Mitogen-activated Protein Kinase Phosphorylates and Negatively Regulates Basic Helix-Loop-Helix-PAS Transcription Factor BMAL1*

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In vertebrates, mitogen-activated protein kinase (MAPK) exhibits circadian activation in several clock structures and likely participates in the timekeeping mechanism of the circadian clock. Here we show that MAPK associates with a basic helix-loop-helix-PAS transcription factor BMAL1, a positive regulator for the autoregulatory feedback loop of the circadian oscillator. MAPK phosphorylates BMAL1 at multiple sites, including Ser-527, Thr-534, and Ser-599, in vitro, and BMAL1: CLOCK-induced transactivation from the E-box element is inhibited by expression of a constitutive active form of MAPK kinase in 293 cells. The inhibitory effect is reversed by coexpression of the kinase-dead mutant of MAPK or by mutation of BMAL1 at Thr-534. These results indicate that BMAL1:CLOCK-induced transcription is negatively regulated by MAPK-mediated phosphorylation of BMAL1 at Thr-534 and suggest a molecular link between circadian-activated MAPK and the clock oscillator.

A variety of organisms show circadian rhythms in physiology and behavior under the regulation of the endogenous circadian clock. In mice, a heterodimer of two basic helix-loop-helix-PAS transcription factors, CLOCK and BMAL1, binds to CACGTG E-box elements for transcriptional activation of Period (Per) and Cryptochrome (Cry) genes (1–3). Products of Per and Cry genes feed back to inhibit BMAL1:CLOCK-induced transcription, resulting in a decrease in levels of these gene products. This then allows the molecular cycle to start again with the activation of the BMAL1:CLOCK heterodimer, forming a transcription/translation-based negative feedback loop. Behavioral and molecular analyses of mutant mice for Clock, Per2, Cry1, and Bmal1 genes have clarified their circadian function (3), and in particular, Bmal1 knock-out mice exhibit a complete loss of the circadian rhythm immediately upon placement in constant darkness, indicating a central role of BMAL1 in the circadian oscillator (4).

In addition to the transcriptional regulation, posttranslational modifications such as phosphorylation of clock gene products seem to regulate the stability and period length of the circadian cycle by generating an appropriate time lag (5, 6). We demonstrated previously that a transient inhibition of circadian activation of mitogen-activated protein kinase (MAPK) induced a phase shift of the oscillator in the chick pineal gland (7, 8) and bullfrog retina (9), both of which are the sites of the circadian clock system (10, 11). In the chick pineal gland, MAPK appears to be regulated via the Ras-Raf-1-MEK pathway, and this classical MAPK cascade seems to form a secondary loop interconnected to the core feedback loop (7). Noticeably, the photic signal down-regulates pineal MAPK via light-activated MAPK phosphatase without affecting the Ras-MEK pathway (8). These observations indicate that MAPK activity is regulated by the clock and photic signals via independent pathways and suggest a pivotal role of MAPK in maintenance of the circadian rhythm and its photic entrainment. Despite growing evidence supporting an important contribution of MAPK to the clock system, less is known about its downstream pathway. One such candidate is the cAMP response element-binding protein/cAMP-response element transcriptional pathway (12–14), but an in vivo involvement of MAPK in the transcriptional activation of cAMP response element-binding protein for the circadian oscillator is still controversial (13–15). In this study, we examined another stream of MAPK signaling and found a direct interaction of MAPK with the clock component BMAL1 for negative regulation of BMAL1:CLOCK-induced transcription via BMAL1 phosphorylation.

