Ser-557-phosphorylated mCRY2 Is Degraded upon Synergistic Phosphorylation by Glycogen Synthase Kinase-3β*S

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Cryptochrome 1 and 2 act as essential components of the central and peripheral circadian clocks for generation of circadian rhythms in mammals. Here we show that mouse cryptochrome 2 (mCRY2) is phosphorylated at Ser-557 in the liver, a well characterized peripheral clock tissue. The Ser-557-phosphorylated form accumulates in the liver during the night in parallel with mCRY2 protein, and the phosphorylated form reaches its maximal level at late night, preceding the peak-time of the protein abundance by ~4 h in both light-dark cycle and constant dark conditions. The Ser-557-phosphorylated form of mCRY2 is localized in the nucleus, whereas mCRY2 protein is located in both the cytoplasm and nucleus. Importantly, phosphorylation of mCRY2 at Ser-557 allows subsequent phosphorylation at Ser-53 by glycogen synthase kinase-3β (GSK-3β), resulting in efficient degradation of mCRY2 by a proteasome pathway. As assessed by phosphorylation of GSK-3β at Ser-9, which negatively regulates the kinase activity, GSK-3β exhibits a circadian rhythm in its activity with a peak from late night to early morning when Ser-557 of mCRY2 is highly phosphorylated. Altogether, the present study demonstrates an important role of sequential phosphorylation at Ser-557/Ser-553 for destabilization of mCRY2 and illustrates a model that the circadian regulation of mCRY2 phosphorylation contributes to rhythmic degradation of mCRY2 protein.

The physiology and behavior of living organisms from bacteria to humans show daily fluctuations, and those controlled by autonomous clocks are termed circadian rhythms (1, 2). These rhythms are synchronized with (entrained to) environmental time cues such as light, and the rhythms are sustained with a period of ~24 h even in the absence of the time cues. In mammals, the suprachiasmatic nucleus in the anterior hypothalamus serves as the central clock of the circadian timing system (3–5). Peripheral tissues throughout the body also have circadian clocks, and both the central and peripheral clocks generate the 24-h rhythm with molecular machinery very similar to each other (6–8).

The molecular mechanism of the circadian oscillator has been investigated extensively by genetic and molecular studies on Drosophila and mice. In the mouse molecular clock, a heterodimer of the two transcription factors, CLOCK and BMAL1, activates E-box-dependent transcription of two cryptochrome genes, mCry1 and mCry2, and three period genes, mPer1, mPer2, and mPer3 (9, 10). Translated mCRY and mPER proteins translocate to the nucleus where mCRY proteins act as predominant negative regulators by interacting directly with CLOCK/BMAL1 heterodimer to inhibit the transactivation from the E-box (10, 11). The negative regulation in turn results in decrease of the protein levels of mCRYs and mPERs and allows the molecular cycle to start again with the activation of the E-box-dependent transcription. Importantly, mice lacking both mCry1 and mCry2 exhibit arrhythmic behavior immediately after being placed in constant darkness (12), indicating their critical role in generating the circadian rhythm in mammals.

In addition to the transcriptional regulation of the clock-related genes, protein phosphorylation plays important roles for regulation of the phase and period length of the molecular clock (12–21). The clock proteins including CLOCK, BMAL1, mPER1, and mPER2 undergo temporal change in phosphorylation in the mouse liver (22), a well characterized peripheral tissue that contains the circadian clock. It was unclear as to whether mCRYs are phosphorylated in vivo. Mammalian PERs are phosphorylated by casein kinase Iε (CKIε), and the phosphorylation reduces the stability of PER (23). A defect in hamster CKIε corresponds to the short period tau mutation (24). Similarly, PER in Drosophila is phosphorylated and destabilized by Doubletime (DBT), a Drosophila homolog of CKIε, and the mutant alleles of dbt result in alteration of the period length of the circadian rhythm (14, 17). Mutant alleles of Shaggy (SGG)/glycogen synthase kinase-3 (GSK-3) also affect the period length of molecular oscillation of the clock in Drosophila (25), and SGG phosphorylates TIM (25), an essential clock component serving as a binding partner of PER (13). Interestingly, mammalian GSK-3 has been related to circadian clockwork in mice (26), but its target protein(s) yet remains to be elucidated. In every case no information is available about the in vivo phosphorylation site(s) in these clock proteins.

We have recently found that mCRY2 is phosphorylated at Ser-265 and Ser-557 by mitogen-activated protein kinase (MAPK) in vitro (27). Likewise, Ser-247 of mCRY1, which corresponds to Ser-265 of mCRY2, is phosphorylated by MAPK in vitro (27), whereas mCRY1 does not have the Ser or Thr resi-

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due which corresponds to Ser-557 of mCRY2. Ser-247/Ser-265 lie in the mCRY central region, which contains the FAD-binding site, and is highly conserved among photolyase/cryptochrome flavoprotein family. Ser-557 of mCRY2, in contrast, lies in the carboxyl-terminal tail, a region that is characterized by diverged structure among CRYs (28). Functionally, phosphorylation of mCRYs at Ser-247/Ser-265 perturbs their activities to suppress CLOCK/EMA1-mediated transactivation (27), but functional significance of Ser-557 phosphorylation was not identified. Interestingly, mCRY1 knock-out and mCRY2 knock-out mice display accelerated and delayed free-running periodicity, respectively (12). This observation suggests that mCRY1 and mCRY2 participate in the clockwork in different ways, and therefore, it is intriguing to elucidate the mechanism how each mCRY protein is regulated and how they contribute to the molecular oscillation.

