

Circadian and photic regulation of MAP kinase by Ras- and protein phosphatase-dependent pathways in the chick pineal gland

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Abstract Chick pineal mitogen-activated protein kinase (MAPK) exhibits circadian activation and light-dependent deactivation at nighttime. Here we report that, in the chick pineal gland, levels of active forms of MAPK, MEK, Raf-1 and Ras exhibited synchronous circadian rhythms with peaks during the subjective night, suggesting a sequential activation of components in the classical Ras-MAPK pathway in a circadian manner. In contrast, the light-dependent deactivation of MAPK was not accompanied by any change of MEK activity, but it was attributed to the light-dependent activation of protein phosphatase dephosphorylating MAPK. These results indicate that the photic and clock signals regulate MAPK activity via independent pathways, and suggest a pivotal role of MAPK in photic entrainment and maintenance of the circadian oscillation. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Circadian rhythm; Photic entrainment; Pineal gland; Mitogen-activated protein kinase; Protein phosphatase; Chicken

1. Introduction

Almost all organisms from prokaryotes to humans have circadian rhythms [1]. Traditionally, the circadian clock system has been depicted to consist of three components: a circadian oscillator, an input pathway responsible for the entrainment of the oscillator, and an output pathway mediating the time-signal transmission. The chick pineal gland contains all the three components within a single cell, and these properties are maintained in cell culture [2–5]. Thus, it represents an excellent model system for the study of the circadian clock system. In several circadian clock-containing tissues, such as the mouse suprachiasmatic nucleus (SCN) [6], chicken pineal gland [7] and bullfrog retina [8], circadian activation/deactivation cycles of mitogen-activated protein kinase (MAPK) are observed even under the constant darkness. More interestingly, the photic signal induces either a rapid activation of MAPK in the mouse SCN [6] or its deactivation in the chick pineal gland [7]. In addition, a transient inhibition

of MAPK activity during the subjective night delayed the phase of the oscillator in the chick pineal gland [7] and bullfrog retina [8], indicating that MAPK also plays an important role in the input pathway. Taken together, MAPK seems to mediate feedback of the output signal back to the input, forming an interconnected loop, and this loop is likely to contribute to maintenance of the timekeeping of circadian clock oscillation.

In the present study, we investigated the upstream regulators responsible for circadian activation of MAPK in the chick pineal gland, and found a synchronous circadian activation of components in the classical Ras-MAPK pathway. On the other hand, the photic signal down-regulates MAPK via light-activated protein phosphatase independent of the Ras-Raf-1-MAPK/ERK kinase (MEK) pathway. These results suggest a key role of MAPK in the clock system as one of converging points receiving the two important signals of time and light.

2. Materials and methods

2.1. Animals

Animals were treated in accordance with the guidelines of The University of Tokyo. Newly hatched male chicks were raised under 12 h light–12 h dark lighting conditions. The light intensity was 200–300 lux at the level of the cages. All the manipulations during the dark period were performed under a dim red light (> 640 nm).

2.2. Immunoblot analysis

The activated states of MEK and MAPK were estimated by immunoblotting with antibodies which detect their active forms. That is, eight pineal glands were homogenized with 500 µl of sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer, and subjected to immunoblotting with anti-phospho-MEK antibody (1:800, New England Biolabs) and anti-phospho-MAPK antibody (1:5000, New England Biolabs). The protein levels of MEK and MAPK were evaluated by using anti-MEK antibody (1:5000, Santa Cruz Biotechnology) and monoclonal anti-MAPK antibody (1:10 000, Transduction Laboratories), respectively. On the other hand, the protein level of MKP-1 was evaluated by using anti-MKP-1 antibody (1:1000, Santa Cruz Biotechnology). Immunoblotting was performed as described [7], and the immunoreactivities were developed by CDP-Star detection system (New England Biolabs) using alkaline phosphatase-conjugated secondary antibody (1:5000, New England Biolabs).

