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Induction of Two Mammalian PER Proteins is Insufficient to Cause Phase Shifting of the Peripheral Circadian Clock

Joon-Woo Lee, Sang-Gil Cho, Jun-Hyung Cho, Han-Gyu Kim, and Kiho Bae*

Department of Life Science, College of Sciences, Yonsei University, Wonju 220-710, Korea

Abstract: Most living organisms exhibit the circadian rhythm in their physiology and behavior. Recent identification of several clock genes in mammals has led to the molecular understanding of how these components generate and maintain the circadian rhythm. Many reports have implicated the photic induction of either *mPer1* or *mPer2* in the hypothalamic region called the suprachiasmatic nucleus (SCN) to phase shift the brain clock. It is now established that peripheral tissues other than the brain also express these clock genes and that the clock machinery in these tissues work in a similar way to the SCN clock. To determine the role of the two canonical clock genes, *mPer1* and *mPer2*, in the peripheral clock shift, stable HEK293EcR cell lines that can be induced and stably express these proteins were prepared. By regulating the expression of these proteins, it could be shown that induction of the clock genes, either *mPer1* or *mPer2* alone is not sufficient to cause clock phase shifting in these cells. Our real-time PCR analysis on these cells indicates that the induction of mPER proteins dampens the expression of the clock-specific transcription factor *mBmal1*. Altogether, our present data suggest that *mPer1* and *mPer2* may not function in clock shift or take part in differential roles on the peripheral circadian clock.

Key words: Circadian clock, *mPer*, *mBmal1*, HEK293EcR cells, real-time PCR

Most living organisms have circadian rhythm or clocks, ways of coordinating their physiology and behavior to the rotation of the Earth. The best known example of this behavior is the human sleep-wake cycle that occurs with about-a-day (~24 h) periodicity. The circadian rhythm in mammals is controlled by pacemaker neurons that are located in the anterior hypothalamic region called the suprachiasmatic nucleus (SCN). In recent decades, researchers have isolated many genes that function in generating and maintaining the circadian rhythm from a variety of model organisms. It is now well established that the circadian rhythm is composed of interacting transcriptional and translational feedback loops in which the expression of clock genes is regulated by their proteins (Weaver, 1998; Reppert and Weaver, 2001; Sehgal, 2004).

The first clock gene, *period* (*per*), has been identified in *Drosophila* by using classic genetic screening (Konopka and Benzer, 1971). The same genetic approach also led to the identification of *Clock* (*Clk*) in mice, which is a basic helix-loop-helix (bHLH) type transcription factor that can bind to E-box (CANNTG) sequences present in the promoters of target genes. The CLOCK protein partners with another bHLH type protein named mBMAL1 and functions as a dimer to promote gene expression (King et al., 1997; Gekakis et al., 1998; Bunger et al., 2000). Among their targets are three mammalian *per* genes, named *mPer1*, *mPer2*, and *mPer3*, that show very similar sequences to *Drosophila per* (Zylka et al., 1998; Bae et al., 2001). Considering their important roles in rendering organisms to adapt to the cyclic environment, it is not surprising that the circadian clock is conserved throughout species during the evolutionary process. Indeed, based on the structural and functional homology with others, many clock genes are identified in mammals. For example, two circadian clock-specific photoreceptors, *mCryptochrome* (*mCry1* and *mCry2*), are isolated and found to be strong negative regulators of mCLOCK-mBMAL1 dimer. Proteins responsible for the circadian clock also undergo progressive phosphorylation by the action of specific kinases such as casein kinase and glycogen synthase kinase (GSK) (Kume et al., 1999; van der Horst et al., 1999; Camacho et al., 2001; Sehgal, 2004).

