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Direct Association between Mouse PERIOD and CKIε Is Critical for a Functioning Circadian Clock

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The mPER1 and mPER2 proteins have important roles in the circadian clock mechanism, whereas mPER3 is expendable. Here we examine the posttranslational regulation of mPER3 in vivo in mouse liver and compare it to the other mPER proteins to define the salient features required for clock function. Like mPER1 and mPER2, mPER3 is phosphorylated, changes cellular location, and interacts with other clock proteins in a time-dependent manner. Consistent with behavioral data from *mPer2/3* and *mPer1/3* double-mutant mice, either mPER1 or mPER2 alone can sustain rhythmic posttranslational events. However, mPER3 is unable to sustain molecular rhythmicity in *mPer1/2* double-mutant mice. Indeed, mPER3 is always cytoplasmic and is not phosphorylated in the livers of *mPer1*-deficient mice, suggesting that mPER3 is regulated by mPER1 at a posttranslational level. In vitro studies with chimeric proteins suggest that the inability of mPER3 to support circadian clock function results in part from lack of direct and stable interaction with casein kinase Iε (CKIε). We thus propose that the CKIε-binding domain is critical not only for mPER phosphorylation but also for a functioning circadian clock.

Circadian rhythms have been observed in organisms from cyanobacteria to humans (11). These rhythms are under the direct influence of environmental cues, most notably the day-night cycles and by a genetically determined, endogenous clock (2, 6). In mammals, a master clock located in the suprachiasmatic nuclei of the anterior hypothalamus controls peripheral clocks through neuronal and humoral connections (19).

A major area of interest in circadian biology is to understand the time-keeping mechanism at the molecular level. Transcriptional feedback loops constitute a central feature of most, if not all, circadian clocks (2, 6, 18). In the mouse, two basic-helix-loop-helix–PAS-containing transcription factors, CLOCK and BMAL1, form heterodimers that bind to E-box enhancer sequences and drive the rhythmic transcription of three *Period* genes (*mPer1*, *mPer2*, and *mPer3*) and two *Cryptochrome* genes (*mCry1* and *mCry2*) (9, 12). As mPER and mCRY are translated in the cytoplasm, they form mPER-mCRY complexes, which then translocate into the nucleus to inhibit their own transcription by directly interacting with the CLOCK-BMAL1 complex (14, 15, 17, 21). Based on in vitro and in vivo studies, it appears that the CRY proteins play a major role in this inhibition (8, 10, 14, 21).

At the posttranslational level, the mPER1 and mPER2 proteins are temporally phosphorylated in a circadian manner (15). Casein kinase Iε (CKIε) and CKIδ are important kinases for PER and possibly other clock proteins in mammals (4, 7, 15, 16). Phosphorylation of clock proteins may affect their stability, cellular location, and interactions with one another (1, 13, 15, 22, 25, 26).

Recently, mutant mice with targeted disruption of all three *mPer* genes have been generated and characterized (3, 5, 20, 27, 28). Studies of these mutant mice reveal that mPER1 and mPER2 have critical roles in the mammalian circadian clock, whereas mPER3 is dispensable for a functional clock. The molecular basis for the difference in clock-relevant functions among the mPER proteins is unknown, however.

We therefore sought to determine why mPER3 cannot sustain circadian clock function by comparing its posttranslational regulation with that of mPER1 and mPER2 in wild-type mice and in mice with various *mPer* mutations. In this way, the functions of each mPER protein could be inferred by correlating biochemical defects with the disruption of specific *mPer* gene(s). Our results show that mPER1 and mPER2 are redundant for the rhythmic, posttranslational regulation of clock proteins, including the phosphorylation program, the rhythmic assembly of clock protein complexes, and nuclear translocation. In vitro studies with chimeric proteins show that the inability of mPER3 to support circadian clock function results in part from lack of direct and stable interaction with CKIε. The data suggest that the CKIε-binding domain (CKBD) is an essential feature for mPER1 and mPER2 function in the circadian clock mechanism.

MATERIALS AND METHODS

Mouse strains and tissue collection. The homozygous *mPer* single-mutant (*mPer1*^{ldc}-, *mPer2*^{ldc}-, and *mPer3*-deficient) and *mPer* double-mutant (*mPer1/2*-, *mPer1/3*-, and *mPer2/3*-deficient) mice used in the present study were described previously (3, 20). The wild-type mice were of the same SV129 genetic background. BALB/c mice were used in the experiments depicted in Fig. 1B to D, 2, 4, and 5. Mice were entrained to a 24-h light-dark cycle (12-h light and 12-h dark) and sacrificed on the first day in constant darkness. Tissues were dissected and frozen on dry ice.

Recombinant plasmids. mPER1, mPER2, mPER3, mCRY1, and mCRY2 plasmids were described previously (15). To generate CKIε expression plasmid, the open reading frame of mouse CKIε (AB028736) was cloned into *Kpn*I and *Xho*I sites of pcDNA3.1/myc-His(A) vector (Invitrogen). For chimeric mPER2 (chPER2) and mPER3 (chPER3) plasmids, the CKBD of mPER2 (amino acids 555 to 754) (1) was replaced with that of mPER3 (amino acids 509 to 710) (29)

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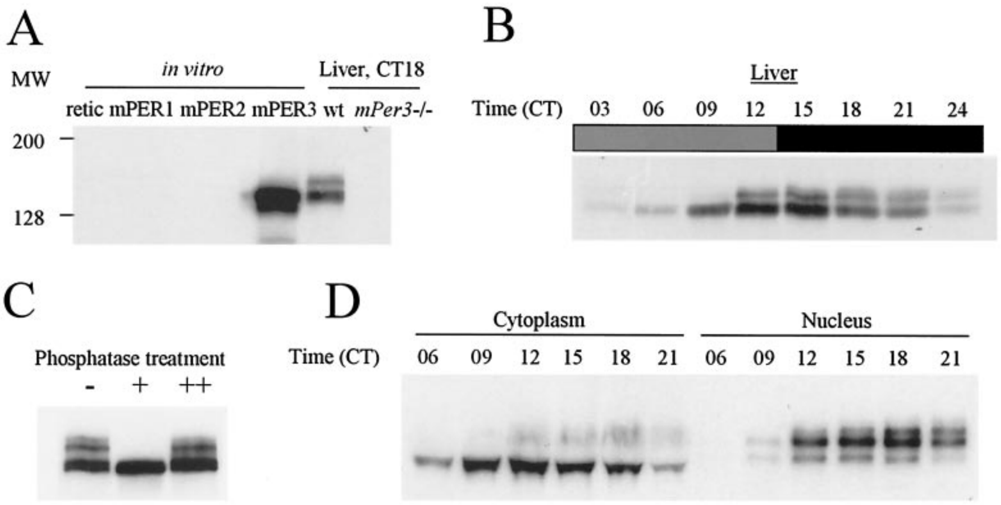

A
MW
in vitro
Liver, CT18
retic mPER1 mPER2 mPER3 wt mPer3-/-
200
128
B
Liver
Time (CT) 03 06 09 12 15 18 21 24
C
Phosphatase treatment
- + ++
D
Cytoplasm
Nucleus
Time (CT) 06 09 12 15 18 21 06 09 12 15 18 21

