Involvement of Stress Kinase Mitogen-activated Protein Kinase Kinase 7 in Regulation of Mammalian Circadian Clock*§

The stress kinase mitogen-activated protein kinase kinase 7 (MKK7) is a specific activator of c-Jun N-terminal kinase (JNK), which controls various physiological processes, such as cell proliferation, apoptosis, differentiation, and migration. Here we show that genetic inactivation of MKK7 resulted in an extended period of oscillation in circadian gene expression in mouse embryonic fibroblasts. Exogenous expression in cultured mammalian cells of an MKK7-JNK fusion protein that functions as a constitutively active form of JNK induced phosphorylation of PER2, an essential circadian component. Furthermore, JNK interacted with PER2 at both the exogenous and endogenous levels, and MKK7-mediated JNK activation increased the half-life of PER2 protein by inhibiting its ubiquitination. Notably, the PER2 protein stabilization induced by MKK7-JNK fusion protein reduced the degradation of PER2 induced by casein kinase 1ε. Taken together, our results support a novel function for the stress kinase MKK7 as a regulator of the circadian clock in mammalian cells at steady state.

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§ This article contains supplemental Figs. 1-4.

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3 The abbreviations used are: MKK, mitogen-activated protein kinase kinase; CHX, cycloheximide; CRE, Cre recombinase; Dex, dexamethasone; DKO, double knockout; ES, embryonic stem; KN, kinase negative; MEF, mouse embryonic fibroblast; NLS, nuclear localization signal; Ab, antibody; WB, Western blot; pTPr, phosphorylated threonine-proline.

**Background:** MKK7 is a kinase involved in the cellular stress response.

**Results:** MKK7 regulates circadian gene expression and the stability of an essential circadian component in unstressed mammalian cells.

**Conclusion:** MKK7 functions as a circadian clock regulator.

**Significance:** Our identification of role of MKK7 in the circadian clock provides insight into the importance of stress-responsive molecules in the maintenance of cellular homeostasis.

The stress kinase mitogen-activated protein kinase kinase 7 (MKK7) is a specific activator of c-Jun N-terminal kinase (JNK), which controls various physiological processes, such as cell proliferation, apoptosis, differentiation, and migration. Here we show that genetic inactivation of MKK7 resulted in an extended period of oscillation in circadian gene expression in mouse embryonic fibroblasts. Exogenous expression in cultured mammalian cells of an MKK7-JNK fusion protein that functions as a constitutively active form of JNK induced phosphorylation of PER2, an essential circadian component. Furthermore, JNK interacted with PER2 at both the exogenous and endogenous levels, and MKK7-mediated JNK activation increased the half-life of PER2 protein by inhibiting its ubiquitination. Notably, the PER2 protein stabilization induced by MKK7-JNK fusion protein reduced the degradation of PER2 induced by casein kinase 1ε. Taken together, our results support a novel function for the stress kinase MKK7 as a regulator of the circadian clock in mammalian cells at steady state.

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been translated, they form heterodimers that can then translocate to the nucleus to repress CLOCK (NPAS2)-BMAL1-mediated transcription through direct protein–protein interaction. These interactions then set up the rhythmic oscillations of gene expression that drive the circadian clock.

The functions of various clock proteins, including CLOCK, BMAL1, PER1, PER2, PER3, CRY1, and CRY2, are regulated via phosphorylation by various enzymes, including casein kinase-1 ε (CK1ε), CK1δ, glycogen synthase kinase-3β (GSK3β), and casein kinase-2 (CK2) (12–14). Genetic studies have revealed the important role this phosphorylation plays in mammalian clock function (12, 13). For example, in the Syrian hamster, the tau mutation causing a short period phenotype affects the gene encoding CK1ε. CK1ε was subsequently demonstrated to be phosphorylated by CK1ε in vitro than is wild type (WT) PER2 (16). At the molecular level, CK1ε-mediated phosphorylation has been shown to decrease the stability of PER2 protein by promoting its ubiquitination (17, 18). Notably, changes in PER2 stability have been linked to changes in the period length of circadian rhythms (19, 20).