The time at which a rhythmic activity occurs defines the "phase" of that particular rhythm and a change in the timing results in a "shift" in the phase of the rhythm. A graph that

*To whom correspondence should be addressed.
Tel: 82-33-760-2280, Fax: 82-33-760-2183
E-mail: kbae@dragon.yonsei.ac.kr

plots the magnitude of the shift relative to the time of day that the shifting stimulus is applied is called phase-response curve (PRC) (Sehgal, 2004). Among the clock genes, *mPer1* and *mPer2* were implicated to light-induced clock shifts in the SCN. Transcription of these two genes is increased in the SCN when the organism is exposed to light pulse during nighttime. The induction of *mPer* genes in the SCN is known to have correlations with the direction, either delaying or advancing, and magnitude of the clock phase shift (Albrecht et al., 1997; Shigeyoshi et al., 1997). The rapid shift or even the reversal of the clock phase forces the body to adjust its endogenous clock to the new phase of the changed clock. This malfunctioning of the clock will result in many disorders such as jet-lag, sleep phase syndromes, hormonal imbalances, and seasonally affected depression (SAD) (Toh et al., 2001). In addition, mPER2 proteins are known to enhance the rate of *mBmal1* transcription indirectly via negatively regulating REV-ERBα, a nuclear orphan receptor that binds to the *mBmal1* promoter and represses transcription (Shearman et al., 2000; Preitner et al., 2002).

Recent studies have proved that circadian clock genes are ubiquitously present even in non-neural cells in the organism and are rhythmically expressed with each tissue-specific phase of oscillations (Zylka et al., 1998; Balsalobre et al., 2000; Storch et al., 2002). In this study, to address the possible involvement of mPER1 and mPER2 expression in the initiation or phase shift of the circadian clock in non-neural, peripheral cells, which are distinct from the SCN clock, we prepared two stably inducible cell lines of these proteins and compared the expression profiles of a key clock gene, *mBmal1*, as an indicator of clock functioning in these cells. Our data indicated that induction of either *mPer1* or *mPer2* alone is not sufficient to cause phase shifting the peripheral clock. Moreover, the ectopic induction of mPER proteins in cells caused the reduction in the expression of *mBmal1*.

MATERIALS AND METHODS

Construction of plasmids

mPer1 and *mPer2* cDNAs were obtained from pCDNA3.1 *mPer1*.V5.His_6 and pCDNA3.1 *mPer2*.V5.His_6, as described previously (Kume et al., 1999). To place *mPer1* under the control of ecdysone response promoters, we digested pCDNA3.1 *mPer1*.V5.His_6 with Hind III and Age I (NEB) and ligated the piece into the Ecdysone expression vector, pIND V5.His_6 type A (Invitrogen) that contains the same restriction sites. For *mPer2*, Xho I and Bst BI restriction sites were utilized with pIND V5.His_6 type B (Invitrogen) instead. These plasmids, named as pIND *mPer1*.V5.His_6 and pIND *mPer2*.V5.His_6, respectively, were then used to transform chemically competent *E. coli* DH5α cells. Plasmid minipreps (Qiagen) prepared from these transformed cells were digested using appropriate restriction enzymes and further verified by sequencing.

Cell culture and transfections

Cells used in this study were maintained in Dulbecco's modified eagle medium (DMEM, catalog no. 11995-065, Gibco) supplemented with 10% fetal bovine serum (FBS, catalog no. 14-501F, BioWhittaker) at 37°C with 5% CO_2 in a humidified incubator. To prepare mPER-inducible cell lines, transfection of plasmids into HEK293EcR cells (Invitrogen) was carried out by mixing the plasmid DNA with 5 μL of Lipofectamine reagent (Gibco), incubating for 15 min at room temperature, and applying the mixture to the cells in serum-free medium. Two hours later, the cells were fed with an equal volume of 10% FBS and incubated for 48 h before use. Cells stably expressing either mPER1.V5.His_6 or mPER2.V5.His_6 were selected by growing them in DMEM with 10% FBS and appropriate antibiotics, Zeocin and G418 (400 μg/mL) for generations. The expression of chimeric proteins was identified by immunoblotting with anti-V5 antibodies (Invitrogen).