In the present study we demonstrate circadian fluctuation of in vivo phosphorylation of mCRY2 at Ser-557 peaking at late subjective night in the mouse liver. Interestingly, Ser-557 phosphorylation of mCRY2 allows subsequent phosphorylation by GSK-3β, leading to proteasome-dependent degradation of mCRY2. These results demonstrate an important role of Ser-557 phosphorylation as the primary step of clearance of mCRY2 and provide the first evidence for the molecular link between GSK-3β and the clock protein of vertebrates.

EXPERIMENTAL PROCEDURES

Animals—The animal experiments were conducted in accordance with the guidelines of University of Tokyo. Five-week-old male mice (C57BL/6) were housed individually at 23 ± 1 °C in cages with food and water available ad libitum, and they were entrained to 12-h light/12-h dark (LD) cycles. After the entrainment at least for 2 weeks, the animals were maintained in constant dark (DD) condition. mCRY2, the mouse liver lysate (200 μg of proteins) thus prepared was preincubated with 20 μl of protein G-Sepharose (Amersham Biosciences) for 1 h at 4 °C and then incubated with anti-CRY2 antibody for 8 h at 4 °C following by incubation with 20 μl of protein G-Sepharose for 2 h at 4 °C. The precipitated materials were washed 5 times with buffer B and mixed with SDS-PAGE sample solution followed by boiling for 8 min.

Immunocytochemistry—Under deep ether anesthesia, DD-housed male mice (C57BL/6) were perfused with saline (20 ml) and subsequently with 20 ml of 4% paraformaldehyde in phosphate buffer (pH 7.4). The livers were isolated from these animals, post-fixed in 4% paraformaldehyde in phosphate buffer (pH 7.4) for 4 h, cryoprotected successively with 10, 20, and 30% sucrose in phosphate buffer, embedded in the OCT mounting medium (Sakura, Japan), and frozen by using liquid nitrogen. Then, 10-μm-thick sections were cut out from the embedded tissues, mounted on gelatin-coated glass slides, and air-dried. The sections on the glass slides were rinsed with TBS, incubated with 0.3% H2O2 in methanol, rinsed with TBS, pretreated with a blocking solution (5% bovine serum albumin, 0.1% goat normal serum, 0.01% (w/v) Triton X-100 in TBS (pH 7.4)) for 30 min at room temperature, and then incubated with anti-pS557-mCRY2 antibody (1:50) diluted in the blocking solution at 4 °C for 24 h. After rinsing with TBS, the sections were treated with a biotin-conjugated goat anti-rabbit IgG antibody (Vector Laboratories) for 24 h at 4 °C and then with avidin-biotin peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories) at room temperature followed by rinsing with TBS. The immunoreactive sites were developed by incubating with 0.4 mg/ml diaminobenzidine and 0.01% H2O2 in 50 mM Tris-HCl buffer (pH 7.6) for 3 min at room temperature.

Preparation of Nuclear and Cytoplasmic Extracts from Liver—Fractionation was performed as described by Gorski et al. (30) with some modifications. The mouse liver was homogenized in 4 volumes of buffer C (10 mM HEPES-KOH, 15 mM KCl, 0.1 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 2.2 M sucrose, 0.5 mM DTT, 50 mM NaF, 1 mM Na3VO4, 4 μM myc epitope-tagged Myc-MAPK (a kind gift of Dr. Louis J. Ptacek) were used.

Antibodies—To generate polyclonal antibody against the Ser-557-phosphorylated form of mCRY2, a synthetic phosphopeptide (CSGPA-557) was conjugated to keyhole limpet hemocyanin (Pierce) using imidobenzoyl-hydrosuccinimide ester (Sulfo-MBS; Pierce) according to a protocol supplied by the manufacturer. Two rabbits were injected subcutaneously with the keyhole limpet hemocyanin-conjugated phosphopeptide. To generate polyclonal antibody against the Ser-557-phosphorylated form of mCRY2, a synthetic phosphopeptide (CSGPA-557) was conjugated to keyhole limpet hemocyanin (Pierce) using imidobenzoyl-hydrosuccinimide ester (Sulfo-MBS; Pierce) according to a protocol supplied by the manufacturer. Two rabbits were injected subcutaneously with the keyhole limpet hemocyanin-conjugated phosphopeptide emulsified in Freund’s complete adjuvant (Difco). For the purification of the antibody, the phosphopeptide or its non-phosphorylated form was conjugated to EAH-Sepharose (Amersham Biosciences) using Sulfo-MBS. Sera obtained from the immunized rabbits were applied to the phosphopeptide-coupled column, and the antibodies bound to the column were eluted with 0.1 M glycine (pH 2.5) and neutralized with 1 M Tris-HCl (pH 8.0). After being dialyzed against TBS, the eluted fraction was applied to the non-phosphopeptide-coupled column, and the flow-through fraction was collected (anti-pS557-mCRY2 antibody).