FIG. 1. Rhythmic posttranslational modification of mPER3. (A) Characterization of antisera against mPER3. Equal amounts of in vitro translated mPER1, mPER2, mPER3, and reticulolycyte lysate were resolved by SDS-PAGE, along with liver extracts from wild-type (wt) and *mPer3*-deficient mice. The proteins were transferred onto nitrocellulose membrane and Western blotted. Representative Western blots with one of our strongest antisera (P3-28R) are shown. The antibody only recognized in vitro-translated mPER3 and not equal amounts of mPER1 or mPER2. Anti-mPER1 (PER1-1-R) and -mPER2 (PER2-1-R) antibodies only recognized in vitro-translated mPER1 and mPER2, respectively (data not shown). Similar sized bands were detected only in liver extracts from wild-type mice and not *mPer3*-deficient mice. (B) Circadian rhythms of mPER3. Liver extracts prepared from mice collected at 3-h intervals were Western blotted with P3-28R and anti-β-actin antibodies. Even loading of total protein was assured by Western blot with anti-β-actin antibody (data not shown). (C) Electrophoretic mobility shift of mPER3 is due to phosphorylation. Liver extract from CT15 was subjected to IP with anti-mPER3 antibody (P3-28GP). The immune complexes were split into three aliquots and incubated in the presence (+) or absence (−) of λPP or with both λPP and 40 mM vanadate (++). (D) mPER3 exhibits nuclear accumulation in a circadian manner. Mouse liver extracts were collected at the indicated times, fractionated into cytoplasmic and nuclear portions, and blotted for mPER3. Both cytoplasmic and nuclear fractions for each time point were derived from the same number of cells.

and vice versa. To replace the endogenous CKBD with the heterologous CKBD, *Not*I and *Xho*I were introduced into the N terminus of CKBD and the C terminus of CKBD, respectively. This resulted in the introduction of four exogenous amino acids in the chimeric PER proteins. The chimeric full-length cDNAs were cloned into *Kpn*I and *Xho*I sites of pcDNA3.1/V5-His(A) vector.

Generation of anti-mPER3, anti-mCRY1, and anti-mCRY2 antibodies. We used PCR to amplify DNA sequences that encodes amino acids 4 to 227 for mPER3 (GenBank accession no. AF050182), amino acids 486 to 606 for mCRY1 (AB000777), and amino acids 504 to 592 for mCRY2 (AB003433). The PCR product for mPER3 was cloned into *Bam*HI and *Hin*dIII sites of PET23b, and those for mCRY1 and mCRY2 were cloned into *Bam*HI and *Eco*RI sites of PET23b (Novagen). Antibodies were produced as described previously (15). Representative antisera to each clock protein were affinity purified and designated P3-28R and P3-28GP (R, raised in rats; GP, raised in guinea pigs) for mPER3, C1-R and C1-GP for mCRY1, and C2-R and C2-GP for mCRY2. Antibodies to mCRY1 and mCRY2 were tested by using in vitro translated proteins and liver extracts prepared from wild-type and mCRY-deficient mice (24). The anti-mCRY1 and mCRY2 antibodies reacted with a band present in liver extracts from wild-type mice but not in liver extracts from mCRY-deficient mice (data not shown). Similar results were obtained with anti-mCRY antibodies from Alpha Diagnostic International.

RNase protection assay. RNase protection assays were performed as described previously. The antisense probes for *Bmal1* and *mPer1* were described previously (15). To make antisense probe for *mPer3*, we used PCR to amplify nucleotide sequences 462 to 678 of the *mPer3* open reading frame (GenBank accession no. AF050182). The PCR product was cloned into *Eco*RI and *Hin*dIII sites of the pCR II-TOPO vector (Invitrogen). The plasmid was linearized with *Eco*RI and in vitro transcribed by Sp6 RNA polymerase.

In vitro translation. In vitro translated proteins were produced in the presence of L-[^{35}S]methionine as described previously (15).

Nuclear and cytoplasmic extracts from liver. Subcellular fractionation of liver extracts was performed as described previously (15). Fractionation was evaluated by examining distribution of RNA polymerase II between cytoplasmic and nuclear fractions as described previously (15). More than 95% of RNA polymerase was found in nuclear fractions in all cases (data not shown).

IP and phosphatase treatment. For frozen liver tissue, immunoprecipitation (IP) was performed as described previously (15), and the immune complexes were analyzed by Western blotting as described below. The antibodies used for IP were P3-28GP for mPER3, C1-GP for mCRY1, and C2-GP for mCRY2. Other antibodies used for IP were described previously (15). For transfected NIH 3T3 cells, IP was performed with the following modifications. NIH 3T3 cells were washed with phosphate-buffered saline and harvested 24 h posttransfection. The cells were homogenized in an Eppendorf tube with a minihomogenizer (Kontes) at 4°C in 5 volumes of extraction buffer (EB; 20 mM HEPES [pH 7.5], 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.05% Triton X-100, 10 μg of aprotinin/μl, 5 μg of leupeptin/μl, and 1 μg of pepstatin A/μl) and centrifuged at 12,000 × *g* for 10 min, and the supernatant was transferred to a new tube. Protein concentration was measured by Coomassie protein assay reagent (Pierce), and equal amounts of total protein were used for each experiment. IP was performed as described previously by using the clarified supernatant (15). Anti-hemagglutinin (HA) and V5 antibodies were purchased from Invitrogen. The antibody to immunoprecipitate CKIε was CKIε-GP (15). For in vitro-translated proteins, reticulocyte lysates containing known amounts of target proteins were mixed in different combinations as indicated in figure legends and incubated in the presence of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 10 μg of aprotinin/μl, 5 μg of leupeptin/μl, 1 μg/μl of pepstatin A, and 1 mM dithiothreitol) at room temperature for 30 min. Subsequently, 300 μl of EB was added. The samples were precleared and subjected to IP as described previously (15). For phosphatase treatment of mPER3, liver extract was subjected to IP with anti-mPER3 antibody (P3-28GP) to obtain mPER3-containing immune complexes. The immune complexes were divided into three aliquots. Then, 200 U of lambda protein phosphatase (λPP; New England Biolabs) was added to one aliquot. To a second aliquot, 40 mM vanadate was added before the addition of λPP. No addition was made to the last aliquot. All three aliquots were incubated at room temperature for 20 min and analyzed by Western blotting as described below.

WB. For frozen tissues, Western blotting (WB) was performed as described previously (15). To visualize mPER3, 6% sodium dodecyl sulfate (SDS)-polyacrylamide gels were used. The primary antibodies used for mPER3, mCRY1, and mCRY2 were P3-28R, C1-R, and C2-R, respectively. Other primary antibodies and a secondary antibody were as described previously (15). For transfected cells, cells were homogenized as described above, and equal amounts (~5 μg) of total protein were analyzed by Western blotting as described previously (15).