2.3. Assay of Raf-1 kinase activity

The activity of Raf-1 was assayed by measuring the phosphorylation rate of a catalytically inactive form of MEK1, as described by Duckworth and Cantley [9] with some modifications. In brief, eight pineal glands were homogenized (10 strokes) with 500 µl of buffer A (250 mM Tris–HCl, 100 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 5 mM NaF, 1% (v/v) Triton X-100, 0.05% (w/v) SDS, 0.03% (w/v) sodium deoxycholate, 10 µg/ml aprotinin and

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Abbreviations: SCN, suprachiasmatic nucleus; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; PAGE, polyacrylamide gel electrophoresis; RBD, Ras-binding domain; GST, glutathione *S*-transferase; CT, circadian time

10 $\mu\text{g}/\text{ml}$ leupeptin; pH 7.6 at 4°C). Raf-1 in the homogenate (350 μg) was immunoprecipitated with 2 μg of anti-Raf-1 antibody (Santa Cruz Biotechnology), and the immunoprecipitate was incubated at 30°C for 40 min with substrates, 1 μg of glutathione *S*-transferase (GST)-K97A MEK1 (Upstate Biotechnology) and 100 μM ATP in 50 μl of kinase buffer (25 mM Tris-HCl, 10 mM MgCl_2 , 1 mM dithiothreitol and 0.5 mM EGTA; pH 7.4). The reaction was terminated by the addition of SDS-PAGE sample buffer, and the phosphorylation level of GST-K97A MEK1 was estimated by immunoblotting with anti-phospho-MEK antibody. The protein level of Raf-1 was measured by immunoblotting with anti-Raf-1 antibody (1:8000).

2.4. Quantification of active form of Ras

The amount of active (GTP-bound) form of Ras was quantified by the protein level of Ras capable of binding with Ras-binding domain (RBD) of Raf-1, as described by Verheijen et al. [10] with some modifications. In brief, eight pineal glands were homogenized with 500 μl of buffer B (25 mM HEPES-NaOH, 150 mM NaCl, 1% (v/v) Nonidet P-40, 10 mM MgCl_2 , 4 $\mu\text{g}/\text{ml}$ aprotinin and 4 $\mu\text{g}/\text{ml}$ leupeptin; pH 7.5 at 4°C), and the GTP-bound form of Ras was precipitated with 5 μg of GST-RBD coupled to glutathione agarose beads (Upstate Biotechnology). The precipitate was subjected to immunoblotting with monoclonal anti-Ras antibody (1:250, Calbiochem) for quantification.

2.5. Assay of protein phosphatase activity in pineal homogenate

Protein phosphatase activity in the chick pineal homogenate was assessed by using a phosphorylated form of recombinant MAPK as a substrate. For this purpose, cDNAs for chicken MAPK and MEK were isolated from a chick pineal cDNA (to be described elsewhere), and each cDNA for chicken MAPK and a constitutive active form of chicken MEK2 (termed DE-MEK2) was cloned into a GST gene fusion vector, pGEX-5X-1 (Amersham Pharmacia Biotech). *Escherichia coli* strain BL21(DE3)pLysS was transformed with the constructs, and recombinant MAPK and DE-MEK2 were expressed as GST fusion proteins and purified as described [11]. GST-MAPK (150 μg) was incubated with DE-MEK2 (100 ng), which was obtained by cleaving GST-DE-MEK2 with factor Xa, in 1 ml of kinase buffer (50 mM Tris-HCl, 20 mM MgCl_2 , 150 μM ATP; pH 7.8) for 2 h at 25°C. The reaction mixture was applied to a glutathione-Sepharose 4B column (Amersham Pharmacia Biotech) to remove DE-MEK2, and then phosphorylated GST-MAPK was eluted by an elution buffer (50 mM Tris-HCl, 20 mM glutathione, 1 mM dithiothreitol, 1 mM benzamide, 0.03% (w/v) Brij 35, 4 $\mu\text{g}/\text{ml}$ aprotinin and 4 $\mu\text{g}/\text{ml}$ leupeptin; pH 7.4 at 4°C). On the other hand, five pineal glands were homogenized with 500 μl of buffer C (50 mM Tris-HCl, 10 mM dithiothreitol, 2 mM glutathione, 100 μM benzamide, 0.003% (w/v) Brij 35, 2 mM phenylmethylsulfonyl fluoride, 4 $\mu\text{g}/\text{ml}$ aprotinin and 4 $\mu\text{g}/\text{ml}$ leupeptin; pH 7.4), and the aliquot (100 μl) was incubated with 100 ng of GST-MAPK at 30°C for 40 min for dephosphorylation reaction. The reaction was terminated by the addition of SDS-PAGE sample buffer, and the phosphorylation level of GST-MAPK was estimated by immunoblotting with anti-phospho-MAPK antibody or anti-phosphotyrosine antibody (1:500, Transduction Laboratories).