In Vitro Phosphorylated mCRYs—Bacterially expressed GST-mCRYs and the phosphorylated (activated) form of Myc-MAPK were prepared as described (27, 29). For phosphorylation reactions, GST-mCRYs and the phosphorylated (activated) form of Myc-MAPK were suspended with 600 μl of kinase buffer (10 mM HEPES-KOH, 15 mM KCl, 0.15 mM spermine, 0.05 mM spermidine, 1 mM EDTA, 2.2 M sucrose, 5% (v/v) glycerol, 0.5 mM DTT, 50 mM NaF, 1 mM Na3VO4, 0.5 mM phenylmethylsulfonyl fluoride, 14 μg/ml aprotinin, and 14 μg/ml leupeptin, pH 7.4, at 4 °C) using a glass-alkali homogenizer for 6 strokes. The homogenate was filtered through 2 layers of cheesecloth, and 4.5 ml of the filtrated homogenate was layered over a 7.5 ml cushion of buffer D (10 mM HEPES-KOH, 15 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 2.2 M sucrose, 5% (v/v) glycerol, 0.5 mM DTT, 50 mM NaF, 1 mM Na3VO4, 0.5 mM phenylmethylsulfonyl fluoride, 14 μg/ml aprotinin, and 14 μg/ml leupeptin, pH 7.6, at 4 °C) and centrifuged for 5 min at 100,000 × g at 4 °C. Only a transparent portion was collected from the 4.5-ml upper fraction and further centrifuged at 100,000 × g for 1 hr. The supernatant was collected from the 100,000 × g centifuged supernatant and used as substrate for the kinase reaction.

Cell Culture, Transfection, and Immunoprecipitation—Costell cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum in wells of 6-well plates and were transiently transfected by using Lipofectamine PLUS (Invitrogen) with a total of 1.0 μg of protein expression plasmids. The cells were then cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum for 24–36 h, washed with ice-cold phosphate-buffered saline, and visualized by an enhanced chemiluminescence detection system (PerkinElmer Life Sciences). When reprobed, the blots were stripped according to the manufacturer’s protocol.
solubilized for 30 min on ice in 300 μl of buffer E (20 mM HEPES-KOH, 1% (w/v) Triton X-100, 100 mM KCl, 2.5 mM EDTA, 5 mM DTT, 10% (w/v) glycerol, 4 μg/ml leupeptin, 4 μg/ml aprotinin, 50 mM NaF, and 1 mM Na3VO4 at pH 7.5 at 4 °C). The cell extracts were then centrifuged for 30 min at 20,000 × g. For immunoprecipitation, the supernatant (20 μg of proteins) was incubated with 1.0 μg of anti-Myc antibody (clone 9E10, Santa Cruz Biotechnology) at 4 °C for 2 h followed by incubation with 20 μl of protein G-Sepharose at 4 °C for 1 h. The beads were washed 4 times with buffer E.

In Vitro Phosphorylation by GSK-3β—After immunoprecipitation of Myc-mCRY2 from the COS7 lysates as described above, the beads were washed 4 times with kinase buffer F (20 mM Tris-HCl, 5 mM DTT, 10 mM MgCl2, 150 μM Na3VO4, 1 mM ATP (pH 7.5)), and the final suspension (50 μl) was incubated with 250 units of recombinant GSK-3β (New England Biolabs) for 1 h at 30 °C.

Phosphatase Treatment—Myc-mCRY expressed in the COS7 cells or endogenous mCRY2 in the mouse liver lysate was immunoprecipitated by anti-Myc or anti-CRY2 antibody, respectively, and the beads were washed 4 times with phosphatase buffer P (50 mM HEPES-KOH, 0.1 mM EDTA, 5 mM DTT, 2 mM MnCl2, 0.01% Brij 35 (pH 7.5)). The final suspension (50 μl) was incubated with 200 units of λ-protein phosphatase (Sigma) for 30 min at 30 °C.

Degradation Assay—The mouse liver was homogenized with 20 volumes of buffer H (50 mM HEPES-KOH, 5 mM MgCl2, 40 mM KCl, 1% (w/v) Nonidet P-40, 0.01% Brij 35 (pH 7.5)), followed by centrifugation for 10 min at 2400 × g, and the unsedimented materials were collected. The lysate (50 μg of proteins) was mixed with or without 10 mM ATP and incubated at 30 °C for the indicated times in the presence or absence of MG132 (50 μM; Calbiochem) and/or GSK-3β (125 units or 500 units). After the incubation, the lysate was subjected to the immunoblot analysis with anti-CRY2 antibody.