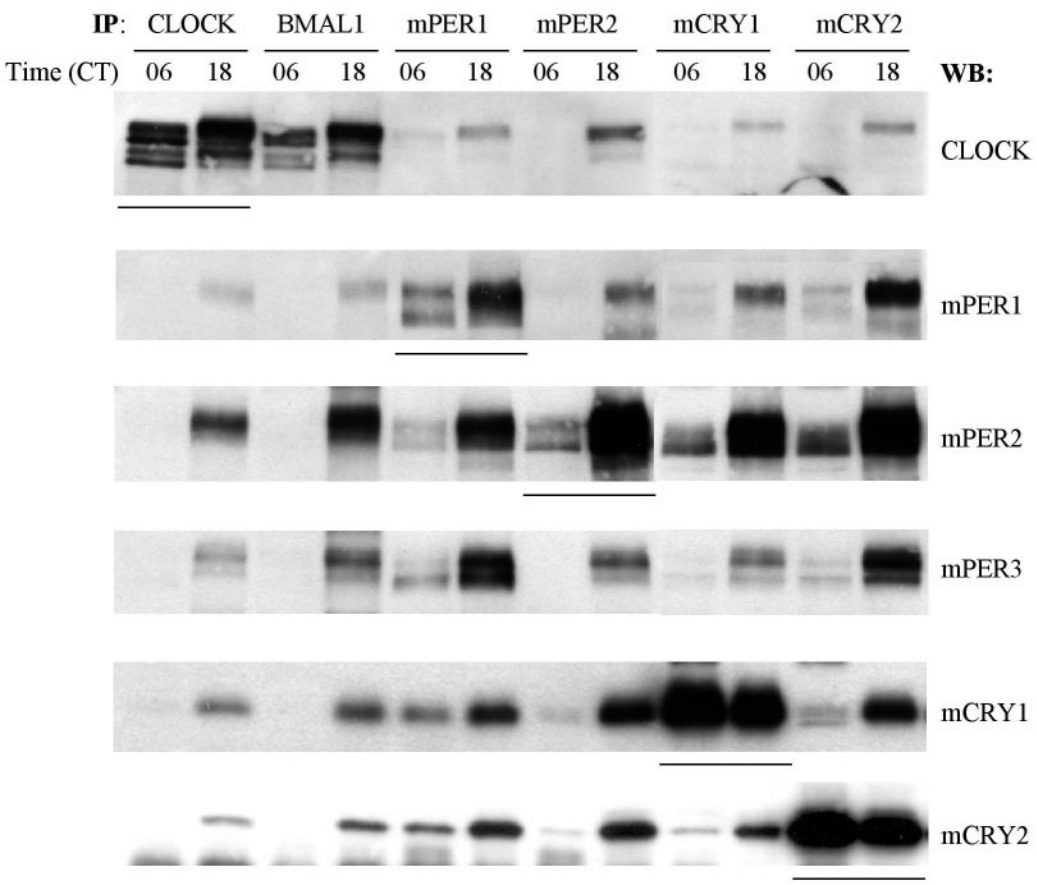


FIG. 2. mPER3 interacts with other clock proteins in a circadian manner. Liver extracts from CT06 and CT18 were immunoprecipitated with antibodies to CLOCK, BMAL1, mPER1, mPER2, mCRY1, and mCRY2. The immune complexes were Western blotted for CLOCK, mPER1, mPER2, mPER3, mCRY1, and mCRY2. Underlined lanes indicate the primary target proteins of IP.

Cell culture and transfection. NIH 3T3 cells were grown and transfected as described previously (14). Then, 6-cm dishes were used for straight WB, and 10-cm dishes were used for IP experiments.

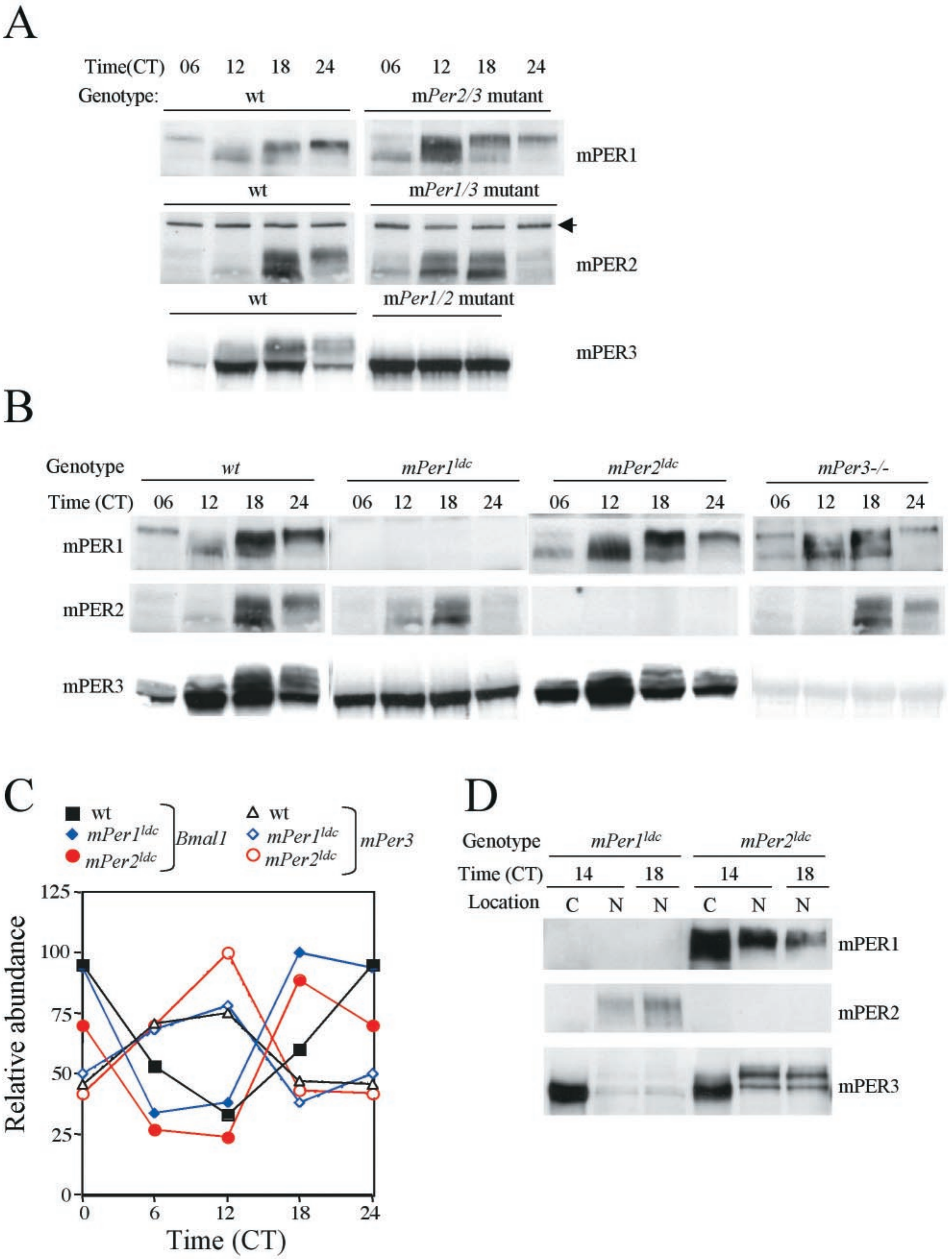
In vitro kinase assay. mPERs, chPERs, and CKIε were synthesized in vitro as described above. Each of the native and chimeric PERs was mixed with CKIε or the same volume of unprogrammed reticulocyte lysate. The molar ratio of PER to CKIε was 1:3. These mixtures were incubated in the presence of protease inhibitors (see above) on ice for 30 min. Subsequently, 100 μl of EB and 0.1 mM ATP were added, and these mixtures were further incubated at room temperature with gentle shaking. The reactions were terminated at 60 min after the addition of ATP for experiments depicted in Fig. 8E, upper panel. For results shown in Fig. 6E, lower panel, small aliquots from the mixtures were taken and mixed with 2× sample buffer at the indicated times after the addition of ATP. These samples were resolved by SDS-polyacrylamide gel electrophoresis (PAGE; 6% gels) and visualized by autoradiography.

RESULTS

Rhythmic phosphorylation and nuclear accumulation of mPER3. We first examined the posttranslational regulation of mPER3 in the livers of wild-type mice. We characterized mPER3 protein in vivo by using novel antisera raised against a fragment of mPER3. One antisera (P3-28R) recognized in vitro-translated mPER3 by WB analysis but did not cross-react with equivalent amounts of mPER1 or mPER2 (Fig. 1A). Furthermore, the antibody reacted with a band whose size ranges between 135 to 150 kDa, which is present only in liver extracts from wild-type mice, but not *mPer3*-deficient mice.