Immunocytochemistry

To further localize the expressed proteins, transfected HEK293EcR cells were seeded onto glass coverslips in 6-well tissue culture plates, grown to 60% confluence, and induced by adding 10 μM of ecdysone (Invitrogen) into the culture media for 12 h. The cells were washed in phosphate buffered-saline (PBS, Gibco) and fixed onto the coverslips with 4% paraformaldehyde in PBS for 10 min at room temperature. The coverslips were then processed with blocking solution (10% normal goat serum and 0.2% Triton X-100 in PBS, 30 min), a primary antibody (mouse monoclonal anti-V5 [Invitrogen], 1 : 500 dilution in blocking solution diluted in 1 : 4 in PBS, 1 h), three PBS washes, a secondary antibody (Cy3-conjugated goat anti-mouse IgG [Jackson] 1 : 200 in blocking solution, 1 h), two PBS washes, DAPI stain (bis-Benzimide [Sigma] 1 : 500 in PBS, 2 min), two brief PBS washes, and a rinse in ddH_2O as previously described (Chang and Reppert, 2003). The coverslips were mounted onto slides with mounting medium for fluorescence microscopy (Kierkegaard and Perry), and the slides were viewed under a fluorescence microscope (Olympus IX70). For each slide, cells with strong signals were classified as having staining in the nucleus, cytoplasm, or both. Cell mixtures were serially diluted and at least 10 or more single cell lines were selected and used for the experiment.

Immunoblotting

HEK293EcR cells were grown in DMEM supplemented with 10% FBS. The cells were then induced either mPER1.V5.His_6 or mPER2.V5.His_6 by adding 10 μM of ecdysone (Invitrogen) for 12 h, harvested using trypsin

every 4 h, washed in PBS, and lysed in cell lysis buffer (20 mM Hepes [pH 7.4], 100 mM KCl, 2.5 mM EDTA, 5% glycerol, 1% Triton X-100, 0.5 mM dithiothreitol [DTT] and protease inhibitors [2.5 mM PMSF, 10 mg/mL leupeptin, 10 mg/mL pepstatin]). Homogenates were centrifuged twice (10 min at 12,000×g) and the supernatants were transfered to new tubes. The protein concentration of the samples was determined using a Coomassie protein assay kit according to the manufacturer's instructions (Pierce). An equal volume of 2×SDS-sample buffer was added to the samples and an equal amount of total protein (~50 μg total) was separated by 6% SDS-polyacrylamide gels and transferred onto nitrocellulose membrane using a transblot SD semi-dry transfer apparatus (Bio-Rad). The blots were then treated with blocking solution (5% skim milk [Bio-Rad] in Tris-buffered saline containing 0.05% Tween 20 [TBST], 30 min), a primary antibody (mouse anti-V5 [Invitrogen], 1 : 5000 dilution in blocking solution, 1 h), six TBST washes, a secondary antibody (HRP-conjugated rabbit anti-mouse IgG [Sigma] 1 : 1000 in blocking solution, 1 h), and six more TBST washes. Immunoblots were visualized using the enhanced Chemiluminescence kit (ECL, Amersham) and ECL Hyperfilm (Amersham) as previously described (Bae et al., 2000). Scanned images of autoradiographs were prepared using Adobe Photoshop 7.0 software.

Real-time PCR

HEK293EcR cells stably expressing either mPER1.V5.His_6 or mPER2.V5.His_6 were grown in DMEM supplemented with 10% FBS and induced proteins, and were harvested using trypsin at indicated time points. Total RNA was prepared using the Trizol Reagent (Sigma). The optical density of the prepared total RNA was measured in a spectrophotometer and used to determine RNA concentration. Two micrograms of total RNA from each sample were used for cDNA synthesis (Takara #RR019A). To perform real-time PCR (Rotor-Gene 3000, Corbett Research), a QuantiTect SYBR Green PCR Kit (Qiagen #204143) was used according to the manufacturer's instructions. Briefly, each 10 μL mix containing 5 μL of 2×QuantiTect SYBR Green PCR Master Mix, 300 nM each of forward and reverse primers, 1 μL of template cDNA, and RNAse-free water was prepared. Human GAPDH primers were used as internal control primers for the amount of template in each sample. Primer sequences used in the reaction were as follows: mouse *Bmal* forward 5'-CAGGAAAAATAGGCC GAATG-3', mouse *Bmal1* backward 5'-GCGATGACCCT CTTATCCTG-3'. Cycle conditions for PCR were composed of one cycle of 94°C for 5 min, followed by 45 cycles of 94°C for 20 sec, 54°C for 30 sec, and 72°C for 30 sec. Each reaction was run in triplicate and in two independent trials with the results averaged and plotted using Microsoft Excel software. Differences between levels of gene expression data were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