RESULTS

Rhythmic Phosphorylation of mCRY2 at Ser-557 in the Mouse Liver—To investigate in vivo phosphorylation of mCRY2 at Ser-557, we generated an antibody specific to the Ser-557-phosphorylated form of mCRY2. The specificity of the antibody (anti-pS557-mCRY2 antibody) was examined by immunoblotting of recombinant GST-mCRY2 and its Ser-265/Ser-557-phosphorylated form (P-GST-mCRY2), which was produced by incubation of GST-mCRY2 with activated MAPK in vitro (27). The anti-pS557/mCRY2 antibody detected the Ser-265/Ser-557-phosphorylated form of GST-mCRY2, and no detectable cross-reaction was observed against the unphosphorylated GST-mCRY2 nor Ser-247-phosphorylated GST-mCRY1 (Fig. 1A, upper panel). The latter observation eliminated any cross-reaction of this antibody to the central region of mCRY2 near phosphorylated Ser-265 because of the fully conserved primary structure of this region between mCRY1 and mCRY2. When Myc-mCRY2 was expressed in COS7 cells and immunoprecipitated with anti-Myc-antibody from the unstimulated cells, the anti-pS557/mCRY2 antibody recognized a band corresponding to Myc-mCRY2 (Fig. 1B, lane 1). The immuno-reaction to the band was abolished by treatment of the immunoprecipitate with λ-protein phosphatase (Fig. 1B, lane 2), whereas in the presence of a phosphatase inhibitor the same treatment had no effect on the immunoactivity of the band (Fig. 1B, lane 3). Finally, we confirmed no detectable immuno-reaction to the Ser-to-Ala mutant at Ser-557 position of Myc-mCRY2 (Myc-mCRY2 S557A-mutant), verifying the sequence specificity of the immuno-reaction (Fig. 1B). These results together demonstrate high specificity of the antibody to the Ser-557-phosphorylated form of mCRY2 and concomitantly indicate the presence of intrinsic protein kinase capable of phosphorylating Ser-557 of Myc-mCRY2 expressed in unstimulated COS7 cells (Fig. 1B).

By using the phospho-specific antibody, we examined the phosphorylation state of mCRY2 Ser-557 in the mouse liver, in which many clock proteins except mCRYs showed obvious shifts in electrophoretic mobility due to phosphorylation (22). The liver lysate was prepared at ZT 22 from mice entrained to LD cycles and subjected to immunoprecipitation with anti-CRY2 antibody followed by immunoblotting. Anti-pS557-mCRY2 antibody recognized a 68-kDa band corresponding to mCRY2 protein (Fig. 1C, lane 1), and the immuno-positive band became invisible after treatment of the immunoprecipitate with phosphatase (Fig. 1C, lane 2), indicating that mCRY2 is phosphorylated at Ser-557 in vivo.

We then pursued any temporal change in phosphorylation of mCRY2 Ser-557 in the liver throughout the day. In LD cycles, the protein level of mCRY2 showed a robust daily variation with a peak at ZT 2 and a trough at ZT 14 (Fig. 1D, open circles) as reported (22). With a similar degree of amplitude, the Ser-557-phosphorylated form of mCRY2 exhibited a daily change with a peak at ZT 22 (Fig. 1D, closed circles), which preceded the zenith of the protein level by ~ 4 h (Fig. 1D). We further examined circadian fluctuation of mCRY2 Ser-557 phosphorylation in DD conditions and then the levels of the Ser-557-phosphorylated form (Fig. 1E, closed circles) and mCRY2 protein (Fig. 1E, open circles) showed robust circadian rhythms with profiles of a 4-h difference in their peak times, as observed in LD cycles.

MAPK in the mouse liver also showed robust daily variation in phosphorylation with a peak at ZT 10 and a nadir at the early night without any significant change in the protein level (Supplemental Fig. S1A). This temporal profile of MAPK phosphorylation was almost antiphase to that of the Ser-557 phosphorylation of mCRY2, raising the possibility that the other kinase(s) is responsible for the phosphorylation of mCRY2 at Ser-557 in vivo. In fact, MAPK was unlikely to contribute to the Ser-557 phosphorylation in COS7 cells because neither activation nor inhibition of the MAPK pathway altered the phosphorylation state of mCRY2 Ser-557 in vivo. After immunoprecipitation of mCRY2 antibody followed by immunoblotting. Anti-pS557-mCRY2 antibody (Fig. 1, F–I) and nuclei staining by hematoxylin (Fig. 2G). At CT 2 and CT 20, the majority of the cell nuclei showed strong immunoreactivity to anti-pS557-mCRY2 antibody, whereas very weak signals were observed in the cell nuclei at CT 8 and CT 14 (Fig. 2, A–D), and this profile of the temporal variation was consistent with that observed in the immunoblot analysis (Fig. 1E). These positive signals detected in the nuclei of the hepatocytes (Fig. 2D) became undetectable when the antibody was preabsorbed with the phosphopeptide that was used as the antigen (Fig. 2E), whereas preincubation with the control (non-phosphorylated) peptide had no effect on the immunoreactivity in the nuclei (Fig. 2F). These observations verified that the positive signals in the nuclei indeed represent the presence of the Ser-557-phosphorylated form of mCRY2. To further confirm the nuclear localization of Ser-557-phosphorylated mCRY2, nuclear and cytoplasmic fractions were prepared from the mouse liver dissected at CT 18. The immunoblot analysis (Fig. 2H) demonstrated that mCRY2 was present in both cytoplasmic and nuclear fractions (top panel) as reported previously (22). In contrast, a significant amount of Ser-557-phosphorylated mCRY2 was detected in the nuclear fraction (second panel). Taken together, it is concluded that the Ser-557-phosphorylated form of mCRY2 is localized in the cell nuclei, although mCRY2 protein is located in both the cytoplasm and nuclei of the mouse liver.