In this study, we present evidence that PER2 may also be regulated by MKK7-JNK-mediated phosphorylation, establishing a role for the stress kinase MKK7 in controlling the mammalian circadian clock. Importantly, we demonstrate that the MKK7-JNK signaling pathway has an effect opposite to that of CK1ε-induced PER2 destabilization. Thus, MKK7-JNK signaling may provide a balancing influence on clock protein functions that helps to maintain the normal periodicity of the circadian clock machinery.

EXPERIMENTAL PROCEDURES

Plasmids, Reagents, Cells, and Transfection—An EcoRI fragment of full-length nuclear localization signal-fused CRE recombinase (NLS-CRE) (4) was inserted in the corresponding site of pCLNCX (IMGENEX, San Diego, CA) retrovirus vector, generating NLS-CRE-pCLNCX. A HindIII-EcoRI fragment of the gene encoding CK1ε (Invitrogen) containing 15% FBS, 0.1% 2-mercaptoethanol (Sigma), and leukemia inhibitory factor (propagation medium). Cultured cells were transfected with FuGene (Roche Applied Science) according to the manufacturer’s protocol. SP600125 and calyculin A were purchased from Calbiochem and Wako Pure Chemical Industries, respectively.

Generation of Stable Reporter Cell Lines and Monitoring of Real-time Luciferase Activity—To obtain lines of MKK7flox/flox MEFs stably expressing firefly luciferase from a 1.8-kb Per2 promoter fragment, MKK7flox/flox MEFs were co-transfected with linearized mouse Per2 promoter-pGL3basic (7 μg) and pcDNA3.1 (1 μg) vectors. After 1 day in standard culture, 0.5 mg/ml G418 was added, and cultures were selected for 2 weeks. Colonies were picked, and their real-time luciferase activities were determined using a Kronos system (ATTO). Two cell lines that showed robust circadian patterns of luciferase activity were selected for further experimentation.

Antibodies—Antibodies (Abs) recognizing the following proteins were used in this study: Myc (9E10), JNK (sc-571), JNK (sc-137018), CLOCK, and actin (all from Santa Cruz Biotechnology, Inc.); ERK, c-Jun, phosphorylated c-Jun, and phosphorylated JNK (Cell Signaling); CK1ε (Abcam); mouse PER2 (Alpha Diagnostic International Inc.); HA (Immunology Consultants Laboratory); and FLAG (Sigma). All other Abs have been described elsewhere (22, 23).

Co-immunoprecipitation—Co-immunoprecipitation assays were performed as described previously with some modifications (22). 293T cells or MEFs were washed twice with phosphate-buffered saline (PBS) and homogenized in binding buffer (150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM EGTA, 5% glycerol, and 20 mM Tris-HCl, pH 7.4) containing protease inhibitor mixture tablets. Extracts were clarified by centrifugation for 10 min at 15,000 × g, and supernatants were incubated with 15 μl of protein G-agarose beads (GE Healthcare) for 1 h at 4 °C. The bead mixture was centrifuged, and the supernatant was incubated for 12 h at 4 °C with the Abs described in each figure legend plus 20 μl of protein G-agarose beads. The beads were washed three times with binding buffer, boiled in SDS sample buffer, and centrifuged. The supernatant was fractionated by SDS-PAGE and analyzed by Western blotting, as described below.

Western Blotting—Immunoprecipitated materials and total cell extracts obtained as described above were fractionated by SDS-PAGE and transferred electrophoretically onto polyvinylidene difluoride membranes. Membranes were blocked with 2 or 5% nonfat milk and incubated for 10 h at 4 °C with the Abs indicated in each figure legend. The blots were incubated with the appropriate secondary Ab plus peroxidase-conjugated antimouse or anti-rabbit IgG Ab (Santa Cruz Biotechnology, Inc.) and developed with the ECL Western blotting detection system (Amersham Biosciences).

Ubiquitination Assay—Myc-PER2 was co-expressed with HA-tagged ubiquitin in 293T cells. The cells were lysed in 1% SDS buffer (1% SDS in TBS), boiled for 10 min, and diluted 10-fold in incubation buffer (1% Triton X-100 in TBS) prior to immunoprecipitation with anti-Myc.