RESULTS AND DISCUSSION

It has been recently discovered that the circadian clock is ubiquitously present in life forms on the Earth. Circadian clock genes that are responsible for the clock in the brain have now been identified even in non-neural cells in the organism and are rhythmically expressed with each tissue-specific phase of oscillations. In the present report, to figure out any possible role(s) of mPER1 and mPER2 in the initiation or phase shift of the clock in non-neural cells, which are distinct from the brain clock, two stably inducible cell lines of mPER1 and mPER2 were prepared. In addition, the expression profiles of a key clock gene, *mBmal1*, were compared as an indicator of circadian oscillation in these cells. Our data showed that induction of either *mPer1* or *mPer2* alone is not sufficient to cause phase shifting in the peripheral cellular clock. Moreover, induction of *mPer* genes in these cells caused reduction in the level of *mBmal1* expression.

Selection of cell lines stably expressing mPER-V5 proteins

To ectopically induce mammalian PER proteins in cells, the Ecdysone inducible system (Invitrogen) was adopted. Two constructs were prepared that can freely regulate the expression of proteins by placing either *mPer1* or *mPer2* cDNAs under the promoter elements that respond to the insect molding hormone, ecdysone. This plasmid also has coding sequences for V5 epitope tag and six histidine stretches in frame to make the detection of proteins easier (Fig. 1A). To localize the induced proteins in cells, immunocytochemisty was then performed by using mouse anti-V5 and Cy3-conjugated anti-mouse IgG antibodies. Consistent with other reports on HEK293 cells (Vielhaber et al., 2000), both of the mPER-V5 proteins were stably induced and mainly localized in the nucleus when induced. The nuclear localization was confirmed by staining cells with DAPI (Fig. 1B). Our data strongly suggest that these two chimeric proteins can participate in the negative feedback regulation of their target gene expression in the nucleus of these cells, known as their main roles in the brain clock (Reppert and Weaver, 2001).

At least ten single cells were then isolated by serially diluting these cell mixtures and the level of protein expression of each cell line was measured by immunoblotting with anti-V5 antibodies. Two cell lines that give strong signals of induction were chosen and named sc-f for *mPer1* and sc-2 for *mPer2*, respectively (Fig. 2A). Moreover, the mobility of both proteins on gel also changed, especially for mPER2-V5, implying that an event observed *in vivo* also