EXPERIMENTAL PROCEDURES

Plasmids—The open reading frame (ORF) of chicken MAPK (GenBankTM accession no. AY033635) was amplified from chicken pineal cDNA by PCR using LA Taq polymerase (Takara) and a pair of primers (5'-GGAGGAGTTCGACTCATGCGCCGCGTGG-3' and 5'-CCGTGCAGCAAGAGTTACTGCGATACTCGGCTGG-3'). The PCR product was digested with SalI and ligated into SalI-digested pCMV-Tag3C (Stratagene). For bacterial expression of myc epitope-tagged MAPK, the 1.2-kbp-NeoXho1 fragment of MAPK-pCMV-Tag5C was ligated into Ncol/SauI-digested PET21d (Novagen). In reporter gene assay, we used ERK2(K54R)-pEF-BOS plasmid (a kind gift of Masato Ogata at Osaka Medical School, Osaka, Japan) for expression of human K54R-ERK2 (KR-MAPK, kinase-dead mutant). For the yeast two-hybrid assay, the ORF of human K54R-ERK2 was amplified by PCR using primers (5'-GGTCGACTCTTACATGGCCGCGCGCGTGG-3' and 5'-GGTTCGACTTTACATGGCCGCGCGCGTGG-3'). The PCR product was digested with NdeI and SalI and then ligated into NdeI/SalI-digested pGBKTT (CLONTECH).

The ORF of chicken CLOCK and BMAL1 (16) was ligated into Xho1-digested pGAD77 (CLONTECH) for the yeast two-hybrid assay. The ORF of chicken BMAL1 was ligated into SalI-digested pGEX-5X-1 (Amersham Biosciences, Inc.) for its bacterial expression as a glutathione S-transferase; HPLC, high performance liquid chromatography; MS, mass spectrometry; MALDI-TOF/MS, matrix-assisted laser desorption/ionization time-of-flight MS; ORF, open reading frame.

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one S-transferase (GST) fusion protein. For reporter gene assay, the ORF of chicken BMAL1 was ligated into SalI-digested pCMV-Tag3C. Mutations (Ser/Thr to Ala) were introduced into BMAL1 by using a site-directed mutagenesis kit (Stratagene). Construction of a plasmid for expression of chicken CLOCK in mammalian cells was described previously (16).

The ORF of chicken MEK (GenBank accession no. L28703) was amplified from chicken pineal cDNA by PCR with a pair of primers (5′-GCGGATCCCGCCATGGCGGCGAAC-3′ and 5′-GATGCTGAA-GACTCAGGGCACTCGGGTGGT-G3′). The PCR product was then digested with BamHI and ligated into BamHI-digested pGEX-5X-1 or pCMV-Tag3C. A constitutive active form of chicken MEK (DE-MEK) was produced by mutagenesis kit (Stratagene) and used as bait in the two-hybrid screening of yeast. The GST-fusion proteins in the supernatant were then purified as described above. For preparing the same gel.

Preparation of Recombinant Proteins—GST-BMAL1 and its mutants were expressed in Escherichia coli strain BL21(DE3) cultured with 30 μM isopropyl-β-thiogalactopyranoside at 25 °C for 15–20 h. The bacteria in a 1-liter culture were then suspended with 50 ml of a lysis buffer (50 mM Tris-HCl, 250 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol, 1 mM benzamidine, 4 μg/ml aprotinin, 4 μg/ml leupeptin, and 1% (w/v) Triton X-100, pH 7.8, at 4 °C) containing 2 mg/ml lysozyme, and the suspension was incubated for 30 min on ice for lysis followed by centrifugation (45,000 × g for 30 min). GST-fusion proteins in the supernatant were purified by a glutathione-Sepharose column (Amersham Biosciences, Inc.), and the glutathione used for the elution was removed by passing through a PD-10 column (Amersham Biosciences, Inc.) pre-equilibrated with buffer A (50 mM Tris-HCl, 1 mM dithiothreitol, 1 mM benzamidine, 4 μg/ml aprotinin, 4 μg/ml leupeptin, and 0.03% (w/v) Brij 35, pH 7.8, at 4 °C). After concentration by a Centricon YM-100 concentrator (Amicon), the protein content was estimated by the densitometry of SDS-PAGE gel bands in comparison with those of serial dilutions of bovine serum albumin standards in the same gel.