Retroviral Transduction—Retroviruses used in this study were produced using the RetroMax expression system (IMGENEX) according to the manufacturer’s instructions.
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NLS-CRE was expressed in Mkk7floxflox MEFs using the pCLNCX-NLS-CRE vector, with pMD.G/vsv-g as the enveloping vector. The high infection efficiency (95–100%) of this system was confirmed by infecting MEFs with pCLNCX vector expressing GFP (data not shown).

RESULTS

Genetic Inactivation of Mkk7 Gene Alters Circadian Gene Expression—To investigate whether MKK7 was involved in circadian regulation, we examined cultured cells under conditions that provide a good model of the cell-autonomous circadian oscillation that occurs in mammalian peripheral tissues (24). When cultured human and mouse cells are treated with dexamethasone (Dex), a rhythm of circadian gene expression is entrained (24). To visualize circadian rhythms in cultured MEFs, we used previously generated mice carrying a conditional Mkk7 allele (Mkk7floxflox mice) (25, 26) to establish cultures of mutant MEFs (Mkk7floxflox MEFs) in which MKK7 could be genetically inactivated by expression of NLS-CRE (Fig. 1A, left). We therefore generated two independent lines of Mkk7floxflox MEFs that stably expressed firefly luciferase from a 1.8-kb DNA fragment containing the mouse Per2 promoter (Per2-Luc/Mkk7floxflox line 1, Per2-Luc/Mkk7floxflox line 2). Retroviral transduction of NLS-CRE resulted in genetic inactivation of MKK7 in both cell lines (Fig. 1A, middle and right). When we treated these cells with Dex and monitored real-time Per2 promoter-driven luciferase bioluminescence, we found that NLS-CRE-mediated inactivation of MKK7 significantly lengthened the circadian period of bioluminescence in both Per2-Luc/Mkk7floxflox line 1 and Per2-Luc/Mkk7floxflox line 2 MEFs (Fig. 1, B and C, and supplemental Fig. 1, A and B). Thus, MKK7 influences circadian gene expression in unstressed cells.

Because MKK7 is a specific activator of JNK (1, 2), we speculated that the altered circadian periodicity we observed following MKK7 inactivation might be associated with impaired JNK function. We therefore examined the effect of MKK7 inactivation on JNK phosphorylation. Previous reports have established that, even in the absence of external stress, cultured cells exhibit a low level of JNK phosphorylation (5, 6), a result we confirmed in our Mkk7floxflox MEFs (Fig. 1D, left). When we treated Mkk7floxflox MEFs with the phosphatase inhibitor calyculin A, JNK phosphorylation was markedly increased. However, when MKK7 was genetically deleted, JNK phosphorylation was significantly reduced both in untreated and calyculin A-treated MEFs (Fig. 1D, right), showing that the stress-independent JNK activation observed in cultured cells depends on the kinase activity of MKK7. We then examined the effect of the kinase inhibitor SP600125 (which blocks JNK activity) on circadian gene expression and found that SP600125 treatment lengthened the circadian period of bioluminescence in both Per2-Luc/Mkk7floxflox line 1 and 2 MEFs (supplemental Fig. 2). Importantly, the effects of SP600125 on circadian gene expression were strikingly similar to those of MKK7 genetic inactivation (Fig. 1, B and C). In addition, our results are consistent with previous studies showing that SP600125 extends the period of circadian transcription in cultured cells and in ex vivo organ culture systems (6, 27). Thus, our findings provide evidence that the MKK7-JNK signaling pathway is involved in the circadian regulation of gene expression.

MKK7-JNK Fusion Protein Induces PER2 Phosphorylation—To identify the MKK7-JNK target(s) involved in regulating the mammalian circadian clock, we overexpressed an MKK7-JNK (WT) fusion protein in 293T or HeLa cells and performed Western blotting (WB). It has been previously demonstrated that expression of this MKK7-JNK (WT) fusion protein, but not of a kinase-negative (KN) version (MKK7-JNK [KN]), results in trans-phosphorylation of JNK by the fused MKK7 and a subsequent marked increase in JNK catalytic activity (21). Consistent with these results, we found that phosphorylation of endogenous c-Jun, a well known JNK target (1), was strongly induced...
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A particular threonine-proline motif is a consensus site for JNK phosphorylation (28). We overexpressed Myc-PER2 or Myc-BMAL1 in the presence or absence of MKK7-JNK (WT) in cultured cells and examined whether these proteins were phosphorylated on this motif. We immunoprecipitated Myc-tagged clock proteins with anti-Myc Ab followed by WB analysis using an Ab specifically recognizing proteins bearing the phosphorylated threonine-proline (pTP) motif. We found that PER2 was modestly phosphorylated on its threonine-proline motif(s) in control cells but that this PER2 phosphorylation was greatly enhanced by MKK7-JNK (WT) (Fig. 2E). However, no band corresponding to BMAL1 phosphorylation was detected in either control or MKK7-JNK (WT)-expressing cells (data not shown). We therefore speculated that PER2 is the more likely circadian target of MKK7-JNK signaling and thus focused our subsequent analyses mainly on PER2.