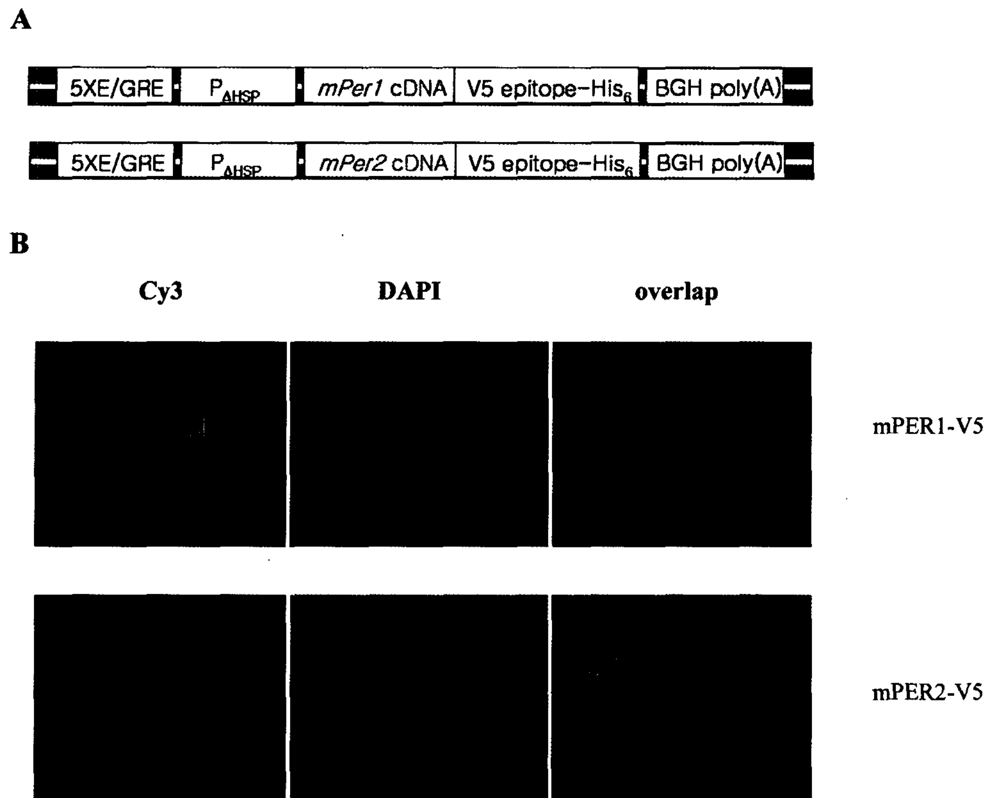


Fig. 1. A, Schematic representation of plasmid vectors used for transfection into cell lines. Either *mPer* cDNAs are placed under the control of enhancer elements (5XE/GRE) and minimal promoters. pIND *mPer1*.V5.His$_6$ for mPER1 expression (top), pIND *mPer2*.V5.His$_6$ for mPER2 expression (bottom). B, Cellular localization of mPER proteins by immunocytochemistry. Cells were processed for immunocytochemistry by using a monoclonal anti-V5 primary antibody, and a Cy3-conjugated anti-mouse IgG secondary antibody. The cells were also DAPI stained to localize the nuclei. Slides were viewed under a fluorescence microscopy. mPer1 cell mixtures (top low), mPer2 cell mixtures (bottom low).

occured in these cells, indicative of post-translational modification. For example, phosphorylation of PER is known to be very important for protein turnover and regulation of circadian clock (Edery, 1999). The optimal protein induction conditions were further defined in detail in sc-f and sc-2 cells. First, cells were treated with a range (2.5-20 μM) of ecdysone as an inducer for 12 h and the level of protein expression was measured. The maximum level of proteins was induced at a concentration of 5-10 μM for sc-f cells and of 10 μM for sc-2 cells (Fig. 2B). Then, the incubation time for the induction was determined by culturing cells with 10 μM inducer and varying incubation periods from 30 min to 12 h. This showed that both mPER-V5 proteins were expressed from 3 h after induction and increased continuously, reaching their maximum by 12 h (Fig. 2C).

Effect of ectopic induction of mPER proteins on the *Bmal1* transcription

To further determine the roles of ectopically induced mammalian PER proteins in phase shifting the cellular clock, we induced either mPER-V5 proteins in cells, and then measured temporal expression profiles of the canonical clock gene *mBmal1*. Cells were grown and treated with 10 μM of ecdysone for 12 h and then removed. Cells were collected every 4 h before and after the induction and the extracts were prepared. Induction of chimeric proteins was confirmed by immunoblotting using antibodies directed against V5 epitope tag (Fig. 3A top middle and bottom). The oscillation of *mBmal1* expression was analyzed by performing real-time PCR using specific primers. The results showed that ectopic induction of either mPER1-V5 (top) or mPER2-V5 proteins (bottom) were not capable of initiating or phase-shifting the cellular circadian clock, as measured by changes in the expression level of a key clock gene, *mBmal1* (Fig. 3B).

In mammalian brain SCN, two bHLH-type transcription factors, mCLOCK and mBMAL1 bind each other and transcribe target genes including circadian clock genes. Three *mPer* and two *mCry* genes are identified in mammals. In the SCN, all of these genes show their peak expression in the morning and trough at night. mPER and mCRY proteins interact with each other and move into the nucleus. Once in the nucleus, they interact with mCLOCK-mBMAL1 and inhibit their action. Previously, it has been suggested that mPER1 appears to be involved in post-translational stabilization of mPER2 and mCRY1, while