We next examined the expression profile of mPER3 at 3-h

FIG. 3. mPER3 is posttranslationally regulated by mPER1. (A) mPER profile in *mPer* double-mutant mice. Liver extracts from wild-type (wt), *mPer1/2*, *mPer1/3*, and *mPer2/3* double-mutant mice collected at indicated times were subjected to Western blot for mPER1, mPER2, and mPER3. The black arrow indicates a nonspecific band. The results from one of two independent experiments are shown. Similar results were obtained from the other experiment (data not shown). (B) mPER profile in wild-type (wt) and *mPer* single-mutant mice. Liver extracts prepared from wild-type and *mPer1*^{ldc}, *mPer2*^{ldc}, and *mPer3*-deficient mice collected at the indicated times were subjected to Western blotting for mPER1, mPER2, and mPER3. (C) *mPer3* RNA levels in *mPer* mutant mice. RNA levels were analyzed at 6-h intervals. One of two independent experiments is shown. Duplicate values varied by no more than 20% from each other. (D) mPER3 is mostly cytoplasmic in *mPer1*^{ldc} mice. Liver extracts from *mPer1*^{ldc} and *mPer2*^{ldc} mice collected at CT14 and CT18 were fractionated and analyzed as in Fig. 1D. C and N, cytoplasmic and nuclear fractions, respectively.


A
Time(CT) 06 12 18 24 06 12 18 24
Genotype: wt
mPer2/3 mutant
mPER1
wt
mPer1/3 mutant
mPER2
wt
mPer1/2 mutant
mPER3
B
Genotype
wt
mPer1ldc
mPer2ldc
mPer3-/-
Time (CT) 06 12 18 24 06 12 18 24 06 12 18 24 06 12 18 24
mPER1
mPER2
mPER3
C
■ wt
◆ mPer1ldc
● mPer2ldc
Bmal1
△ wt
◇ mPer1ldc
○ mPer2ldc
mPer3
Relative abundance
125
100
75
50
25
0
0
6
12
18
24
Time (CT)
D
Genotype
mPer1ldc
mPer2ldc
Time (CT)
14
18
14
18
Location
C N N C N N
mPER1
mPER2
mPER3

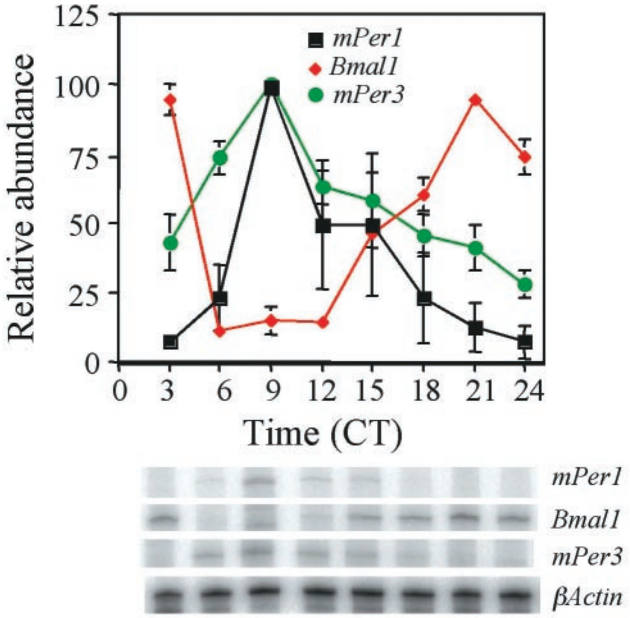


FIG. 4. Circadian oscillation of *mPer3* mRNA. *mPer1*, *mPer3*, and *Bmal1* RNA rhythms in liver were determined by RNase protection assay at 3-h intervals on the first day in constant darkness (DD). Each value was normalized to β-actin and converted to the percentage of maximal value for each gene. Mean values and standard errors from three separate experiments are shown. A representative autoradiogram is shown at the bottom of the figure.

intervals on the first day in constant darkness (DD). Like mPER1 and mPER2, mPER3 exhibited a circadian rhythm in abundance and electrophoretic mobility (Fig. 1B) (15). Rapidly migrating forms start to accumulate at circadian time 06 (CT06). Both abundance and size increase with time, peaking between CT12 and CT15. The mRNA peak of mPER3 in liver occurs at CT09, indicating a delay between mRNA peak and protein peak, as observed for the other mPERs (Fig. 4) (15). The electrophoretic mobility shift was due mainly to phosphorylation, as treatment with λPP altered the mobility of the slowly migrating forms to more rapidly migrating forms (Fig. 1C).

We showed previously that mPER1 and mPER2 translocate into the nucleus in a time-dependent manner, and hyperphosphorylated forms of mPER1 and mPER2 are detected predominantly in the nucleus (15). mPER3 also showed a striking circadian rhythm in the nuclear accumulation in liver, with hyperphosphorylated mPER3 found primarily in the nucleus (Fig. 1D).

mPER3 associates with other clock proteins in a time-dependent manner. Our previous studies showed that seven clock proteins (CLOCK, BMAL1, mPER1, mPER2, mCRY1, mCRY2, and CKIε) associate as a multimeric complex in a time-dependent manner in liver (15). To test whether mPER3 is also part of this complex, we used co-IP to examine interactions of mPER3 with other clock proteins before (CT06) and during (CT18) negative feedback. Hyperphosphorylated mPER3 was copurified along with each of the seven clock proteins examined at CT18 (Fig. 2 and data not shown).

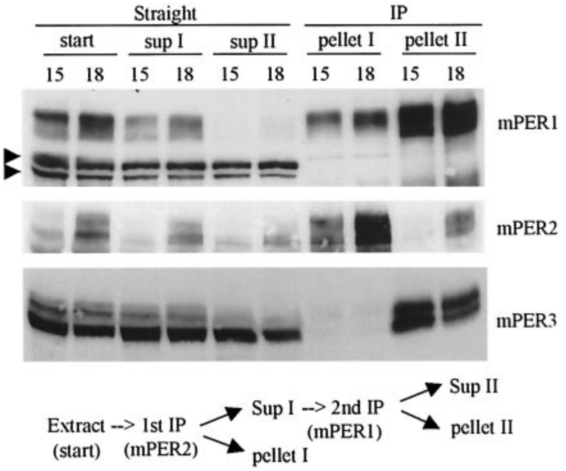


FIG. 5. The mPER1-mPER3 complex is more abundant than the mPER2-mPER3 complex. Liver extracts from CT15 and CT18 were subjected to sequential IP reactions. Antibody to mPER2 was first added, and immune complexes were separated from supernatant. The supernatant was transferred to a new tube and subjected to a second IP with anti-mPER1 antibody. Immune complexes and supernatant from both IP reactions were analyzed by WB for mPER1, mPER2, and mPER3. Two arrowheads indicate nonspecific bands that confirm equal loading of total protein. The majority of mPER3 was copurified with mPER1, indicating that mPER1 and not mPER2 is the major binding partner for mPER3.

mPER1 regulates the rhythmic posttranslational modifications of mPER3. The robust oscillations in mPER1 and mPER2 abundance and phosphorylation allow these rhythms to be used to assess the functional state of the circadian clock. Consistent with the loss of locomotor rhythmicity in *mPer1/2* double-mutant mice, the double mutants lacked rhythmic posttranslational modification of mPER3 (Fig. 3A). In *mPer1/3* and *mPer2/3* double-mutant mice, in which molecular and behavioral rhythmicity persist for several days in DD (3), the remaining PER gene product (mPER2 and mPER1, respectively) underwent rhythmic changes in abundance and phosphorylation, as in wild-type mice, except the rhythm phases appeared advanced compared to those in wild-type mice (Fig. 3A). Thus, either mPER1 or mPER2 is sufficient for its own circadian changes in phosphorylation and abundance, whereas mPER3 is not.