**JNK Physically Interacts with PER2**—We tested whether JNK could physically interact with clock proteins using co-immunoprecipitation assays. First, we co-expressed FLAG-JNK with Myc-PER2, Myc-BMAL1, or Myc-LacZ (negative control) in 293T cells and subjected lysates to immunoprecipitation with anti-FLAG Ab. We found that these exogenous forms of PER2 and BMAL1 both co-immunoprecipitated with FLAG-JNK (Fig. 3A). To confirm an interaction between PER2 and JNK at the endogenous level, we used anti-PER2 Ab to immunoprecipitate endogenous PER2 from Dex-synchronized WT MEFs at various time points and performed WB to detect endogenous JNK. Indeed, endogenous JNK co-immunoprecipitated with endogenous PER2 in a manner that appeared to depend on the abundance of PER2 protein (Fig. 3B).

**MKK7-JNK Signaling Stabilizes PER2 Protein**—During the course of the above experiments, we realized that MKK7-JNK (WT) expression increased the abundance of PER2 protein, suggesting that MKK7-JNK signaling might affect PER2 stability. To test this possibility, we co-expressed increasing amounts of MKK7-JNK (WT) with Myc-PER2 and Myc-GFP in 293T cells and detected a dose-dependent increase in PER2 protein (Fig. 4A). This PER2 up-regulation was almost entirely dependent on the enzymatic activity of JNK because it was not observed when Myc-PER2 was co-expressed with MKK7-JNK (KN). A more modest increase in Myc-PER2 protein was observed in HeLa cells expressing MKK7-JNK (WT) (data not shown), indicating that this phenomenon varies by cell type. We believe that the MKK7-JNK-induced increase in PER2 protein is not due to an elevation in Per2 mRNA because MKK7-JNK co-expression did not affect levels of Myc-GFP protein (Fig. 4A), whose transcription was controlled by the same CMV promoter as that controlling Per2.

We next investigated the effect of MKK7-JNK (WT) on the half-life of PER2 protein. To this end, we co-transfected cultured cells expressing Myc-PER2 plus Myc-GFP with empty vector, MKK7-JNK (WT), or MKK7-JNK (KN) and treated the cells with cycloheximide (CHX) to prevent new protein synthesis. Intriguingly, MKK7-JNK (WT), but not MKK7-JNK (KN), markedly extended the half-life of Myc-PER2 protein (Fig. 4, B and C). Thus, the PER2 protein stabilization attributable to MKK7-JNK (WT) depends on the kinase activity of JNK.

by overexpression of MKK7-JNK (WT) but not by MKK7-JNK (KN) (Fig. 2A). We then examined the effect of MKK7-JNK (WT) on the co-expression of a set of Myc-tagged clock proteins. Interestingly, MKK7-JNK (WT) caused a significant shift in the electrophoretic mobility of Myc-PER2 and Myc-BMAL1 (Fig. 2B) that could be reversed by phosphatase treatment (Fig. 2, C and D). These data indicate that JNK-mediated phosphorylation can induce a mobility shift in clock proteins, implying a concrete interaction between MKK7-JNK signaling and the clock machinery.
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FIGURE 3. PER2 and BMAL1 can physically interact with JNK. A, exogenous level. FLAG-JNK was co-expressed with Myc-BMAL1, Myc-PER2, or Myc-LacZ in 293T cells. Lysates were immunoprecipitated (IP) with anti-FLAG Ab and analyzed by WB with anti-Myc Ab to detect clock proteins or with anti-JNK (sc-571) Ab to detect JNK. Input, total lysates subjected to WB with anti-Myc Ab. B, endogenous level. WT MEFs were synchronized with Dex, and protein extracts were prepared at the indicated times (h). Top, extracts were immunoprecipitated with anti-PER2 Ab, and the immunoprecipitate was analyzed by WB with anti-JNK or anti-PER2 Abs. CNT, Immunoprecipitation using rabbit IgG (negative control). Input, total lysates subjected to WB with anti-JNK or anti-ERK Abs.