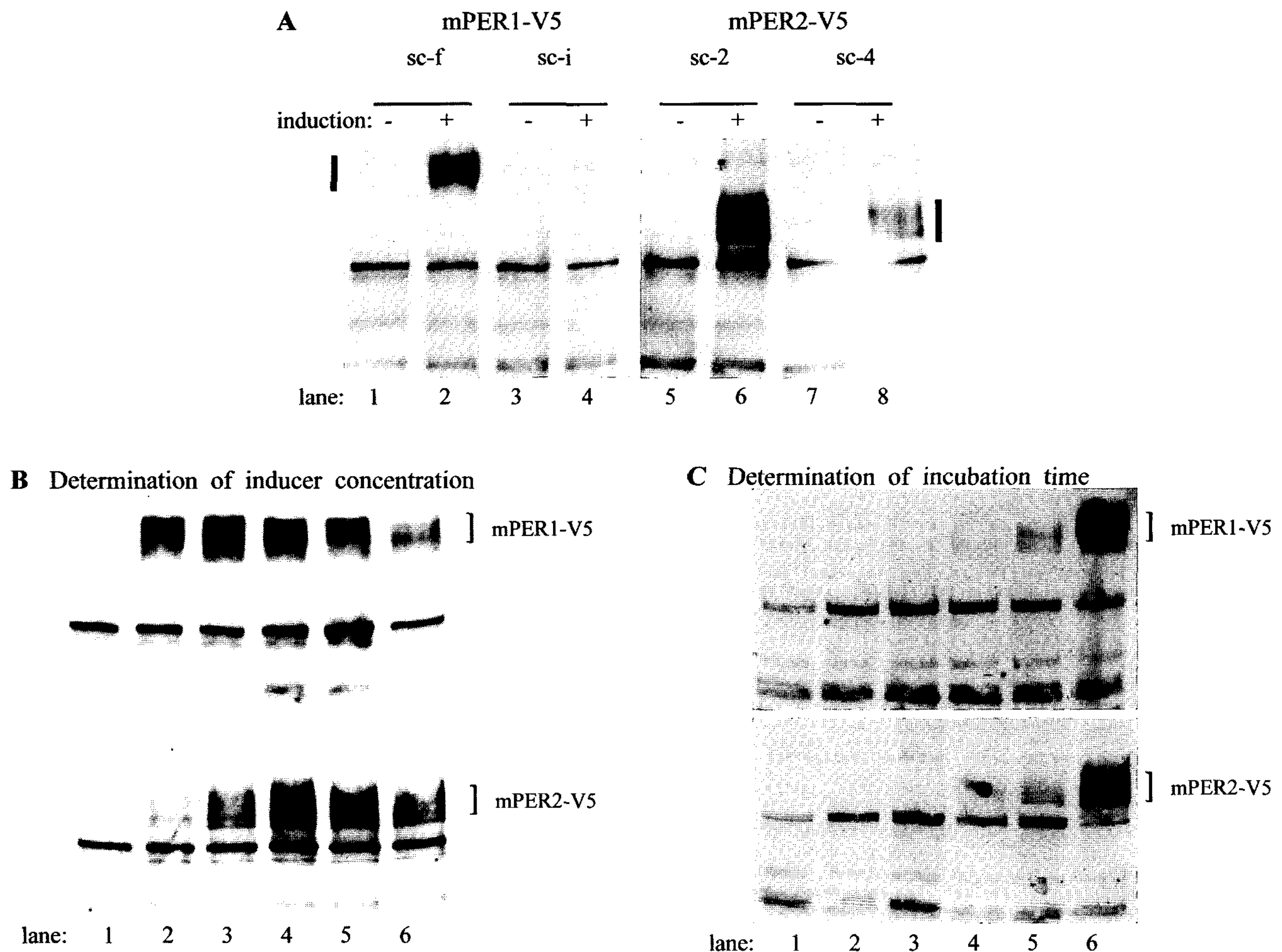
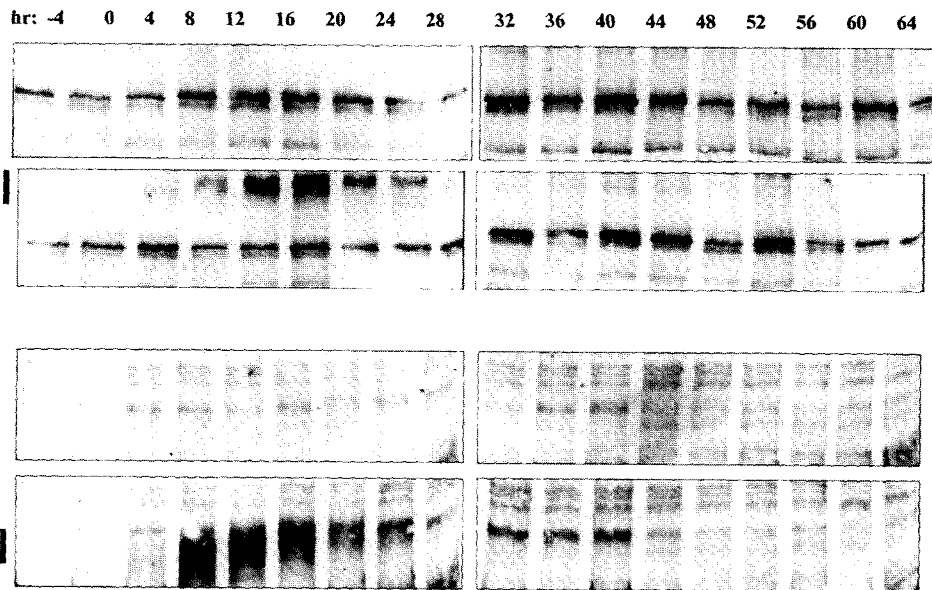


