fig. 2B, lanes 1–3). The mobility shift of mPer1 seems to be caused by its phosphorylation, because treatment with the calf intestine phosphatase (CIP) eliminated the shift (data not shown). Patterns of the mobility shift of mPer1 S/T[653–663]A and S[784]A were similar to that of wild-type mPer1 when they were coexpressed with CK1ε. In contrast, mobility shift of mPer1 S[714–726]A was observed even at time 0 (just after the start of the incubation with radiolabeled methionine) and was similarly observed 3 and 6 h after the start of the pulse labeling (Fig. 2B, lane10), despite the reduction of phosphorylation sites in the mutant of S[714–726]A by rCK1ε. All of these mPer1 mutants interacted with CK1ε at 0 h (Fig. 2B, lower column). These results suggest that mPer1 S[714–726]A is phosphorylated rapidly by CK1ε compared with wild-type mPer1 or other mutants. Furthermore, we constructed the corresponding mutants of mPer1 (mPer1 S[714]G) to hPer2 S[662]G and examined their phosphorylation by the pulse chase method. As shown in Fig. 2C, mPer1 S[714]G exhibited rapid mobility shift (phosphorylation) by rCK1ε which was similar to mPer1 S[714–726]A. These findings suggest that the phosphorylation of the conserved CK1ε-phosphorylation motif cluster corresponding to aa 714–726 in mPer1 is implicated in the control of the phosphorylation in other areas of mPer1 protein.