We previously established mouse ES cells bearing disruptions of both the Mkk4 and Mkk7 genes (MKK-DKO ES cells) (29). Because the circadian machinery is not functional in ES cells (30), we can study this cell type to avoid the effects of time-dependent regulation of clock protein expression and degradation. We confirmed that JNK phosphorylation, and therefore activity, was markedly diminished in MKK-DKO ES cells (Fig. 4D), consistent with our previous report (29). Interestingly, PER2 protein was also dramatically reduced in MKK-DKO ES cells. In contrast, the expression of other clock proteins, including CLOCK, BMAL1, and CRY1, was comparable in WT and MKK-DKO ES cells. These data support our results obtained using the MKK7-JNK fusion protein (Fig. 4, A and B) and indicate that JNK activity has a specific effect on PER2 protein stability.

Ubiquitination is an important step in the targeting of PER2 protein for proteasomal degradation (17, 18, 23). We therefore tested the effect of MKK7-JNK (WT) or MKK7-JNK (KN) expression on PER2 ubiquitination. Myc-PER2 was co-expressed in cultured cells with HA-ubiquitin plus empty vector, MKK7-JNK (WT), or MKK7-JNK (KN). Immunoprecipitation of extracts followed by WB showed that Myc-PER2 underwent prominent ubiquitination in control cultures (Fig. 4E), consistent with previous reports (17, 18, 23). Significantly, this ubiquitination of Myc-PER2 was efficiently inhibited by co-expression of MKK7-JNK (WT) but not by co-expression of MKK7-JNK (KN) (Fig. 4E). These results imply that the enzymatic activity of MKK7-activated JNK is required to block PER2 ubiquitination.

MKK7-JNK Inhibits CK1ε-mediated PER2 Degradation—It has been reported that CK1ε phosphorylates PER2 and induces subsequent PER2 degradation via ubiquitination (17, 18). We found that overexpression of CK1ε caused a significant shift in the mobility of PER2 protein that was consistent with its destabilization (Fig. 5, A (compare lanes 1–3 with lanes 4–6) and B). This electrophoretic retardation resulted from phosphorylation because it could be reversed by phosphatase treatment (supplemental Fig. 3), finding consistent with previous reports (17, 18). Notably, co-expression of MKK7-JNK (WT) did not inhibit the electrophoretic retardation of the PER2 protein band induced by CK1ε. Instead, MKK7-JNK (WT) caused an additional shift in the PER2 band (Fig. 5C) that was also reversed by phosphatase treatment (supplemental Fig. 3). These data suggest that CK1ε and JNK do not compete in phosphorylating PER2. To confirm this hypothesis, we mutated the Ser-659 residue of mouse PER2 protein to alanine (PER2 S659A), thereby altering the residue that corresponds to the best-characterized CK1ε phosphorylation site of the human PER2 protein (16). We found that MKK7-JNK (WT) was still able to efficiently phosphorylate the PER2 (S659A) protein (Fig. 5D), indicating that MKK7-JNK phosphorylates PER2 at amino acid residue(s) other than Ser-659. When we assessed the effect of MKK7-JNK (WT) expression on CK1ε control over PER2 stability, we found that the enzymatic activity of JNK reduced the destabilization of PER2 induced by CK1ε (Fig. 5, A (compare lanes 4–6 with lanes 10–12) and B). Furthermore, MKK7-JNK (WT) co-expression suppressed CK1ε-induced PER2 ubiquitination (Fig. 5E). Thus, our results clearly show that the MKK7-JNK signaling pathway and CK1ε activity oppose each other in regulating PER2 protein stability.