2.6. Data analysis

Densitometric analysis of the immunoreactivity was performed by using a ScanJet IICx (Hewlett Packard) desktop scanner and MacBAS 2.5 (Fujifilm) software.

3. Results and discussion

We first hypothesized that circadian activation of MAPK is mediated by the classical Ras-MAPK pathway. To test this, we examined whether activities of MEK and MAPK exhibit synchronous circadian rhythm or not. Chicks were entrained to light/dark cycles for 14 days and subsequently transferred to constant darkness. On days 15–16, their pineal glands were isolated every 4 h, and activities of both MEK and MAPK in the homogenate were estimated by immunoblotting with antibodies that detect activated MEK (phosphorylated on two regulatory serine residues) and activated MAPK (phosphory-

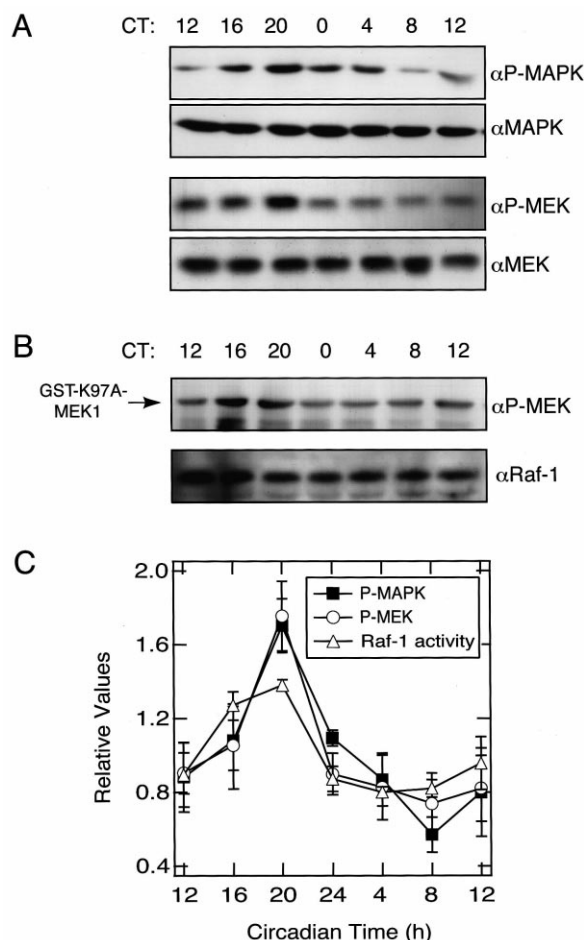


Fig. 1. Circadian activation of MAPK, MEK, and Raf-1. A: The chick pineal homogenate (30 μg) prepared at each time point was immunoblotted with anti-phospho-MAPK antibody (top panel) and then reprobbed with anti-MAPK antibody (second panel). The other aliquot of each sample (30 μg) was immunoblotted with anti-phospho-MEK antibody (third panel) and then reprobbed with anti-MEK antibody (bottom panel). B: Raf-1 kinase activity in the chick pineal homogenate was measured by the immune complex kinase assay employing GST-K97A MEK1 as a substrate (upper panel). Raf-1 protein level was estimated by the immunoblot of chick pineal homogenate (30 μg) with anti-Raf-1 antibody (lower panel). C: The band densities in A ($\alpha\text{P-MAPK}$, $\alpha\text{P-MEK}$) and B ($\alpha\text{P-MEK}$ as a measure for Raf-1 activity) were quantified by the densitometry and were shown as values (means $\pm$ S.D., $n=2$ or 3) relative to the average band density during the entire experimental period.

lated on both regulatory threonine and tyrosine residues). The phosphorylation levels of both MEK and MAPK exhibited overt rhythms with each peak both at circadian time (CT) 20 (Fig. 1A, top and third panels), whereas their protein levels were not altered significantly throughout the period (Fig. 1A, second and bottom panels). These results support the idea that MEK participates in the circadian activation of MAPK.