Ser-557 Phosphorylation-dependent Phosphorylation of mCRY2 by GSK-3β—In the amino acid sequences of mouse, human, and chicken CRY2 (Fig. 3A), a serine residue is con-
or Myc-mCRY2 S557A-mutant (lanes 4–6). Twenty-four hours after transfection, the cell lysate (20 μg each) was subjected to immunoblotting with anti-pS557-mCRY2 antibody (upper panel) and with anti-GST antibody (lower panel). B, COS7 cells were transiently transfected with a plasmid for wild-type (WT) Myc-mCRY2 (lanes 1–3) or Myc-mCRY2 S5557A-mutant (lanes 4–6). Twenty-four hours after transfection, the cell lysate (20 μg of proteins) was immunoprecipitated (IP) with anti-Myc antibody, and the immunoprecipitates were incubated at 30 °C for 30 min without (lanes 1 and 4) or with α-protein phosphatase (αPPase; lanes 2, 3, 5, and 6) in the absence (lanes 1, 2, 4, and 5) or presence of 40 mM vanadate (lanes 3 and 6). C, the mouse liver lysate was prepared at ZT 22, and mCRY2 was immunoprecipitated with anti-CRY2 antibody. The immunoprecipitates were incubated at 30 °C for 30 min without (lane 1) or with λ-protein phosphatase (lanes 2 and 3) in the absence (lanes 1 and 2) or presence of 40 μM vanadate (lane 3). After the incubation, the samples were immunoblotted with anti-pS557-mCRY2 antibody (upper panel) or with anti-CRY2 antibody (lower panel). D and E, the mouse liver lysate was prepared at various time points in LD cycle (D) or on the fifth day in DD (E). mCRY2 in each sample was immunoprecipitated with anti-CRY2 antibody, and the immunoprecipitates were immunoblotted with anti-pS557-mCRY2 antibody (top panels). The same blot was reprobed with anti-CRY2 antibody (second panels). The band densities were quantified by a densitometric scanning of the immunoblotted membrane, and shown in values (mean ± S.E., n = 3) relative to the highest values (third panels). Data at ZT (CT) 22 and ZT (CT) 2 were double-plotted. Before the immunoprecipitation, the samples were immunoblotted with anti-pan ERK antibody as a control, and we detected no significant change in the band densities among the samples within the day. The same sets of the samples were used in the experiments of supplemental Fig. S1, and a representative set of the data is shown in supplemental Fig. S1 (A, second panel, samples in LD; B, second panel, samples in DD).

Fig. 1. Daily variation and circadian rhythm of Ser-557 phosphorylation of mCRY2 in the mouse liver. A, phosphorylated (P) and non-phosphorylated forms of GST-mCRY1 or GST-mCRY2 (0.2 μg each) were subjected to immunoblotting with anti-pS557-mCRY2 antibody (upper panel) and with anti-GST antibody (lower panel). B, COS7 cells were transiently transfected with a plasmid for wild-type (WT) Myc-mCRY2 (lanes 1–3) or Myc-mCRY2 S5557A-mutant (lanes 4–6). Twenty-four hours after transfection, the cell lysate (20 μg of proteins) was immunoprecipitated (IP) with anti-Myc antibody, and the immunoprecipitates were incubated at 30 °C for 30 min without (lanes 1 and 4) or with α-protein phosphatase (αPPase; lanes 2, 3, 5, and 6) in the absence (lanes 1, 2, 4, and 5) or presence of 40 mM vanadate (lanes 3 and 6). C, the mouse liver lysate was prepared at ZT 22, and mCRY2 was immunoprecipitated with anti-CRY2 antibody. The immunoprecipitates were incubated at 30 °C for 30 min without (lane 1) or with λ-protein phosphatase (lanes 2 and 3) in the absence (lanes 1 and 2) or presence of 40 μM vanadate (lane 3). After the incubation, the samples were immunoblotted with anti-pS557-mCRY2 antibody (upper panel) or with anti-CRY2 antibody (lower panel). D and E, the mouse liver lysate was prepared at various time points in LD cycle (D) or on the fifth day in DD (E). mCRY2 in each sample was immunoprecipitated with anti-CRY2 antibody, and the immunoprecipitates were immunoblotted with anti-pS557-mCRY2 antibody (top panels). The same blot was reprobed with anti-CRY2 antibody (second panels). The band densities were quantified by a densitometric scanning of the immunoblotted membrane, and shown in values (mean ± S.E., n = 3) relative to the highest values (third panels). Data at ZT (CT) 22 and ZT (CT) 2 were double-plotted. Before the immunoprecipitation, the samples were immunoblotted with anti-pan ERK antibody as a control, and we detected no significant change in the band densities among the samples within the day. The same sets of the samples were used in the experiments of supplemental Fig. S1, and a representative set of the data is shown in supplemental Fig. S1 (A, second panel, samples in LD; B, second panel, samples in DD).