Fig. 2. A, Biochemical detection of mPER-V5 fusion proteins from inducible cell lines by using a monoclonal anti-V5 antibody. Cells were treated with the inducer ecdysone (10 μm) for 12 h and processed for protein analysis. Vertical bars indicate the position of either mPER1-V5 (left) or mPER2-V5 proteins (right). mPer1 cell lines, sc-f and sc-i (lanes 1-4), mPer2 cell lines, sc-2 and sc-4 (lanes 5-8). B, Determination of inducer concentration for maximal expression of target proteins. Cells were treated with an increasing concentration of ecdysone and the protein induction was analyzed by using immunoblot. lane 1: no addition, 2: 2.5 μM, 3: 5 μM, 4: 10 μM, 5: 15 μM, 6: 20 μM. mPer1 cell line sc-f (top), mPer2 cell line sc-2 (bottom). C, Determination of incubation time for inducer treatment for maximal induction of target proteins. Cells were treated with 10 μM ecdysone with an increasing time intervals and analysed by immunoblot. lane 1: no addition, 2: 30 min, 3: 60 min, 4: 3 h, 5: 6 h, 6: 12 h mPer1 cell line sc-f (top), mPer2 cell line sc-2 (bottom).

mPER2 positively regulate mBMAL1 at the transcriptional level (Shearman et al., 2000; Bae et al., 2001). The role of mPER2 on the expression and circadian clock-specific regulation of mBMAL1 has been well established and was originally suggested by Shearman et al. (2000). Because mPER2 is found to have no known DNA binding motif, they must exert positive regulation of *mBmal1* transcription via an indirect way, probably inhibiting transcriptional repressors or recruiting activators on the *mBmal1* promoter. In accordance with this idea, two transcriptional regulators, named REV-ERBα and RORα, have recently been identified (Preitner et al., 2002; Sato et al., 2004). Interestingly, in our real-time PCR analysis, ectopic induction and the presence of chimeric mPER2-V5 proteins did not cause *mBmal1* activation. Rather, they seemed to dampen the expression, in contrast to their positive role in the brain clock. In mammalian clocks, mCRY1 and mCRY2 proteins are known to be strong negative regulators of circadian clock-specific mCLOCK-mBMAL1 protein dimers. However, it is worth noting that there are precedents of mPER protein's negative role in the feedback regulatory loops in cultured cells (Kume et al. 1999; Yagita et al., 2001).