Among several known kinases phosphorylating MEK, Raf-1 immunoreactivity (72 kDa band) was found in the chick pineal homogenate, whereas only a weak immunoreactivity was detected with B-Raf-specific antibody, which heavily immunostained 94 kDa band in the chick brain homogenate (data not shown). Then we investigated circadian rhythmicity of the chick pineal Raf-1 kinase activity by an immune complex kinase assay employing a catalytically inactive form of

MEK1 as a substrate. As expected, Raf-1 kinase activity, but not the protein level, exhibited an overt rhythm in constant darkness (Fig. 1B), with a peak time similar to those of MEK and MAPK activities (Fig. 1C), suggesting that the rhythmic regulatory process of Raf-1 kinase activity contributes to circadian activation of MEK and MAPK. On the other hand, we were not able to detect B-Raf kinase activity in an immune complex kinase assay of the chick pineal homogenate (data not shown).

Among multiple input signals regulating Raf-1 kinase activity, Ras small GTPase is known as an important regulator [12]. Thus we investigated a possible involvement of Ras in the circadian regulation of the MAPK cascade in the chick pineal gland. For this purpose, temporal change in levels of GTP-bound (activated) Ras in the pineal homogenate was assessed by using GST-RBD pull-down assay. The amount of GTP-bound activated Ras exhibited a circadian rhythm with a peak at CT 18, and this overt rhythm persisted for at least 2 days (Fig. 2A, top panel), whereas the total Ras protein levels in the homogenate did not change significantly throughout the period (Fig. 2A, second panel). The same samples were immunoblotted with anti-phospho-MAPK antibody and anti-phospho-MEK antibody (Fig. 2A, third and bottom panels, respectively), showing that the peak time of the activity rhythm of Ras coincided with those of MAPK and MEK (Fig. 2B). These results support a model that the circadian activation of MAPK originates from rhythmic activation

of Ras via the classical Ras-MAPK pathway which is commonly used in various cellular events. This highly conserved mechanism regulating MAPK activity is likely used in other clock-containing tissues, such as the mammalian SCN. Recently, circadian oscillation of clock genes was also observed in peripheral organs [13,14] and even in cultured cell lines [15]. Interestingly, activation of MAPK cascade triggers several cycles of circadian oscillation of clock gene expression in NIH3T3 cells, but no endogenous rhythmicity of MAPK activity is observed in these peripheral clock cells [16]. These observations suggest that the circadian oscillator in central clock cells is stabilized by contribution of circadian activated MAPK, which is not obvious in peripheral systems. Identification of a component responsible for the circadian activation of Ras may help to understand the difference in mechanism between central and peripheral clock systems.

We previously demonstrated that chick pineal MAPK is dephosphorylated and deactivated by light [7], raising two possibilities: (i) the phosphorylation state of MAPK is determined by a balance between upstream kinase and phosphatase, the former of which is deactivated by the photic signal, and/or (ii) the photic signal activates protein phosphatase dephosphorylating MAPK. To investigate these possibilities, we first assessed the light-dependent fluctuation of MEK activity. Chicks entrained to light/dark cycles for 14 days were transferred to constant darkness, and exposed to white light (200–300 lux) at CT 18:00 on day 15. Their pineal glands were isolated at 10, 20, 30 and 40 min after the light exposure of animals. As shown in Fig. 3A, MAPK was rapidly dephosphorylated within 10 min after the light exposure and then returned nearly to a control level at 40 min in the light (top panel, lanes 2–5). The phosphorylation level of MAPK was unchanged in chicks kept in the darkness from CT 18:00 to CT 18:50 (lanes 1, 6), and the protein level of pineal MAPK was also constant with or without light illumination (Fig. 3A, second panel), confirming light-induced dephosphorylation of MAPK in the chick pineal gland. Under the conditions, the phosphorylation level and the total amount of MEK did not change significantly after exposure of chicks to light (Fig. 3A, third and bottom panels, respectively). It seemed likely that the photic signal activated protein phosphatase dephosphorylating MAPK. To test this, phosphatase activities in the pineal homogenate prepared from chicks before and after the light illumination were measured by mixing with exogenously added substrate, the phosphorylated form of recombinant GST-MAPK. As shown in Fig. 3B (top panel), The phosphorylation level of GST-MAPK evaluated by anti-phospho-MAPK antibody was markedly lowered by the addition of the pineal homogenates prepared at 10, 20, 30 min after the onset of light. This indicates light-dependent rapid activation of MAPK phosphatase activity in the pineal homogenate. The phosphatase activity was gradually reduced to a control level during 40 min of the light exposure, and this apparently paralleled the time-course of dephosphorylation of MAPK after the light exposure (Fig. 3A, top panel).