GST-DE-MEK was microbiologically expressed in E. coli strain BL21(DE3) pLysS, and the bacteria were sonicated in the lysis buffer followed by centrifugation (45,000 × g for 30 min). GST-DE-MEK extracted in the supernatant was then purified as described above. For preparing the phosphorylated form of myc epitope-tagged MAPK (treated P-myc-MAPK), bacterially expressed myc-MAPK (1 mg) was purified to near homogeneity by a phenyl-Sepharose column (Amersham Biosciences, Inc.), dialyzed against buffer B (20 mM Tris-HCl, 2 mM MgCl2, and 1 mM Na3VO4, pH 7.8), and then phosphorylated by incubation at 30 °C for 2 h with GST-DE-MEK (100 μg) in 10 ml of a kinase buffer (50 mM Tris-HCl, 20 mM MgCl2, and 50 μM Na3VO4, pH 7.8) containing 1 mM ATP. This mixture was passed through a glutathione-Sepharose column to adsorb GST-DE-MEK, and the flow-through fraction containing P-myc-MAPK was dialyzed against buffer B. Based on gel shift analysis, we estimated more than 90% of MAPK to be phosphorylated.

In Vitro Binding Assay—GST-BMAL1, its mutant, or GST alone (each 3 μg) was incubated with myc-MAPK or P-myc-MAPK (0.3 μg) in 80 μl of a binding buffer (20 mM Tris-HCl, 10% (w/v) glycerol, 135 mM NaCl, 50 μg NaF, 0.5 mM benzamidine, 1 mM EDTA, and 1 mM Na3VO4, pH 7.8) containing 0.5 mg/ml bovine serum albumin at 4 °C for 1 h. To this mixture was added 10 μl of glutathione-Sepharose beads for sedimentation of GST-fusion protein, which was washed five times with the binding buffer containing 0.5% (w/v) Triton X-100. The bound proteins were resolved by SDS-PAGE followed by immunoblotting with anti-myc antibody (1:1000 dilution, Santa Cruz Biotechnology) or anti-GST antibody as described above. For preparing the phosphorylated fragment of synthetic dextrose medium (lacking Trp and Leu) containing His and Ade. Three independent yeast transformants were assayed for transactivation of the HIS3 and ADE reporter genes, as indicated by growth on minimal medium lacking His and Ade. B, bacterially expressed GST-BMAL1, GST-BMAL1(residues 1–493), or GST alone was incubated with myc-MAPK or P-myc-MAPK and then sedimented with glutathione-Sepharose. The precipitated proteins were subjected to SDS-PAGE followed by immunoblotting with anti-myc (upper panel) or anti-GST antibody (lower panels). As markers, both myc-MAPK and P-myc-MAPK (10% of those used in the binding reaction) were loaded on the same gel (input).

preprotease for further digestion at 37 °C for 18 h, and the digest was subjected to reverse-phase HPLC (18). All the fragments eluted from the column were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Voyager MALDI-TOF/MS spectrometer equipped with a delayed extraction ion source, operated in the linear mode, PerSeptive Biosystems). For sequence analysis of phosphopeptides (19), the HPLC-purified peptide was lyophilized, dissolved in 10 μl of 10% (w/v) HCl for incubation at 50 °C for 6 h, and subjected to MALDI-TOF/MS analysis.

RESULTS AND DISCUSSION

Association of MAPK with BMAL1—MAPK is known to translocate to the nucleus where activated MAPK phosphorylates and regulates nuclear proteins including transcription factors, and in some cases, MAPK forms a complex with its substrate (20). Therefore we performed a yeast two-hybrid assay to examine the possible interaction of MAPK (with kinase-dead MAPK as bait) with a couple of basic helix-loop-helix-PAS transcription factors, chicken BMAL1 and CLOCK, that are important elements in the clock function (16). In the yeast two-hybrid assay, MAPK interacted with BMAL1 (Fig. 1A) but not with CLOCK (data not shown). We further explored this interaction by GST pull-down assay and examined the effect of MAPK phosphorylation on the complex formation with BMAL1. GST-BMAL1 preferentially associated with phosphorylated MAPK (P-myc-MAPK) rather than with myc-MAPK, whereas GST alone interacted with neither of the proteins (Fig. 1B). Deletion of C-terminal 140 amino acids of BMAL1 (GST-BMAL1(1–493)) did not abrogate the interaction, implying that the C-terminal activation domain (21) is not required for the interaction with MAPK. Two analyses suggest that BMAL1 could be one of the in vivo targets of phosphorylated and hence activated MAPK.