A conserved (Ser-553 (**)) at the fourth position amino-terminal to the conserved phospho-acceptor Ser-557 (*). This sequence feature coincides with a consensus motif for Ser-553 to be phosphorylated by GSK-3. To examine GSK-3-catalyzed phosphorylation of mCRY2, Myc-mCRYs expressed in COS7 cells were immunoprecipitated with anti-Myc antibody and incubated with recombinant GSK-3β. This in vitro incubation generated an up-shifted band that was immunoreactive to both anti-Myc antibody (Fig. 3B, lane 5, solid arrowhead) and anti-pS557-mCRY2 antibody (Fig. 3C, lower panel, lane 2), and phosphatase treatment down-shifted the upper band (Fig. 3B, lane 6). Such a striking electrophoretic mobility shift due to phosphorylation was not observed for Myc-mCRY1 when incubated with GSK-3β under similar conditions (Fig. 3B, lane 2). As described, GSK-3β-catalyzed phosphorylation of its substrates, such as glycogen synthase and β-catenin, depends on priming phosphorylation catalyzed by other protein kinase at Ser/Thr residue that is located at the fourth position carboxy-terminal to the target Ser/Thr residue (31–33). We then examined whether GSK-3β phosphorylation of mCRY2 requires priming phosphorylation of Ser-557. First, mCRY2 S553A-mutant exhibited no mobility shift after incubation with GSK-3β (Fig. 3C, lane 6). Second, preincubation of Myc-mCRY2 with phosphatase completely eliminated Ser-557 phosphorylation (Fig. 3C, lane 3), and subsequent incubation with GSK-3β neither phosphorylated Ser-557 of mCRY2 nor altered its mobility (lane 4). These results demonstrate that GSK-3β phosphorylates mCRY2 in a manner perfectly dependent on priming phosphorylation of Ser-557 and strongly suggest Ser-553 as the phosphorylation site of GSK-3β. Indeed, when Myc-mCRY2 S553A-mutant expressed in COS7 cells was incubated with GSK-3β, only a trace of up-shifted band was detected by anti-CRY2 antibody (Fig. 3D, lane 4).

Daily Variation and Circadian Rhythm of the Phosphorylation of GSK-3β—The GSK-3β activity is controlled by phosphorylation at Ser-9, which suppresses its kinase activity (34). A
temporal change in GSK-3β activity in the mouse liver was investigated by using the antibody specifically recognizing the Ser-9-phosphorylated form of GSK-3β. In LD cycles, GSK-3β exhibited a daily variation of the phosphorylation level peaking in the evening (Fig. 4A, solid circles), and similar temporal variation was observed in DD conditions with a peak in the late subjective day (Fig. 4B, solid circles). In both conditions, the protein levels of GSK-3β were almost constant throughout the day (Fig. 4, A and B, open circles), indicating circadian fluctuation of phosphorylation and/or dephosphorylation of GSK-3β Ser-9 in the mouse liver. These data suggest strongly that GSK-3β activity in the liver is elevated from the late (subjective) night to early (subjective) day, and hence, we propose that Ser-553/Ser-557-phosphorylation of mCRY2 is regulated coordinately by multiple kinases whose activities appear circadian-regulated.

GSK-3β-mediated Phosphorylation of mCRY2 Causes Degradation of mCRY2 by a Proteasome Pathway—As described in the in vivo experiments with the mouse liver (Fig. 1), we were unable to detect the up-shifted band that represents GSK-3β-mediated Ser-553 phosphorylation of mCRY2. The up-shifted band was also invisible in cultured COS7 cells and immunoprecipitated with anti-Myc antibody. B, the immunoprecipitates of wild-type (WT) Myc-mCRY1 (lanes 1–3) and wild-type Myc-mCRY2 (lanes 4–6) were incubated in vitro with (lanes 2, 3, 5, and 6) or without (lanes 1 and 4) GSK-3β or treated with λ-protein phosphatase (APPPase) after the incubation with GSK-3β (lanes 3 and 6). C, wild-type Myc-mCRY2 (lanes 1 and 2), phosphatase-pretreated wild-type Myc-mCRY2 (lanes 3 and 4), or Myc-mCRY2 S557A mutant (lanes 5 and 6) was incubated in vitro with (lanes 2, 4, and 6) or without (lanes 1, 3, and 5) GSK-3β. D, wild-type Myc-mCRY2 (lanes 1 and 2) or Myc-mCRY2 S553A-mutant (lanes 3 and 4) was incubated with (lanes 2 and 4) or without (lanes 1 and 3) GSK-3β. The two protein bands of Myc-mCRYs are indicated by solid (up-shifted band) and open arrowheads. The samples were probed with anti-Myc antibody (B and C, upper panel), anti-CRY2 antibody (D, upper panel), or anti-pS557-mCry2 antibody (C and D, lower panels).