So far, MAPK dephosphorylation has been attributed to three classes of phosphatases: (i) serine/threonine-specific phosphatases [17], (ii) tyrosine-specific phosphatases [18,19], and (iii) dual-specificity phosphatases that dephosphorylate both threonine and tyrosine residues [20]. The result described above showed that the light-activated phosphatase dephosphorylates either or both of the regulatory tyrosine and threo-

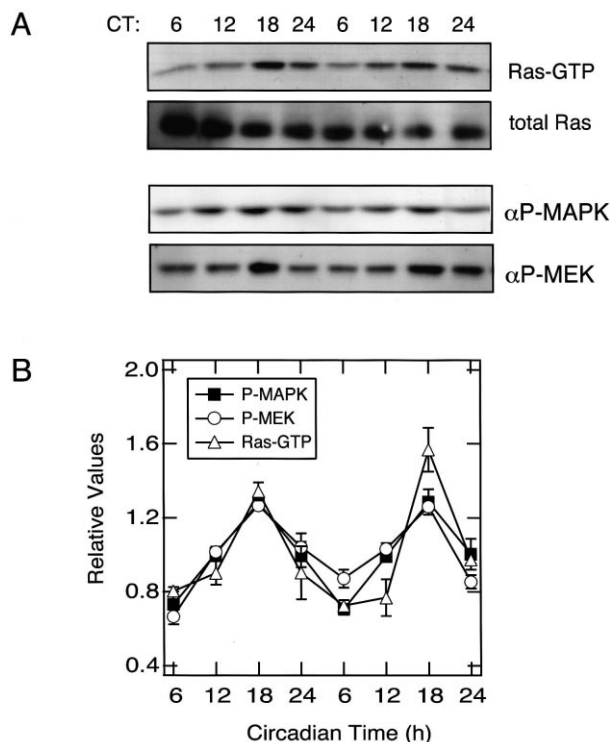


Fig. 2. Synchronous circadian activation of Ras, MEK and MAPK. A: GTP-bound form of Ras was precipitated from the chick pineal homogenate using GST-RBD and the precipitate was immunoblotted using anti-Ras antibody (top panel). The chick pineal homogenate (30 μ g) was immunoblotted with anti-Ras antibody (second panel), with anti-phospho-MAPK antibody (third panel), or with anti-phospho-MEK antibody (bottom panel). B: The band densities in A (Ras-GTP, α P-MAPK and α P-MEK) were quantified by the densitometry and were shown as values (means $\pm$ S.D., $n=3$) relative to the average band density during the entire experimental period.