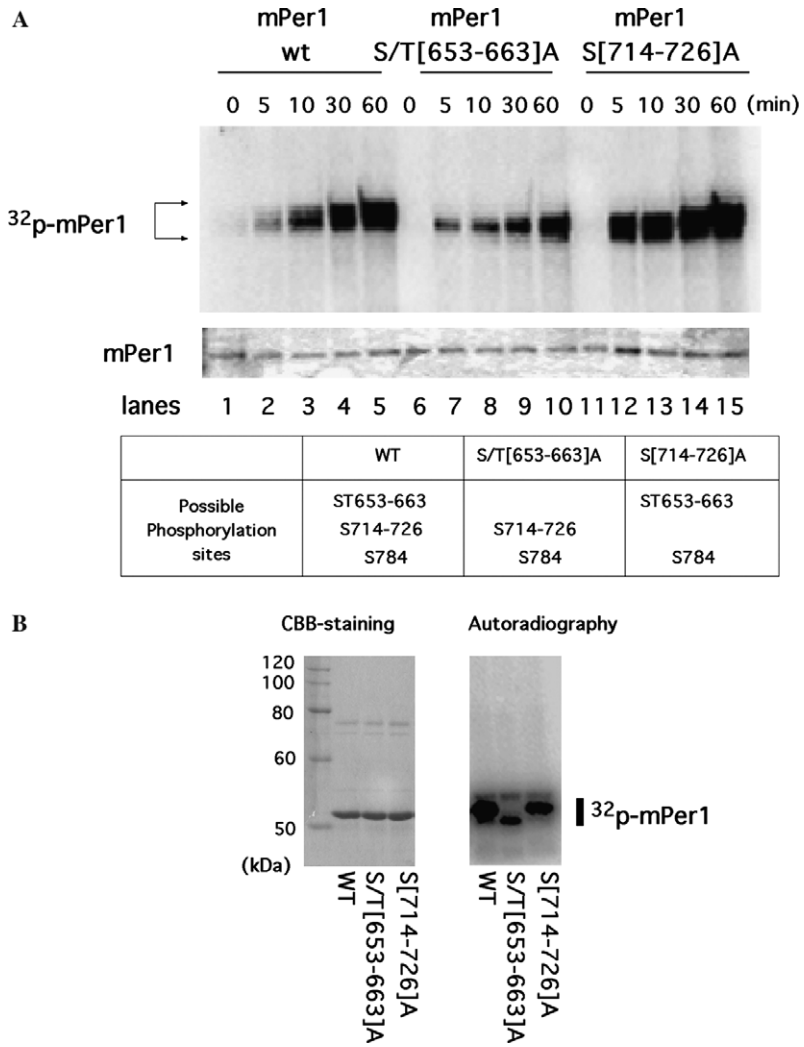


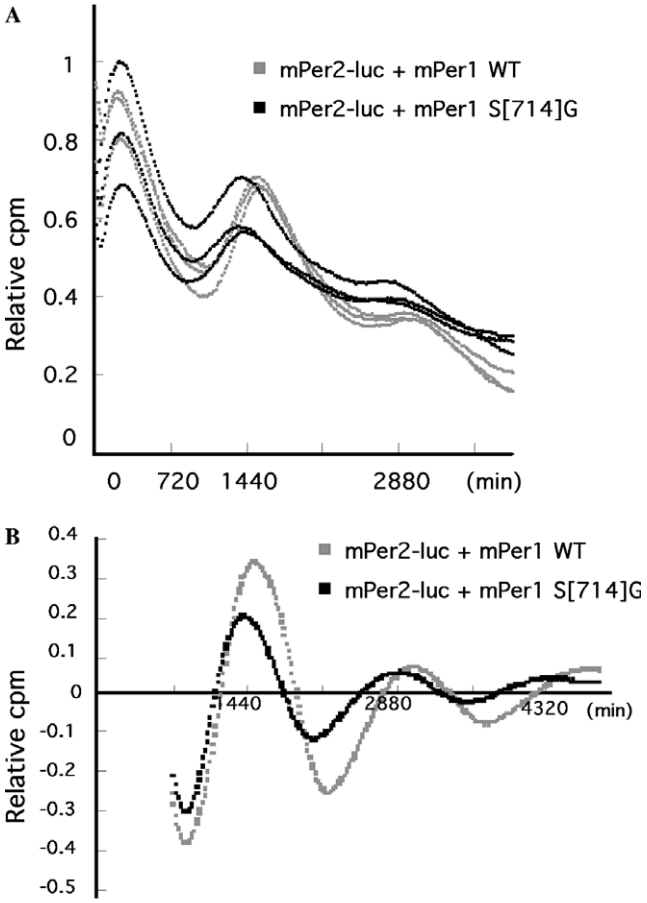
Fig. 3. In vitro kinase assays for analyses of time-course of the phosphorylation kinetics of wild-type mPer1, and its S/T[653–663]A and S[714–726]A mutants. (A) Time-course analyses of mPer1 or its mutants by rCK1ε. As substrates for the kinase assay, HA-mPer1 or its mutants were expressed in COS-7 cells, immunoprecipitated with anti-HA antibody, treated with CIP and 10 mM iodoacetamide. Thirty nanograms of GST-rCK1ε pretreated with trypsin was used as the enzyme. Kinase assays were performed as described in Materials and methods, and the reactions were stopped at indicated times after the start of the incubation and the incorporated radioactivity was analyzed by SDS–PAGE. Upper panel, autoradiography; lower panel, HA-mPer1 or its mutants immunoblotted using an anti-HA antibody. (B) In vitro kinase assays with various substrates. GST-fusion mPer1 or its mutant fragments were incubated with rCK1ε and the radioactivity was analyzed by SDS–PAGE. Kinase assay was performed for 10 min and 200 ng of the substrates was used. Left panel, CBB-staining; right panel, autoradiography.

S[714]G showed higher phosphorylation than wild-type mPer1

Next, to confirm further the role of the CK1ε-phosphorylation motif cluster in mPer1 S714–726 in the phosphorylation kinetics of these proteins and to eliminate the specific condition in COS-7 cells, in vitro kinase assays were performed. The full-length mPer1 or its mutants (S/T[653–663]A and S[714–726]A) were expressed in COS-7 cells and these proteins were immunoprecipitated with an anti-HA antibody. The precipitated proteins were then treated with CIP to dephosphorylate the proteins and with 10 mM iodoacetamide to inactivate the kinase possibly contaminating with the precipitates, and used as substrates for the kinase assay. Bacterially expressed GST-fused rCK1ε was purified, treated with trypsin, and 30 ng of the purified recombinant enzyme was used for kinase assay. As a result, all of these mPer mutants were rapidly phosphorylated by rCK1ε. The phosphorylation of wild-type mPer1 was observed slightly at 5 min and reached a maximum at 30 min (Fig. 3A, lanes 1–5). However, S[714–726]A mutant was more rapidly phosphorylated and the phosphorylation had reached almost the maximum already at 5 min (Fig. 3A, lanes 6–10). The mutant mPer1 S[714–726]A was also hyperphosphorylated by rCK1ε in vitro, even though the reduction of phosphorylation sites in S[714–726]A mutant by rCK1ε. In contrast, the level O-phosphorylation of S/T[653-663]A mutant was lower than that of wild-type mPer1 (Fig. 3A, lanes 11–15). To examine further the results that S[714–726]A mutation caused hyperphosphorylaiton and ST[653–663]A hypophosphorylation, in vitro kinase assays using GST-fusion mPer1 fragments or its mutants as substrates were performed. The GST-mPer1 fragments corresponding to aa 549–799 of wild-type of mPer1, mPer1 S/T[653–663]A, or mPer1 S[714–726]A were used as substrates. After the kinase reaction for 10 min, GST-wild-type mPer1 fragment showed a broad mobility shift by rCK1ε. In contrast, GST-mPer1 S/T[653–663]A fragment exhibited only a slight shift, and GST-mPer1 S[714–726]A fragment revealed only a shifted band probably caused by the hyperphosphorylation (Fig. 3B). This position, aa 714–726, is corresponding site of the mutated site responsible for the human disease of FASPS, and the mutation of S[662]G in hPer2 caused FASPS and the mutation was reported to cause hypophosphorylation of hPer2 by CK1ε [14]. Therefore, the present result seems to be discrepant with that in the previous report. Previous studies indicated three CK1ε-dependent phosphorylation clusters in mPer1 by CK1ε but mPer2 has only one phosphorylation cluster by CK1ε [14,17]. The phosphorylation clusters of S653–663 and S784 in mPer1 are not conserved in mPer2. Considering these facts, the CK1ε-phosphorylation motif cluster S714–726 in mPer1 might be implicated in the negative control effect on the phosphorylation of other sites in mPer1. S714 in mPer1 may have most important role within the clusters, even though single mutation in this site elicited the hyperphosphorylation of mPer proteins. In contrast, S/T[653–663]A mutation of mPer1 caused hypophosphorylation of the mutant. Thus, these data strongly suggest that the phosphorylation of S714–726, especially Ser714 in mPer1, causes suppression of the phosphorylation of Ser/Thr residues in other regions in mPer1by CK1ε.