Fig. 3. Phosphorylation of mCRY2 by GSK-3β. A, amino acid sequence of mCRY2 at the region encompassing Ser-553 and Ser-557. For comparison, the corresponding regions of human CRY2 (hCRY2) and chicken CRY2 (cCRY2) were aligned. B–D, Myc-mCry2s were transiently transfected to COS7 cells and immunoprecipitated with anti-Myc antibody. B, the immunoprecipitates of wild-type (WT) Myc-mCRY1 (lanes 1–3) and wild-type Myc-mCRY2 (lanes 4–6) were incubated in vitro with (lanes 2, 3, 5, and 6) or without (lanes 1 and 4) GSK-3β or treated with λ-protein phosphatase (APPPase) after the incubation with GSK-3β (lanes 3 and 6). C, wild-type Myc-mCRY2 (lanes 1 and 2), phosphatase-pretreated wild-type Myc-mCRY2 (lanes 3 and 4), or Myc-mCRY2 S557A mutant (lanes 5 and 6) was incubated in vitro with (lanes 2, 4, and 6) or without (lanes 1, 3, and 5) GSK-3β. D, wild-type Myc-mCRY2 (lanes 1 and 2) or Myc-mCRY2 S553A-mutant (lanes 3 and 4) was incubated with (lanes 2 and 4) or without (lanes 1 and 3) GSK-3β. The two protein bands of Myc-mCry2s are indicated by solid (up-shifted band) and open arrowheads. The samples were probed with anti-Myc antibody (B and C, upper panel), anti-CRY2 antibody (D, upper panel), or anti-pS557-mCry2 antibody (C and D, lower panels).
mCRY2 protein is preferentially phosphorylated by GSK-3. These results, it is concluded that Ser-557-phosphorylated mCRY2 protein is post-translationally regulated by sequential phosphorylation by GSK-3β and its maximal level 4 h later (ZT/CT 2). It is important to note that the kinase activity of GSK-3β is up-regulated from late Ser-557-phosphorylated form had a peak level at late (subjective) night to morning (ZT/CT 2–3). The ATP-dependent degradation (37) of mCRY2 was almost completely suppressed by the addition of MG132 (squares), whereas it was accelerated by exogenously added GSK-3β in a dose-dependent manner (Fig. 5C, compare triangles and diamonds with circles). It is concluded that stability of mCRY2 is regulated by both the proteasome pathway and GSK-3β in a manner dependent on priming phosphorylation at Ser-557.

**DISCUSSION**

CRYs are members of the DNA photolyase/cryptochrome flavoprotein family. CRY proteins were initially identified in plants as structural homologues of the DNA repair enzyme, DNA photolyase, but CRY proteins lack DNA repair activity (28). Mice have two CRY proteins, mCRY1 and mCRY2, and mutant mice lacking both mCRY1 and mCRY2 exhibit arrhythmic behavior when placed in constant darkness, whereas the mutants for individual mCRY genes showed distinct abnormality with a shorter (mCRY1 knock-out) and longer (mCRY2 knock-out) free-running period (12). Therefore, it has been suggested that mCRY1 and mCRY2 proteins may be differently regulated by and/or contribute to the clockwork in manners that are distinguishable from each other. In the present study, we identified mCRY2-specific regulatory mechanism that should constitute the well orchestrated degradation process in the circadian clockwork.

Many clock proteins such as CLOCK, BMAL1, mPER1, and mPER2 exhibit electrophoretic mobility shift, which is attributed to their intrinsic phosphorylation in the mouse liver (22). In particular, the phosphorylation of PER1 protein accelerated its degradation (23). To date, however, no evidence has been presented for phosphorylation of mCRYs in any intact tissues. In the present study, by taking advantage of our phospho-specific antibody, we found the overt phosphorylation rhythm of mCRY2 at Ser-557 in the mouse liver and demonstrated its important role as priming for the subsequent phosphorylation at Ser-553 by GSK-3β. Importantly, the dually phosphorylated form of mCRY2 was targeted for degradation via the proteasome pathway. These data provide evidence that mCRY2 protein is post-translationally regulated by sequential phosphorylation at Ser-557 and Ser-553, and this is the first study to identify the phosphorylation sites of any clock proteins in mammalian clock tissues.

In the liver of the mice maintained in LD and DD conditions, the amount of the Ser-557-phosphorylated mCRY2 displayed circadian variation (Fig. 1), and the amount of the phosphorylated form starts elevating at the early night (ZT/CT 14) when mCRY2 protein just begins to increase. This observation indicates that synthesized mCRY2 is readily subject to phosphorylation at Ser-557 by the priming kinase. On the other hand, the Ser-557-phosphorylated form had a peak level at late (subjective) night (ZT/CT 22), whereas the protein amount reached its maximal level 4 h later (ZT/CT 2). It is important to note that the kinase activity of GSK-3β is up-regulated from late night to morning (ZT/CT 22–23), as judged from the circadian rhythm of its phosphorylation at Ser-9 which suppresses the kinase activity (34). Now it is most probable that Ser-557-phosphorylated mCRY2 rapidly degraded after subsequent phosphorylation by GSK-3β during the late (subjective) night to early (subjective) day. Such a cooperative activation of GSK-3β along with the active degradation system specific for dually phosphorylated mCRY2 should be responsible for the 4-h advance of the declining phase of the Ser-557-phosphorylated form relative to its protein abundance (Fig. 1, D and E). In support of this idea, the GSK-3β-phosphorylated (up-shifted) protein level of mCRY2 displayed a minimal decrease along with the incubation time to ~80% of the original level after a 6-h incubation (Fig. 5B, open circles). On the other hand, the addition of 10 mM (final) ATP to the lysate induced a substantial decrease of mCRY2 protein to a level of ~50% after a 6-h incubation (solid circles). The ATP-dependent degradation (37) of mCRY2 was almost completely suppressed by the addition of MG132 (squares), whereas it was accelerated by exogenously added GSK-3β in a dose-dependent manner (Fig. 5C, compare triangles and diamonds with circles). It is concluded that stability of mCRY2 is regulated by both the proteasome pathway and GSK-3β in a manner dependent on priming phosphorylation at Ser-557.
form of mCRY2 was not detected in vivo (Fig. 1, D and E), unlike in vitro (Fig. 3). In addition to the circadian rhythm of GSK-3β activity, the priming kinase may similarly exhibit a circadian rhythm in activity. Altogether, the dual kinase system phosphorylating mCRY2 appears to be temporally regulated so as to coordinate the Ser-557/Ser-553 phosphorylation with protein degradation of mCRY2. Because mCRY proteins are the predominant inhibitors of E-box-dependent transcription of the clock (related) genes, the E-box-dependent molecular oscillation should strongly depend on circadian regulation of the activity of the dual kinase system.