Fig. 1. Association of MAPK with BMAL1. A, Saccharomyces cerevisiae strain AH109 (MATA trp1–200 his3–200 ade2–1 tru1–512 gal4Δ gal80A lys2::GAL1-his3 gal2::ADE2 ura3::MEL1-lacZ) was cotransformed with plasmids encoding the indicated proteins fused to either GAL4 BD or GAL4 AD by the standard lithium acetate method with 0.5 μg of each plasmid. Transformants containing two independent fusion proteins were selected by culture at 30 °C on plates of synthetic dextrose medium (lacking Trp and Leu) containing His and Ade. Three independent yeast transformants were assayed for transactivation of the HIS3 and ADE reporter genes, as indicated by growth on minimal medium lacking His and Ade. B, bacterially expressed GST-BMAL1, GST-BMAL1(residues 1–493), or GST alone was incubated with myc-MAPK or P-myc-MAPK and then sedimented with glutathione-Sepharose. The precipitated proteins were subjected to SDS-PAGE followed by immunoblotting with anti-myc (upper panel) or anti-GST antibody (lower panels). As markers, both myc-MAPK and P-myc-MAPK (10% of those used in the binding reaction) were loaded on the same gel (input).

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Negative Regulation of BMAL1 by MAP Kinase

MAPK Phosphorylates BMAL1 at Multiple Pro-Ser/Thr Sites—We then tested whether BMAL1 is phosphorylated by activated MAPK. In the presence of radiolabeled ATP, GST-BMAL1 was efficiently phosphorylated by P-myc-MAPK (Fig. 2A, lane 9) but not by myc-MAPK (lane 8). Quantitative analysis of the time-dependent change of the phosphorylated band revealed that BMAL1 incorporated 6–7 pmol of [γ-32P]ATP/pmol of protein, indicating that MAPK phosphorylates BMAL1 at multiple sites (Fig. 2B). Noticeably, deletion of the C-terminal 140 amino acids of BMAL1 resulted in substantial loss of phosphorylation by P-myc-MAPK (Fig. 2A, lane 7) despite the MAPK binding of the truncated form being comparable with that of full-length BMAL1 (Fig. 1B). We speculate that the C-terminal region of BMAL1 contains the primary site(s) for MAPK phosphorylation.