The lack of rhythmic posttranslational modifications of mPER3 in *mPer1*/*mPer2* double-mutant mice could be secondary to a broken circadian clock; that is, the posttranslational modification of mPER3 could simply be dependent upon a functional circadian clock. Alternatively, there may be a specific dependence of mPER3 on one of the other two mPER proteins for its posttranslational regulation. To dissect these possibilities, we examined the posttranslational regulation of mPER proteins in homozygous single-mutant mice. In *mPer2*^{ldc} or *mPer3*-deficient mice, mPER1 exhibited robust oscillations in phosphorylation and abundance, as in wild-type mice (Fig. 3B). Similarly, circadian rhythms of mPER2 phosphorylation and abundance were not significantly altered in *mPer1*^{ldc} or *mPer3*-deficient mice (Fig. 3B). mPER3 rhythms also appeared normal in *mPer2*^{ldc} mice (Fig. 3B).

Remarkably, however, mPER3 did not show temporal

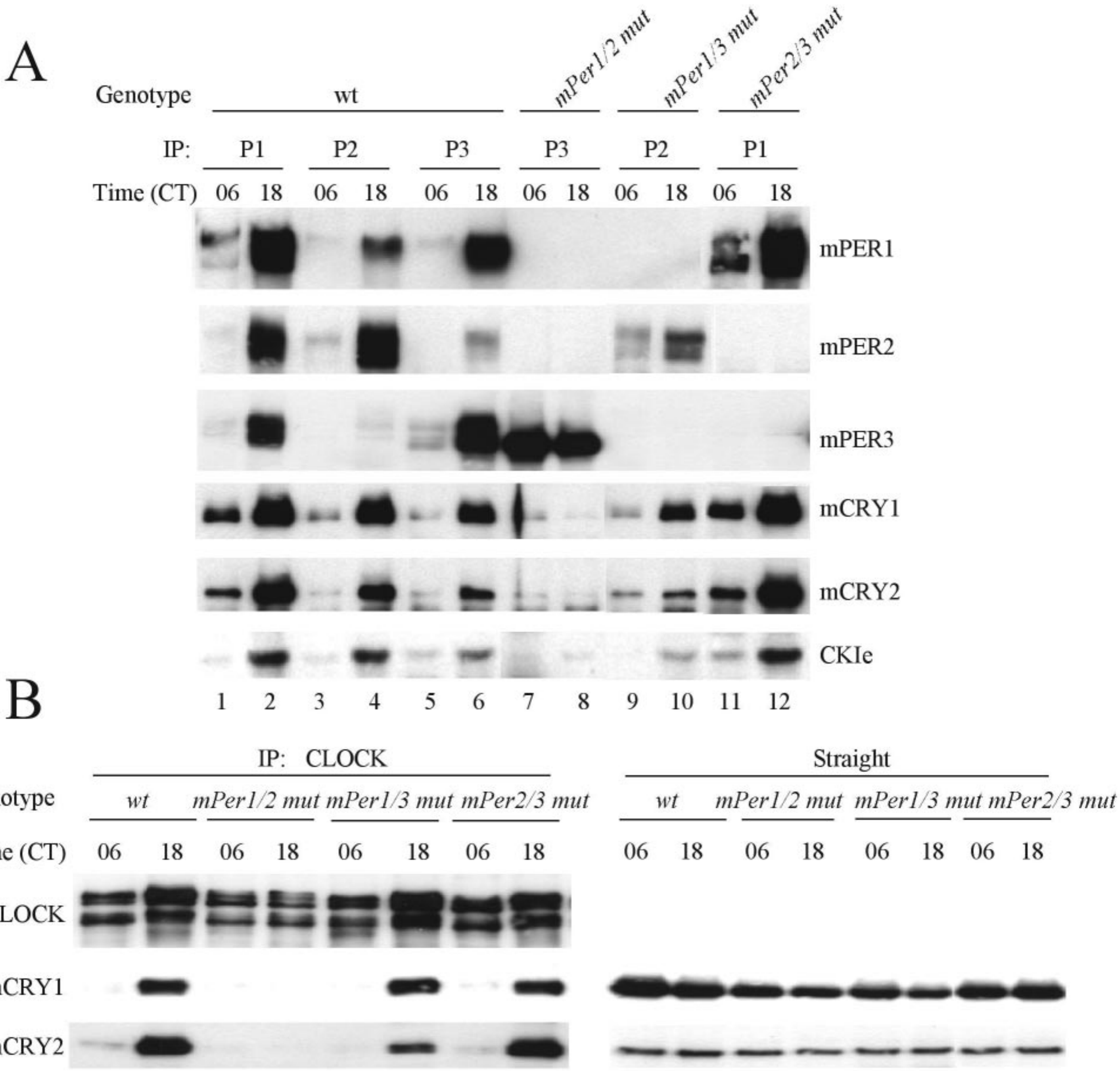
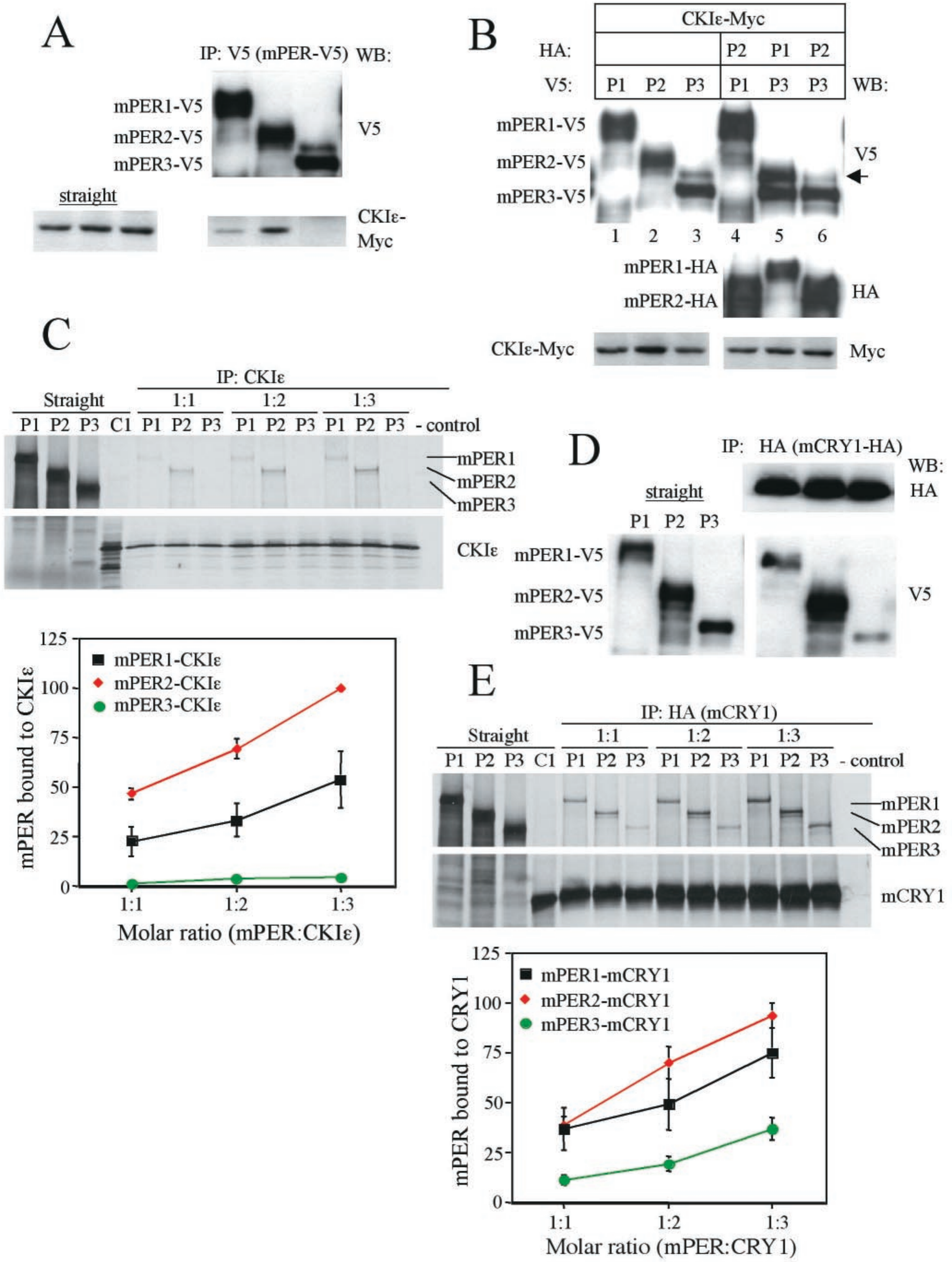