DISCUSSION

Previous studies have shown that phosphorylation levels of JNK, and thus its kinase activity, fluctuate in a circadian manner in both the suprachiasmatic nucleus, the site of the master clock, and in cultured mammalian cells (5, 6). In addition, the use of the JNK inhibitor SP600125 and siRNA-mediated suppression of Jnk have both been reported to lengthen the period of circadian transcription in cultured cells and in ex vivo organ culture systems (6, 27, 31). These findings have provided evidence of the involvement of the JNK signaling pathway in circadian regulation. However, several laboratories, including ours, have demonstrated that SP600125 suppresses the activities of kinases other than JNK, such as phosphatidylinositol 3-kinase and Cdk1 (32, 33). Thus, to examine circadian gene expression in the specific absence of JNK activity, we used...
genetic inactivation of M KK7, which activates JNK alone. We have shown that abrogation of M KK7 function in unstressed cultured cells lengthens the period of circadian gene expression (Fig. 1), identifying for the first time a function for M KK7 as a circadian clock regulator.

Several studies have demonstrated that external stimuli, such as ionizing radiation, UV light, and hydrogen peroxide affect circadian gene expression by cultured cells and tissues (34, 35). We have also confirmed that hydrogen peroxide altered circadian gene expression and the accumulation of PER2 protein (supplemental Fig. 4). Because M KK7-JNK signaling is activated by these physical and chemical stresses (1, 2), this pathway may mediate stress-induced molecular clock regulation, an intriguing possibility to examine in the future. Our present study has focused on the role of M KK7 in unstressed cells, and we have shown that M KK7 contributes to the regulation of the molecular clock in cells at steady state. These findings provide insight into the roles of stress-responsive molecules in the maintenance of normal cellular homeostasis.

In our hands, the effects of SP600125 on the periodicity of circadian gene expression were more severe than those of M KK7 inactivation (Fig. 1, B and C, and supplemental Fig. 2, C and D). The more drastic phenotype achieved with SP600125 may be due to its known effects on several other kinases (32, 33). Indeed, it has been reported that LY294002 can lengthen the period of circadian gene expression in cultured cells by inhibiting phosphatidylinositol 3-kinase (27). On the other hand, our data may indicate that JNK phosphorylation mediated by M KK4, the other key JNK activator (1, 2), can also contribute to circadian clock regulation. This possibility is under investigation.

At the molecular level, our study has provided several lines of evidence that the M KK7-JNK pathway regulates the circadian feedback loop by interacting with and phosphorylating PER2.

FIGURE 4. M KK7-mediated JNK activation stabilizes PER2 protein. A, increased PER2 protein. 293T cells were transfected with Myc-PER2 and Myc-GFP and either empty vector (−); 0.01, 0.04, 0.16, or 0.64 µg of M KK7-JNK (WT); or 0.64 µg of M KK7-JNK (KN). Lysates were analyzed by WB with anti-Myc (Myc-PER2 and Myc-GFP), anti-JNK (M KK7-JNK), anti-phospho-c-Jun (p-c-Jun), and anti-ERK (ERK) Abs. B, enhanced PER2 stability. 293T cells were transfected with vectors expressing Myc-PER2 and Myc-GFP and either empty vector (−), M KK7-JNK1 (WT), or M KK7-JNK1 (KN). Transfected cells were treated with 20 µg/ml CHX at time 0. At the indicated times after CHX treatment, protein extracts were analyzed by WB with anti-Myc Ab to detect Myc-PER2 and Myc-GFP. C, quantitation of B. Relative intensities of the Myc-PER2 and Myc-GFP bands in the blot in B were determined by densitometry. Myc-PER2 values were normalized to the Myc-GFP value for a given time point, and values at time 0 were set to 1. The graph shown is for a single experiment representative of three trials. D, decreased PER2 protein in M KK-DKO ES cells. Protein extracts from WT and M KK-DKO ES cells were subjected to WB using anti-phospho-JNK (p-JNK), anti-JNK (JNK), anti-PER2 (PER2), anti-BM AL1 (BM AL1), anti-CLOCK (CLOCK), anti-CRY1 (CRY1), and anti-ERK (ERK) Abs. E, JNK activity decreases PER2 ubiquitination. 293T cells were transfected with Myc-PER2 and HA-ubiquitin (HA-Ub) and either empty vector (−), M KK7-JNK (WT), or M KK7-JNK (KN). Top, lysates of transfected cells were immunoprecipitated with anti-Myc Ab, and the immunoprecipitate was subjected to WB analysis with anti-HA Ab to detect ubiquitinated PER2 (PER2-(HA-Ub)n). Middle, WB analysis of immunoprecipitate using anti-Myc Ab to detect Myc-PER2. Bottom, WB analysis of total cell lysate with anti-JNK Ab to detect M KK7-J NK (WT) and M KK7-JNK (KN).
First, the MKK7-JNK (WT) fusion protein, which is a constitutively active form of JNK, can phosphorylate PER2 (Fig. 2). Importantly, the threonines in the consensus JNK phosphorylation motifs present in PER2 were phosphorylated in cultured cells, and co-expression of MKK7-JNK (WT) enhanced this phosphorylation (Fig. 2E). Second, we showed that JNK interacts with PER2 at both the exogenous and endogenous levels (Fig. 3). Third, overexpression of MKK7-JNK (WT) inhibited PER2 ubiquitination and stabilized PER2 protein (Fig. 4). These results, together with our observation that the genetic inactivation of MKK7 has an influence on circadian gene expression (Fig. 1), suggest that the MKK7-JNK pathway contributes to circadian rhythm regulation by controlling PER2 protein levels and thus PER2 functions.