Within the C-terminal 140 amino acids of BMAL1 from human, rat, mouse, chicken, and zebrafish, we find six conserved sites for putative MAPK phosphorylation (ψX[S/T]P, in which ψ is aliphatic or proline; see Refs. 22 and 23). To determine the phosphorylation site(s), GST-BMAL1 was incubated with either phosphorylated or nonphosphorylated MAPK in the presence of unlabeled ATP, digested by lysylendopeptidase and V8 protease, and subjected to reversed-phase HPLC for isolating the proteolytic fragments. From nonphosphorylated BMAL1, we obtained two relevant fragments (termed A and B) showing MALDI-TOF/MS signals at m/z 3509.4 and 2333.2, which correspond to the BMAL1 sequences Ile-513–Lys-546 (fragment A; calculated mass 3508.9, assuming that Cys-523 is acrylamidated) and Asn-583–Asp-604 (fragment B; calculated mass 2334.3), respectively (Table I). The assignment of the fragment was confirmed by MS-based sequence analysis (see below). We paid special attention to these fragments due to the accumulation of the putative phosphorylation sites within their sequences (Table I, see footnote a and b). As expected, phosphorylated BMAL1 yielded two peak fractions containing fragment A (Ile-513–Lys-546), and they displayed a MALDI-TOF/MS signal at m/z 3589.7 or 3669.4, which corresponds to the peptide modified by one or two phosphate groups (3509.8 plus 79.9 or 159.8 mass units, respectively; Table I). Fragment B (Asn-583–Asp-604) derived from phosphorylated BMAL1 showed MALDI-TOF/MS signals at m/z 2412.1, corresponding to the peptide with one phosphate group (2333.4 plus 79.9 mass units). This fragment, Asn-583–Asp-604, contains only one putative MAPK phosphorylation site, Ser-599. By contrast, fragment A contains four putative phosphorylation sites, and we performed an MS-based sequence analysis of the doubly phosphorylated fraction of fragment A to determine the phosphorylated residues. This analysis utilizes a sequential C-terminal degradation of a peptide dissolved in 10% (w/v) HCl (Ref. 19; see also “Experimental Procedures”). MALDI-TOF/MS analysis of the mixture of partially degraded fragments yielded an array of signals that were assigned to ions of fragments with 0–2 phosphate groups. As summarized in Table II, the loss of phosphate groups was observed at two distinct degradation steps, one from peptide 513–538 to 513–533 and the other from peptide 513–532 to 513–525, supporting the phosphorylation sites at Thr-534 and Ser-527 (Table II). The three sites for in vitro MAPK phosphorylation, Ser-527, Thr-534 (both in fragment A), and Ser-599 (in fragment B), in BMAL1 were mutated to Ala, and each mutant was subjected to an in vitro kinase assay (Fig. 2C). These BMAL1 mutants, S527A, T534A, and S599A, were phosphorylated by activated MAPK to reduced degrees (by ~24, 24, and 40%, respectively) as compared with wild-type BMAL1, which is consistent with the phosphopeptide analysis predicting in vitro phosphorylation at these sites. The observed multiple phosphorylation of BMAL1 (6–7 pmol phosphate/pmol of protein in average, Fig. 2, B and C) suggests the presence of the other sites to be phosphorylated quantitatively that were not identified in our analyses. Alternatively, the overall phosho-

Table I

| Source         | Observed mass | Predicted peptide structure | Calculated mass |
|----------------|---------------|------------------------------|-----------------|
| GST-BMAL1      | 3509.4        | fragment A                  | 3508.9          |
|                | 2333.2        | fragment B                  | 2334.3          |
| Phosphorylated | 3669.4        | fragment A + 2PO₄           | 3668.7          |
| GST-BMAL1      | 3589.7        | fragment A + 1PO₄           | 3588.7          |
|                | 2412.1        | fragment B + 1PO₄           | 2413.3          |

*a* Fragment A: IHRIRGSSPSSCGSSPLNITSTPPPDTSSPGSKK (513–546).

*b* Fragment B: NSHGIDMINDQGSSPSNDE (583–604).

FIG. 2. Phosphorylation of BMAL1 by MAPK. A, GST (lane 1), GST-BMAL1 (residues 1–493) (lane 2), or GST-BMAL1 (lane 3) was incubated for 30 min with myc-MAPK or P-myc-MAPK in the presence of 400 μM [γ-32P]ATP followed by electrophoresis and autoradiography (right panel). B, BMAL1 and BMAL1(1–493) was phosphorylated by P-myc-MAPK as in panel A, and the incorporation of 32P into BMAL1 protein was quantified by an image analyzer (FLA2000, Fujifilm, Tokyo, Japan) was plotted against incubation time. C, wild-type (WT) BMAL1 or indicated BMAL1 mutant was incubated with P-myc-MAPK as in panel A, and the incorporation of 32P into BMAL1 protein was quantified and plotted as in panel B. The data are representative results of replicate experiments with similar results. CBB, Coomassie Brilliant Blue.
negative regulation may involve, in addition to physiologically important phosphorylation on certain sites, slow reaction of less importance at multiple sites that are phosphorylated to various degrees in vitro. The latter might account for the unsuccessful identification of the other sites in our MS-based peptide analyses.