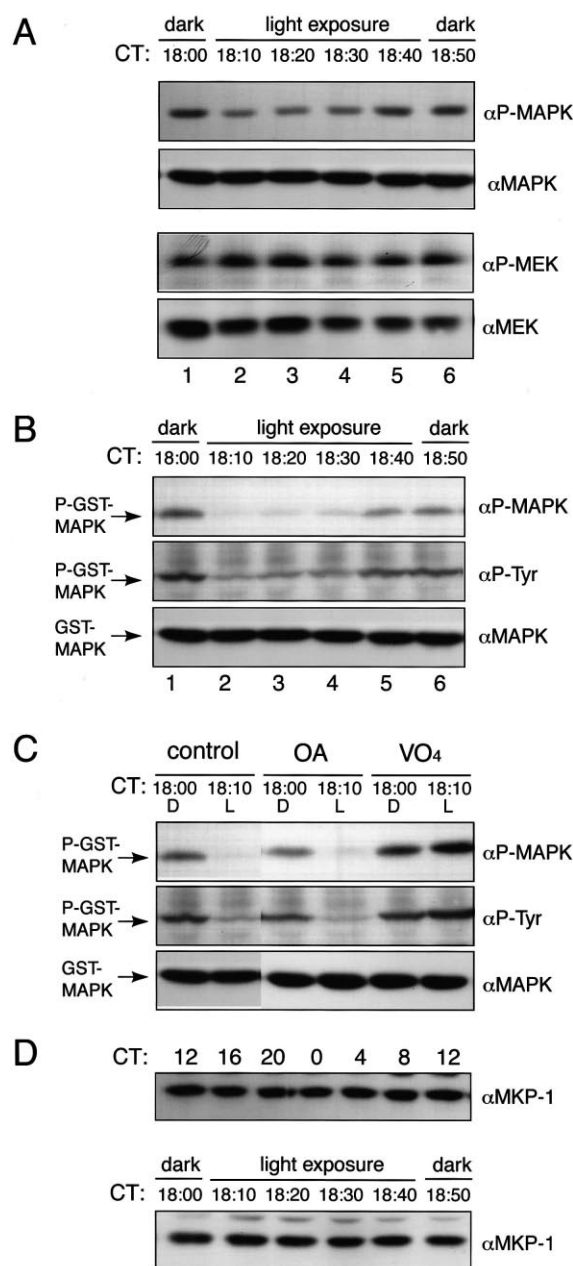


Fig. 3. Photoc induction of protein phosphatase activity against MAPK. A: The chick pineal homogenate (30 μ g) was immunoblotted with anti-phospho-MAPK antibody (top panel) and then re-probed with anti-MAPK antibody (second panel). The chick pineal homogenate (30 μ g) was immunoblotted with anti-phospho-MEK antibody (third panel) and then re-probed with anti-MEK antibody (bottom panel). B: The chick pineal homogenate was incubated with phosphorylated GST-MAPK and the phosphorylation level of GST-MAPK was evaluated by the immunoblotting with anti-phospho-MAPK (upper panel) and with anti-phosphotyrosine antibody (middle panel). Total amount of GST-MAPK was evaluated by immunoblotting with anti-MAPK antibody (lower panel). C: Phosphatase activity was assessed in the absence (control) or presence of 100 nM okadaic acid (OA), or 1 mM sodium orthovanadate (VO₄). D: The chick pineal homogenate (30 μ g) was immunoblotted with anti-MKP-1 antibody (upper and lower panels). The data are representative results of two independent experiments with similar results.

nine residues. To characterize the nature of the light-activated phosphatase, we examined whether or not the tyrosine dephosphorylation is involved, and whether well-known inhibitors suppress the light-dependent activation of the phosphatase. Then, we found that the regulatory tyrosine residue of GST-MAPK was dephosphorylated by the addition of the pineal homogenate prepared after the light exposure (Fig. 3B, second panel). This light-dependent stimulation of MAPK dephosphorylation was not affected by pretreatment of 100 nM of okadaic acid (Fig. 3C, OA), an inhibitor of protein phosphatases 1 and 2A [21], whereas it was completely abolished in the presence of 1 mM of vanadate (Fig. 3C, VO₄) which inhibits both tyrosine-specific and dual-specificity phosphatases [22,23]. Taken together, the light-activated dephosphorylation of MAPK is attributed to tyrosine-specific or dual-specificity phosphatase. Among dual-specificity phosphatases, MKP-1 is known to be transiently synthesized by extracellular stimuli [20]. Strong MKP-1 immunoreactivities were detected at constant levels throughout the day in the chick pineal gland under constant dark conditions (Fig. 3D, upper panel), but no significant change in its protein level was observed upon light exposure of chicks (Fig. 3D, lower panel). MKP-1 might balance the phosphorylation level of MAPK in the chick pineal gland, but it seems to play no significant role in the light-dependent dephosphorylation of MAPK. The molecular identity of the light-activated phosphatase remains to be elucidated, but it probably contributes to photic entrainment of the oscillator, because suppression of chick pineal MAPK activity affects the phase of the circadian oscillator [7].

In the chick pineal cells, calcium influx of intracellular stores has been shown to play a critical role in photic entrainment [24], and thus it is possible that the activity of the light-activated phosphatase is regulated by calcium via calcium-binding protein and/or protein kinase C. Unlike the light-dependent suppression of MAPK activity in chicken pineal gland, MAPK is activated in the SCN and in NIH3T3 cells by light and serum shock, respectively, both of which function as entrainment signals [6,16]. Taken together, it is strongly suggested that extracellular signal-induced fluctuation (activation and deactivation) of MAPK is generally required for resetting the circadian oscillators, and that MAPK represents one of converging points of the entrainment pathway and the circadian oscillator.

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