Consistent with the previous report (Yagita et al., 2001), the level of *mBmal1* transcripts oscillated in a circadian manner in the cell lines with their own specific phase of oscillation, as shown in Fig. 3B. For instance, while the level of *mBmal1* showed its peak expression 4 h after induction in mPer1 control cells, it showed two peaks around 8-16 and 28 h in mPer2 counterpart. Surprisingly, there was no difference in the level of *mBmal1* gene expression before and after the induction of either mPER proteins. Both mPER1 and mPER2 are initially implicated



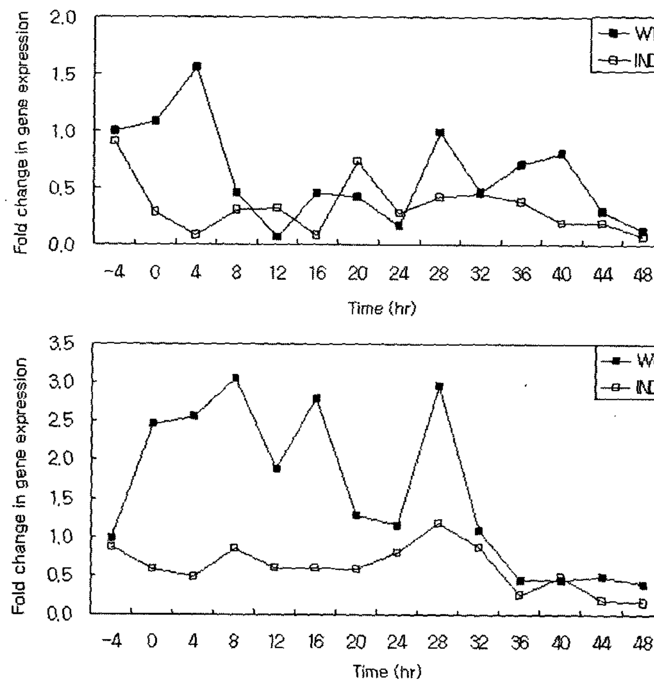


Fig. 3. Time course of clock protein and mRNA cycling. Cells were grown and treated with ecdysone for 12 hours starting at time 0 and then removed. Cells were collected every 4 h before and after induction and the extracts were prepared and analyzed either by immunoblotting (A) or by real-time PCR (B). A, Immunoblots using antibodies directed against V5 epitope tag. Vertical bars indicate the position of either mPER1-V5 (top middle) or mPER2-V5 proteins (bottom). mPer1 cells without induction (top), mPer1 cells with induction (top middle) mPer2 cells without induction (bottom middle), mPer2 cells with induction (bottom). B, Real-time PCR using oligos specific for *mBmal1* gene. Human GAPDH primers were also used as internal control and fold changes in *mBmal1* gene expression were normalized and plotted every 4 h before and after induction. Cells with or without induction are marked as IND (line with open squares) and WT (line with closed squares), respectively. mPer1 cell line sc-f (top), mPer2 cell line sc-2 (bottom).

in clock phase shifts in the SCN to light pulses in a time-of-day specific manner. While *mPer1* mRNA is rapidly induced by a single light pulse both early and late in subjective night, *mPer2* induction is restricted to early

subjective night (Albrecht et al., 1997; Shigeyoshi et al., 1997). Although these induction schemes were introduced because what is known in the SCN, the present results indicate that either of the two mPERs is not sufficient for the rhythmicity of circadian clock genes in cultured cells. This is in stark contrast to their proposed roles on the clock shift in the brain SCN. One possible explanation is that mPER induction may be strictly dependent on the time-of-clock phase to influence the magnitude of the phase shift in cultured cells. The same mPER1 and mPER2 are required for the maintenance of circadian rhythmicity in the SCN (Zheng et al., 1999; Bae et al., 2001).

In conclusion, this study shows that ectopic induction of mPER1 and mPER2 proteins is not sufficient for the rhythmic gene expression in cultured cells. Moreover, mPER2 is not likely to function as a transcriptional activator for *mBmal1* expression in these cells. Therefore, they may function differently in the brain and peripheral cells to manifest the rhythmic oscillation in gene expression. Further studies are required to identify the distinct clockwork in which the two mPER proteins participate in non-neural cultured cells.

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