Effect of MAPK-mediated Phosphorylation of BMAL1 on Its Transactivation Activity—A functional role of MAPK-catalyzed phosphorylation of BMAL1 was examined by a transcriptional assay in 293 cells in which coexpression of BMAL1 and CLOCK stimulated the E-box element-dependent transcription of a luciferase reporter gene (Fig. 3A). Under these conditions, coexpression of DE-MEK (a constitutive active form of MEK) suppressed BMAL1:CLOCK-induced transcription, and the maximal inhibition reached 23–30%. This inhibitory effect of DE-MEK was completely reversed by additional coexpression of KR-MAPK (a kinase-dead mutant of MAPK), suggesting that activated MAPK mediates the inhibition of BMAL1:CLOCK-induced transcription. To evaluate the contribution of BMAL1 phosphorylation to the inhibition, we examined the effects of BMAL1 mutations at the phosphorylation sites (S527A, T534A, and S599A) in the transcription assay. Similar to wild-type BMAL1, every mutant induced a large increase in E-box element-dependent transcription together with CLOCK in 293 cells (Fig. 3B). In addition, as is observed for wild-type BMAL1: CLOCK, DE-MEK-dependent suppression of the transcription was observed for BMAL1(S527A):CLOCK and BMAL1 (S599A):CLOCK (inhibition by 21 and 25%, respectively). On the other hand, the tranactivation induced by BMAL1, CLOCK, and KR-MAPK is denoted, and data are presented as 100% activity (full activity), 50% activity (half activity), 25% activity (quarter activity), and 0% activity (complete inhibition). Under our experimental conditions, activation of MAPK gave a modest inhibitory effect (≤30%) on transcription of a luciferase reporter gene linked in tandem (three copies) to the CACGTG E-box element with its flanking sequences from the promoter/enhancer region of mouse vasopressin gene was linked in tandem (three copies), and it was inserted into pGL3-Promoter plasmid (Promega). The total amount of DNA applied per well was adjusted to 1 μg by adding pCMV-Tag3C empty plasmid.

Role of MAPK in Clockworks—MAPK activity exhibits a circadian rhythm with a peak at mid to late subjective night in various clock structures (7, 9, 24). During the nighttime, protein levels of negative regulatory elements in the circadian feedback loop (mPERs and mCRYs) are in declining phase, but
their mRNA levels do not start to increase until these protein levels reach to their circadian trough at late subjective night (2, 25). BMAL1:CLOCK-mediated transcription of mPer/mCry is kept suppressed during mid to late subjective night despite very low protein levels of negative elements. Therefore the negative element-independent inhibition of BMAL1:CLOCK heteromer seems to delay the restart of the mPer/mCry cycle, generating an appropriate time lag required for the circadian rhythmicity of the oscillator (5). The MAPK-mediated inhibition of BMAL1 activity observed in this study (Fig. 3) is an event capable of explaining well such a time lag for activation of E-box element-dependent transcription during the nighttime, and thereby MAPK is likely involved in the time-keeping mechanism of the circadian oscillation.

Recently a BMAL1 homolog, BMAL2 (also called MOP9 or CLIF), has been identified in various clock structures (16, 26). BMAL1:CLOCK-mediated transcription of mPer/mCry is kept suppressed during mid to late subjective night despite very low protein levels of negative elements. Therefore the negative element-independent inhibition of BMAL1:CLOCK heteromer seems to delay the restart of the mPer/mCry cycle, generating an appropriate time lag required for the circadian rhythmicity of the oscillator (5). The MAPK-mediated inhibition of BMAL1 activity observed in this study (Fig. 3) is an event capable of explaining well such a time lag for activation of E-box element-dependent transcription during the nighttime, and thereby MAPK is likely involved in the time-keeping mechanism of the circadian oscillation.

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