S[714]G mutant of mPer1 showed advanced phase of hPer2-luc expression

S662G mutant in hPer2 was reported to cause shortening of its circadian period. Therefore, we examined whether



C

	WT	S[714]G
first peak	1546±10.2	1382±18.0

Fig. 4. mPer2-luc plasmids were cotransfected with mPer1 or S[714]G mutant and monitored in vitro bioluminescence system. (A) Transcriptional oscillation of *mPer2* was monitored by using the bioluminescence reporter assay. *mPer2*-luc constructs were cotransfected with mPer1 or S[714]G mutant in NIH3T3 cells and then stimulated with a 50 % concentration of serum. After the serum shock, light emission was counted and integrated for 1 min at intervals of 15 min (vertical scale, relative counts per minute; horizontal scale, 1440 min = 1 day). Peak values of the curves were set to 1. A representative result is three independent experiments. Data represent means ± SEM of triplicate samples. (B) The signals obtained in (A) were detrended. (C) The time of the first peak was calculated as a phase marker (means ± SEM; $n = 3$).

S[714]G mutation of mPer1 affects circadian core oscillation using in vitro bioluminescence system. *mPer2*-luc plasmid was cotransfected with wild-type mPer1 or S[714]G mutant of mPer1 and mPer2-luc expression was monitored in a circadian cell-autonomous manner. Treatment with 50 % serum can trigger rhythmical oscillation. After the serum shock, in the presence of luciferin, light emission was measured and the data were integrated for 1 min at intervals of 15 min. *mPer2*-controlled waves of luminescence resulted in a series of readily appreciable peaks and troughs, as exemplified in Fig. 4. Almost the same phases and amplitudes were observed in the cells transfected with wild-type mPer1 (Fig. 4A, 3 gray lines). In contrast, phase advance (approximately 2.5 h earlier) of *mPer2*-luc expression was observed when it was coexpressed with S[714]G mutant of mPer1 compared with the phase of the expression when it was coexpressed with wild-type mPer1 (Fig. 4A, 3 black lines). To better analyze phase differences, wave data were detrended (Fig. 4B). We examined the time of the first peak (Fig. 4C). Coexpressed with S[714]G mutant of mPer1 showed about 2.7 h phase advance in the expression compared with the phase when coexpressed with wild-type mPer1, as expected in Fig. 4A. These data indicated a similar tendency with FASPS [14]. However, it is conceivable that the phosphorylation motif cluster by CK1ε, conserved between mPer1 and mPer2, may have different roles. Thus, the present result suggests that the mutation of S[714]G in mPer1 affects circadian core oscillator. The CK1ε-dependent phosphorylation of the Ser714 of mPer1 seems to cause negative regulation on the phosphorylation of other regions of mPer1. Whether this is the case must be further studied in future.

Acknowledgments

We would like to thank Dr. Toru Takumi (Osaka Bioscience Institute) for providing plasmid of mper2-luc and using photon detection unit (Hamamatsu Photonics). We also thank Dr. Makoto Akashi for helpful suggestions on data analysis.

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