Many kinases, including CK1ε, CK1δ, CK2, and GSK3β, have been shown to regulate PER2 functions (13, 14, 17, 36, 37). Moreover, these kinases can cooperate in this regard because CK2 reportedly decreases PER2 protein stability by enhancing CK1ε-mediated PER2 degradation (36). In our study, we found that co-expression of MKK7-JNK (WT) decreased CK1ε-mediated PER2 degradation (Fig. 5). However, our data show that MKK7-JNK signaling does not directly inhibit CK1ε-mediated PER2 phosphorylation and may instead phosphorylate PER2 residue(s) different from those targeted by CK1ε-dependent phosphorylation. In short, MKK7-JNK signaling and CK1ε appear to have opposing regulatory effects on PER2 protein stability that are mediated via differential phosphorylation.

The control of PER2 stability via phosphorylation is associated with the period length of circadian gene expression (19, 20). Notably, the phosphorylation of PER2 protein regulates its stability in multiple ways, and this phosphorylation depends both on which phosphorylation sites are used and on which kinases are performing the phosphorylation (12, 13). In addition, interference with phosphorylation-dependent PER2 stability controls can lead to either short or long circadian periods, making it difficult to predict how a given level of PER2 stability will affect the period length of circadian gene expression (18–20, 37, 38). For example, both mutation of PER2 Ser-662, which leads to a lower rate of CK1ε/δ-dependent phosphorylation, and mutation of PER2 N-terminal phosphorylation site (Ser-662) for CK2 lead to PER2 instability. However, these mutations have the opposite effect on the circadian period; Ser-662 mutation...
shortens the period, whereas N-terminal mutation extends it (19, 20, 37). Thus, further elucidation of the processes that affect PER2 stability are required to unravel the complex and largely mysterious post-translational controls governing circadian timing in mammals.

Previous work has established that a single kinase is capable of phosphorylating multiple clock components (13, 14). For example, CK1ε can phosphorylate PER1, PER2, PER3, BMAL1, and CRY1, altering their subcellular localization, protein stability, and transcriptional activity and thus contributing to regulation of their functions (17, 39–41). Our results indicate that, in addition to PER2, BMAL1 is also a target for MKK7-JNK-mediated phosphorylation. We have not yet obtained enough data to prove that BMAL1 phosphorylation is required for circadian clock function, and the physiological importance of this phosphorylation remains under investigation.

In addition to their circadian clock functions, PER2 and BMAL1 participate in non-circadian physiological processes, such as tumor suppression (34), aging (42), immune responses (43), and metabolic control (44–46). MKK7-JNK-dependent phosphorylation of circadian regulators, such as PER2 and BMAL1, may therefore be a key integrating event that translates internal and external signals into a variety of physiological cellular responses, including circadian rhythm adjustment.

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