A
Genotype
wt
mPer1/2 mut
mPer1/3 mut
mPer2/3 mut
IP:
P1
P2
P3
P3
P2
P1
Time (CT)
06 18 06 18 06 18 06 18 06 18 06 18
mPER1
mPER2
mPER3
mCRY1
mCRY2
CKIe
1 2 3 4 5 6 7 8 9 10 11 12
B
IP: CLOCK
Straight
Genotype
wt
mPer1/2 mut
mPer1/3 mut
mPer2/3 mut
Time (CT)
06 18 06 18 06 18 06 18
CLOCK
mCRY1
mCRY2

FIG. 6. mPER3 cannot participate in rhythmic assembly of clock protein complexes. (A) mPER complexes in wild-type (wt) and *mPer* double-mutant mice. Liver extracts from wild-type and *mPer* double-mutant mice collected at CT06 and CT18 were immunoprecipitated with antibodies to mPER1, mPER2, and mPER3 (P1, P2, or P3). The immune complexes were Western blotted for mPERs, mCRYs, and CKIε. (B) Interaction between CLOCK-BMAL1 and mCRY is abolished in *mPer1/2* double-mutant mice. Liver extracts from wild-type (wt) and *mPer* double-mutant mice collected at CT06 and CT18 were subjected to IP with anti-CLOCK antibody. The immune complexes were Western blotted for CLOCK, mCRY1, and mCRY2 (left panels). Straight liver extracts were Western blotted for mCRY1 and mCRY2 (right panels) to show that mCRYs are readily detectable in mice of each genotype.

changes in phosphorylation and abundance in *mPer1*^{ldc} mutant mice (Fig. 3B). Instead, mPER3 abundance was constant, and the protein remained hypophosphorylated across the circadian cycle in the absence of mPER1. The loss of rhythmicity in mPER3 was not due to altered transcriptional regulation of *mPer3* because *mPer3* mRNA levels manifested robust oscillations in *mPer1*^{ldc} and *mPer2*^{ldc} mutant mice, as in wild-type mice (Fig. 3C). Examination of the subcellular localization of mPER3 revealed that the protein was trapped in the cytoplasm in the absence of mPER1 (Fig. 3D). Immunodepletion experiments of wild-type livers confirmed that the majority of hyperphosphorylated mPER3 is complexed with mPER1 (Fig. 5).

mPER3 cannot participate in the rhythmic assembly of clock protein complexes. We next examined the ability of mPER3 to direct the rhythmic assembly of non-PER clock proteins by using the *mPer* double-mutant mice. As previously indicated (Fig. 2), mPER proteins associated with mCRY1, mCRY2, and CKIε more at CT18 than at CT06 in wild-type mice (Fig. 6A, lanes 1 to 6). The same interaction pattern was maintained in *mPer1/3* and *mPer2/3* double-mutant mice; as-


A
IP: V5 (mPER-V5)
WB:
mPER1-V5
mPER2-V5
mPER3-V5
V5
straight
CKIε-Myc
B
CKIε-Myc
HA:
P2 P1 P2
V5:
P1 P2 P3 P1 P3 P3
WB:
mPER1-V5
mPER2-V5
mPER3-V5
V5
1 2 3 4 5 6
mPER1-HA
mPER2-HA
HA
CKIε-Myc
Myc
C
IP: CKIε
Straight
1:1
1:2
1:3
P1 P2 P3 C1 P1 P2 P3 P1 P2 P3 P1 P2 P3 - control
mPER1
mPER2
mPER3
CKIε
mPER1-CKIε
mPER2-CKIε
mPER3-CKIε
mPER bound to CKIε
125
100
75
50
25
0
1:1
1:2
1:3
Molar ratio (mPER:CKIε)
D
IP: HA (mCRY1-HA)
WB:
HA
straight
P1 P2 P3
mPER1-V5
mPER2-V5
mPER3-V5
V5
E
IP: HA (mCRY1)
Straight
1:1
1:2
1:3
P1 P2 P3 C1 P1 P2 P3 P1 P2 P3 P1 P2 P3 - control
mPER1
mPER2
mPER3
mCRY1
mPER1-mCRY1
mPER2-mCRY1
mPER3-mCRY1
mPER bound to CRY1
125
100
75
50
25
0
1:1
1:2
1:3
Molar ratio (mPER:CRY1)

sociations between the single remaining mPER member and mCRY1, mCRY2, and CKIε were most apparent at CT18 (Fig.6A, lanes 9 to 12). However, the interactions between mPER3 and mCRY1, mCRY2, and kinase in *mPer1/2* double-mutant mice were greatly reduced and did not appear to be rhythmic (Fig. 6A, lanes 7 and 8), suggesting that mPER3 association with these clock proteins requires mPER1 and/or mPER2. There appeared to be a decrease in mPER2 abundance in the absence of mPER1 in some experiments (Fig. 3D and 6A), but this was not a consistent finding (Fig. 3A and B).

We also examined whether the remaining mPER protein in the double-mutant mice can induce rhythmic interactions between the major transcriptional inhibitors (mCRY1 and mCRY2) and the transcriptional activators (CLOCK-BMAL1). As expected, the interactions between CLOCK and the mCRY proteins were much greater at CT18 than at CT06 in wild-type mice (Fig. 6B). The same rhythmic interactions were maintained in *mPer1/3* and *mPer 2/3* double-mutant mice, but these interactions were not detectable at either time in the *mPer1/2* double-mutant mice (Fig. 6B). The absence of these interactions is not due to low levels of the mCRY proteins in the mutants, since the mCRYs were readily detectable in the *mPer1/2* double-mutant mice and in the other mutant and wild-type mice (Fig. 6B, right panels).

Taken together, our data indicate that either mPER1 or mPER2 alone is sufficient for rhythmic posttranslational regulation of clock proteins and maintaining a functioning clock for a few circadian cycles. In contrast, mPER3 is not sufficient to support rhythmic molecular oscillations for a functioning circadian clock, implying that mPER3 is mechanistically different from mPER1 and mPER2 in clock-relevant functions.

In vitro binding assays reveal a lack of stable interaction between mPER3 and CKIε. Our previous studies suggested that the interactions between mPER proteins and other clock proteins in the cytoplasm trigger nuclear translocation of mPER-containing complexes and subsequent inhibition of CLOCK-BMAL1 transcription (15). Therefore, it is possible that the inability of mPER3 to sustain the circadian clock results from the lack of interactions with CKIε and/or the mCRY proteins in the cytoplasm. We thus examined affinities of each mPER protein for CKIε and the mCRY proteins.

First, the physical interaction between each mPER protein and CKIε were examined in transiently transfected cells. When the kinase and each mPER protein were coexpressed in NIH 3T3 cells, CKIε was able to coprecipitate with mPER1 and mPER2, but not mPER3, a finding consistent with studies reported by Akashi et al. (Fig. 7A). CKIε-induced phosphorylation of mPER3 was enhanced when mPER1, but not mPER2, was coexpressed in NIH 3T3 cells (Fig. 7B, lane 5 versus lane 6). In agreement with our in vivo studies (Fig. 3), the data suggest that mPER3 requires mPER1 for stable interaction with CKIε and efficient phosphorylation.