Recent studies show that GSK-3β is active in unstimulated cells, and external signals inhibit the kinase activity (for review, see Ref. 32). The Ser-9 phosphorylation involved in this inactivation process of GSK-3β appears to be catalyzed by several kinases such as p90 ribosomal S6 kinase (38), an enzyme that is a downstream target of MAPK (39). Because the rhythmic Ser-9 phosphorylation of GSK-3β is synchronous to the activation rhythm of MAPK in the mouse liver (compare Fig. 4 with Supplemental Fig. S1), MAPK might be responsible for the circadian variation of GSK-3β activity (e.g. through the regulation of p90 ribosomal S6 kinase by MAPK; Ref. 40).

Generally, GSK-3β phosphorylates its substrates in a manner dependent on priming phosphorylation at Ser/Thr, located four residues carboxyl-terminal to the target Ser/Thr (32). Individual substrates of GSK-3β appear to be phosphorylated by a variety of specific priming kinases such as CKII for glycogen synthase (31), CKII for β-catenin (33), and protein kinase A for cAMP-response element-binding protein (41). Although Ser-557 of mCRY2 was phosphorylated by MAPK in in vitro experiments (27), neither activation nor inhibition of the MAPK pathway in COS7 cells affected the level of Ser-557 phosphorylation relative to its protein level (Supplemental Fig. S1, C and D). MAPK may play a minimal role as the priming kinase in vivo. mCRY2 is known to be phosphorylated by CKIε in culture (36), but cotransfection of CKIε caused no significant change of the phosphorylating state at the priming site, Ser-557 (Supplemental Fig. S1C). Ser-557 may be phosphorylated by a protein kinase that has not been referred to as a clock-related protein, or alternatively, a unique regulatory mechanism may underlie Ser-557 phosphorylation in vivo. Here, the nuclear localization of Ser-557-phosphorylated mCRY2 (Fig. 2) prompted us to speculate that the priming kinase may be located in the nucleus. But it is equivalently possible that mCRY2 (Ser-557) is phosphorylated in the cytoplasm and then translocates into the nucleus. Even in the latter case, phosphorylated Ser-557 may not be the prerequisite for the nuclear entry of mCRY2, because not only wild-type mCRY2 but also its S557A mutant was detected in the nucleus when expressed in COS7 cells. 2 It is intriguing to determine the identity of the priming kinase and its intracellular localization for a better understanding of molecular regulation of mCRY2 in vivo.

In the present study we found that Ser-557-phosphorylated mCRY2 is subsequently phosphorylated by GSK-3β and then degraded in a proteasome-dependent manner (Fig. 5). In circadian clockworks, such a phosphorylation-dependent protein degradation mechanism is well studied in Drosophila PER, 2 Y. Harada, M., Sakai, M., and Y. Fukada, unpublished observation.
which is phosphorylated by Doubletime (14, 17) and degraded by the proteasome upon interaction with Slimb, a member of the F-box/WD40-repeat protein family of ubiquitin ligase SCF (Skp1, Cul1, F-box protein) complex (42, 43). Recently it has been shown that mPER2 is regulated in a manner similar to Drosophila PER (44). Generally, F-box proteins specifically recognize a short phosphopeptide motif “phosphodegron,” and it is known that β-Trcp, a mammalian homolog of Slimb, interacts with a phosphodegron, which contains the sequence Asp-Ser(P)-Gly-δ-X-Ser(P), where Ser(P) is phosphoserine, and δ is a hydrophobic amino acid (45). Noticeably, the amino acid sequence surrounding phosphorylation sites of mCRY2 (Ser(P)-553–Gly–Pro–Ala–Ser(P)-557) is similar to the phosphodegron for β-Trcp, and it is possible that the phosphorylated carboxyl-terminal region of mCRY2 serves as a recognition site of β-Trcp. Recently, the carboxyl-terminal region of CRY has been shown to contain a nuclear localization signal that is necessary for CRY to translocate into the nucleus (46, 47), and it is known that TIM and mCRY2 are the dimerization part-

In Drosophila, the increased function of sgg, a Drosophila ortholog of GSK-3β, resulted in period shortening, whereas decreased sgg function caused substantial period lengthening of the circadian clock (25). SGG phosphorylates TIM in vitro and appears to regulate the nuclear translocation of the PER/TIM heterodimer (25). Apparently, GSK-3β phosphorylates and regulates different molecules in different species, namely, TIM in the fly and mCRY2 in the mouse. It should be noted, however, that both TIM and mCRY2 are the dimerization partners for PER proteins in the clock structures of the fly and mouse, respectively (10, 13), and it is conceivable that GSK-3β may contribute to the clockwork through the regulation of components that are operative together with PER proteins in a variety of animals.

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Ser-557-phosphorylated mCRY2 Is Degraded upon Synergistic Phosphorylation by Glycogen Synthase Kinase-3 β

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