To determine the affinities of the mPER proteins for CKIε quantitatively, we performed binding assays with in vitro translated proteins. Each of the mPER proteins and kinase were mixed at various molar ratios and then subjected to IP with an anti-CKIε antibody. Subsequently, the affinity of the mPER for CKIε was measured by quantifying the amount of copurified mPER.

The amounts of mPER2 bound to CKIε were about twofold higher than those of mPER1 (Fig. 7C). Consistent with the results in Fig. 7A, we could not detect stable interaction between mPER3 and CKIε despite numerous attempts (Fig. 7C). These data demonstrate that mPER3 is intrinsically defective in its ability to bind to CKIε.

A set of similar experiments was conducted to examine interactions between the mPER and mCRY proteins. As previously reported (14), all three mPER proteins associated with mCRY1 in transiently transfected NIH 3T3 cells (Fig. 7D). The relative binding affinity of mPER2 for mCRY1 appeared to be greater than that of mPER1 or mPER3. Similar results were obtained when mCRY2 was cotransfected with each of the mPER proteins (data not shown). The affinities of the mPER proteins for mCRY1 were quantitatively determined with in vitro binding assays (Fig. 7E). The rank order of mPER affinity for mCRY1 was mPER2 > mPER1 > mPER3. At all molar ratios, the amounts of mPER3 bound to mCRY1 were two- to threefold less than those of mPER2.

Although the affinity of mPER3 for mCRY1 is lower than that of mPER1 or mPER2, the interactions between mPER3 and the mCRY proteins are readily observed in cultured cells (Fig. 7D) (14). Thus, mPER3 is not intrinsically defective in binding to the mCRY proteins. Furthermore, mPER3 is more abundant than mPER1 in liver extracts, since immunodepletion of mPER1 removed less than 50% of mPER3 from liver extracts (Fig. 5 and data not shown). Higher levels of expression would mitigate the impact of a slightly reduced affinity. We therefore conclude that the inability of mPER3 to maintain molecular or behavioral rhythms is due primarily to a deficiency in interaction with CKIε. The reduced affinity of

FIG. 7. Quantitative difference of mPER protein interactions with CKIε and mCRY1. (A) mPER3 does not stably associate with CKIε in NIH 3T3 cells. NIH 3T3 cells were transfected with CKIε-Myc and mPER1-V5, mPER2-V5, or mPER3-V5. The cell lysates were subjected to IP with anti-V5 antibody and the immune complexes were Western blotted with anti-V5 and Myc antibodies. CKIε-Myc was comparably expressed in the three cell lysates (left panel). (B) CKIε-induced phosphorylation of mPER3 is enhanced by mPER1 but not by mPER2. CKIε-Myc and various combinations of mPER were expressed in NIH 3T3 cells. The cell lysates were subjected to Western blotting with anti-V5, HA, and Myc antibodies. The arrow indicates hyperphosphorylated mPER3. (C) Binding of mPER with CKIε. mPERs and CKIε were synthesized in vitro in the presence of L-[^{35}S]methionine. Each of the mPER proteins and CKIε were mixed at a molar ratio of 1:1, 1:2, or 1:3. The mixtures were subjected to IP with CKIε antibody. The immune complexes were resolved by SDS-PAGE and visualized by autoradiography or quantified by Fuji phosphorimager. All IP reactions recovered more than 95% of input primary antigens (data not shown). A representative autoradiogram is shown (upper panel). Coimmunoprecipitated mPER was quantified from three independent experiments. Each value was converted to the percentage of the maximal value of the experiment. Mean values with standard errors are shown (lower panel). (D) NIH 3T3 cells were transfected with mCRY1-HA and mPER1-V5, mPER2-V5, or mPER3-V5. The cell lysates were subjected to IP with anti-HA antibody, and the immune complexes were Western blotted with anti-HA and V5 antibodies. The left panel shows the amounts of mPER proteins present in the three cell lysates. (E) Experiments with mPER proteins and mCRY1 were performed as in panel C to determine mPER affinity for mCRY1.

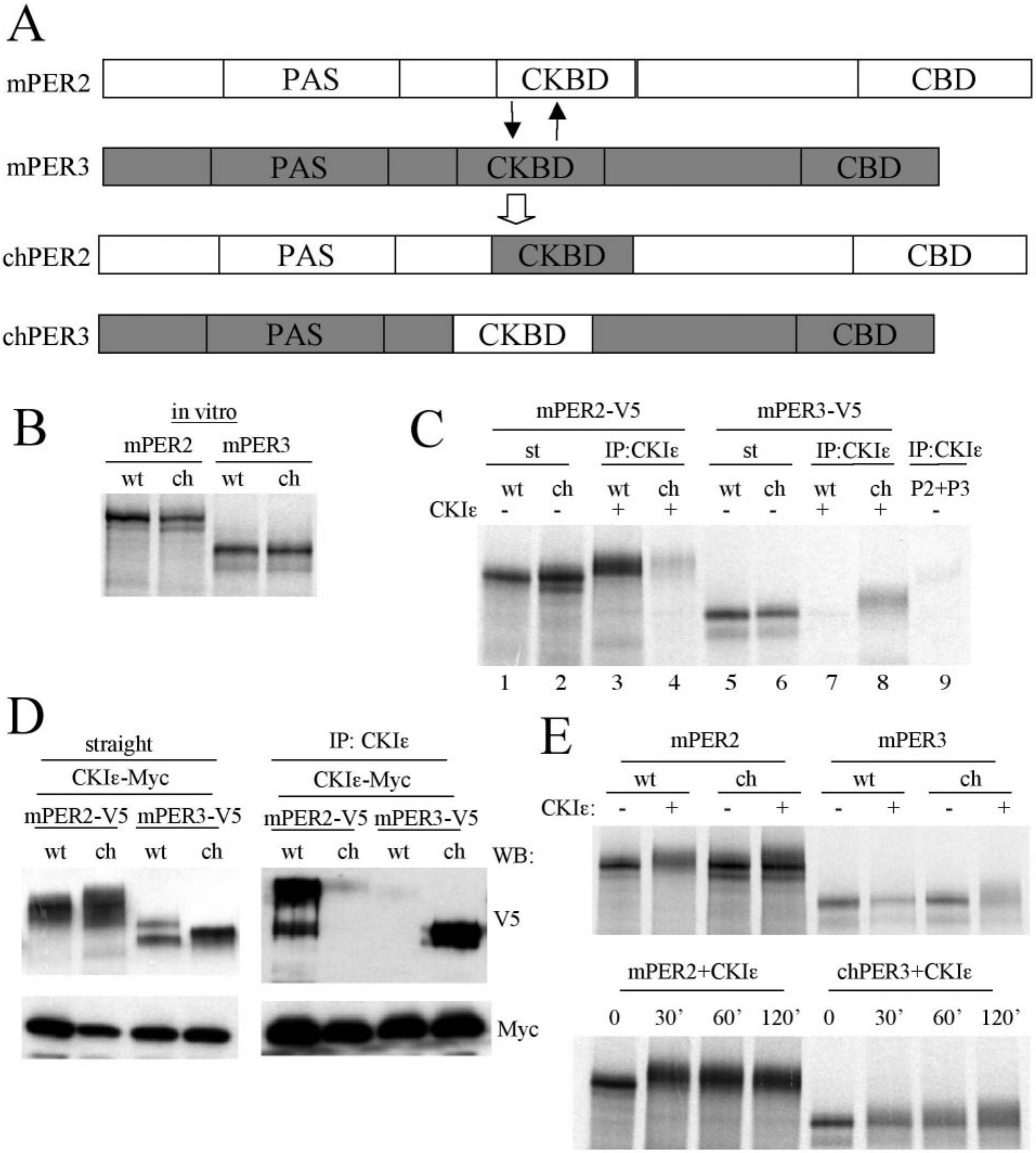

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FIG. 8. CKBD of mPER is important for interaction with CKIε and efficient phosphorylation. (A) Diagram of CKBD swapping between mPER2 and mPER3. Amino acids 510 to 709 of mPER3 were exchanged for amino acids 551 to 750 of mPER2. (B) The apparent sizes of chPERs are the same as their wild-type counterparts. mPER2, mPER3, chPER2, and chPER3 were synthesized in vitro in the presence of L-[^{35}S]methionine, and they were resolved by SDS-PAGE. (C) chPER3 binds to CKIε more efficiently than mPER3, whereas chPER2 binds to CKIε much less efficiently than mPER2. The PERs and CKIε were synthesized in vitro in the presence of L-[^{35}S]methionine. Each of the PERs was mixed with CKIε at a molar ratio of 1:3. The mixtures were subjected to IP with CKIε antibody, and the immune complexes were resolved by SDS-PAGE and visualized by autoradiography. CKIε antibody immunoprecipitated >95% of input CKIε in all cases (data not shown). When CKIε was absent from the mixtures, mPER2 and mPER3 were not significantly immunoprecipitated by anti-CKIε antibody (last lane). Slow-migrating forms were visible for copurified PER proteins (lanes 3, 4, and 8) compared to input PERs (lanes 1, 2, and 6). (D) Results similar to those in panel C were obtained with transiently expressed proteins in NIH 3T3 cells. Native and chimeric PERs were coexpressed with CKIε in NIH 3T3 cells. The cell lysates were subjected to IP with anti-CKIε antibody, and the immune complexes were Western blotted with anti-V5 and Myc antibodies (right panel). The left panel indicates the amounts of mPERs and CKIε present in the cell lysates. (E) Phosphorylation of chPER3 is enhanced, but that of chPER2 is attenuated compared to their wild-type counterparts in vitro. In vitro-translated PER proteins were incubated with or without CKIε for 60 min. at room temperature (upper panel) or incubated with CKIε for the indicated times (lower panel). The mixtures were then resolved by SDS-PAGE and visualized by autoradiography. Representative autoradiograms are shown.

mPER3 for the mCRY proteins may also contribute, but to a lesser degree.

The CKBD of mPER is essential for kinase interaction and phosphorylation. If the direct association of mPER with CKIε is essential for clock-relevant functions of mPER, exchanging the CKBDs between mPER2 and mPER3 should cause predictable alterations in chimeric protein function. We thus generated the chimeric chPER2 and chPER3 proteins containing each other's CKBD (Fig. 8A). The apparent sizes of chPER2 and chPER3 were similar to their wild-type counterparts (Fig. 8B).

To determine whether the CKBD alters the functions of the hybrid proteins, we performed in vitro binding assays between the chimeric proteins and CKIε. As expected, the amount of chPER3 bound to CKIε greatly increased compared to that of native mPER3 (Fig. 8C). In contrast, the binding of chPER2 to CKIε was barely detectable. When the interactions were examined in transiently transfected NIH 3T3 cells, mPER2 and chPER3 were copurified with CKIε, whereas mPER3 and chPER2 were not (Fig. 8D). These data indicate that the CKBD determines the binding affinity of mPER proteins for CKIε. Even although mPER3 and chPER2 did not appreciably bind CKIε in cell culture, the proteins were highly phosphorylated, suggesting endogenous kinase activity not involving CKIε.

Next, we examined the efficiency of chPER phosphorylation by CKIε. Wild-type and chimeric proteins and CKIε were synthesized in vitro, and each of the PER proteins was mixed with CKIε to compare phosphorylation efficiency of the chimeric and native mPERs by CKIε in the absence of other interacting proteins. mPER2 and chPER3 were more readily phosphorylated by CKIε than were their counterparts, chPER2 and mPER3, respectively (Fig. 8E).

Taken together, these data suggest that the CKBD of mPERs not only determines kinase binding affinity but also phosphorylation efficiency. Since it has been suggested that mPER phosphorylation plays important roles in regulating interactions with other clock proteins, nuclear translocation, and degradation, our data suggest that the mPER CKBD is essential for clock-relevant functions of mPER1 and mPER2.

DISCUSSION

As in other organisms, a negative feedback loop constitutes the backbone of the molecular circadian clock in mammals (18). The mPER-mCRY complex plays an essential role in the negative feedback loop by exerting inhibitory activity on CLOCK-BMAL1-driven transcription in a time-dependent manner (14, 15). Although mCRYs play a major role in the inhibition, the timing of this inhibition seems to be regulated by mPERs (15). The present study suggests that the CKBD of an mPER protein dictates its clock-relevant functions.

Although *mPer3* has been placed outside of the core feedback loop based on previous genetic studies (3, 20), we show here that mPER3 is in fact part of the transcriptional inhibitory complex and is regulated in a manner similar to the regulation of mPER1 and mPER2. Like mPER1 and mPER2, mPER3 is rhythmically phosphorylated and translocated into the nucleus, and it interacts rhythmically with other clock proteins. Among clock proteins, mPER3 is most strongly associated with mPER1. In addition, in the absence of mPER1 (*mPer1*^{ldc} and *mPer1/2* double-mutant mice) mPER3 phosphorylation is abolished. Thus, mPER3 is regulated by mPER1 at a posttranslational level. Consistent with these observations, phosphorylation of mPER3 by CKIε is enhanced in the presence of mPER1 in NIH 3T3 cells (Fig. 7B), and the nuclear translocation of mPER3 is promoted by mPER1 in NIH 3T3 cells (14). The presence of mPER3 in circadian clock protein complexes is likely mediated by interaction with mPER1.

mPER1 and mPER2 in *mPer2/3* and *mPer1/3* double-mutant mice, respectively, show apparently normal phosphorylation, interaction with other clock proteins, and nuclear translocation, suggesting that the single remaining mPER can support the molecular clock. The levels of the remaining mPER protein in the mutant mice were comparable to those of the mPER proteins in wild-type mice, suggesting that circadian changes of mPER abundance in liver were not significantly altered by disruption of *mPer1/3* or *mPer2/3* genes.

Unlike mPER1 or mPER2, mPER3 alone cannot support the molecular clock even for one cycle. mPER3 in *mPer1/2* double mutant mice is apparently not phosphorylated and interacts very weakly with other clock proteins. In addition, mPER3 in the double-mutant mice is always cytoplasmic. Our data suggest that the lack of physical association of mPER3 with CKIε might explain the functional inadequacy of mPER3 in terms of supporting the circadian clock. In fact, CKIε has been implicated in the nuclear translocation of the mPER proteins (1, 22).

Our results suggest that stable interactions between CKIε and mPERs are important for effective phosphorylation and likely lie upstream of nuclear translocation of mPERs. If mPER3 alone cannot support the circadian clock due to the lack of stable interaction with CKIε, then replacement of CKBD of mPER3 with that of mPER2 should rescue the inability of mPER3 to support the clock. Our study indeed shows that chPER3 physically interacts with CKIε and is efficiently phosphorylated in vitro. Taken together, our data strongly suggest that the CKBD of mPERs is essential for stable interaction with CKIε, effective phosphorylation by CKIε, and possibly other clock-relevant functions, such as nuclear translocation. The importance of the CKBD for a functioning circadian clock is supported by a human genetic study showing that a mutation in the CKBD of hPER2 is associated with a severe sleeping disorder known as familial advanced sleep phase syndrome (23).

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