The human *PER1* gene is transcriptionally regulated by multiple signaling pathways

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Abstract The mammalian period (*Per*) genes are components of the circadian clock and appear to be regulated via an autoregulatory feedback loop. Here we show that the human *PER1* (*hPER1*) gene is synergistically activated by protein kinases A and C (PKA, PKC) and cAMP responsive element binding protein. Activators and inhibitors of PKA as well as PKC modulate endogenous *hPER1* expression and *hPER1* promoter-driven reporter gene activity in a dose-dependent manner. Our results suggest that the *hPER1* promoter acts as a sensor for multiple signaling molecules thereby integrating different physiological parameters. This regulation of *hPER1* appears to be significant for rapid adaptation to changing environmental conditions.

Key words: Circadian rhythm; Chronobiology; Human *Per* gene; Signaling pathway; Clock resetting

1. Introduction

The circadian clock is a self-sustaining oscillator with a periodicity of approximately 24 h and controls many physiological and behavioral systems [1]. On the molecular level, the oscillator consists of an autoregulatory feedback loop involving a set of clock genes (see [2]). Central components of the mammalian circadian clock are the period (*Per*) genes which are expressed in a diurnal manner in various tissues on the RNA and protein levels [3–9] and also in cell culture [10]. Because the intrinsic circadian rhythm of an organism is not exactly 24 h the clock needs to be reset periodically. Light induces *Per1* and *Per2* gene expression [5,6,8,9,11]. In vitro, rhythmic *Per1* and *Per2* expression is inducible by high serum [10], forskolin [12,13] and phorbol 12-myristate 13-acetate (PMA) [14]. In the SCN circadian regulation of gene expression can be mediated by cAMP analogs [15] through cAMP response elements (CREs) [16]. Recent results indicate that expression of *Per1* is regulated in a species-dependent manner. Alternative usage of promoter elements can elicit different responses [17–19]. Since the read-outs of reporter gene assays with *hPER1*–luciferase constructs depend on the promoter fragment used [17–19], we compared endogenous activation of *hPER1*, measured by quantitative PCR (TaqMan) analysis, with *hPER1*–luc reporter gene assays in HUH-7 human hepatoma cells. Our results indicate that our reporter gene assay reflects endogenous *hPER1* expression. Moreover, we found that at least two independent signaling pathways are involved in the acute induction of the human *PER1* gene.

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Abbreviations: CREB, cAMP responsive element binding protein; PBGD, porphobilinogen deaminase; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; *Rp*-8-CPT-cAMPS, 8-(4-chlorophenylthio)adenosine-3′,5′-cyclic monophosphorothioate, *Rp*-isomer; *Sp*-5,6-DCl-cBiMPS, 5,6-dichlorobenzimidazole-1-β-D-ribofuranosyl-3′,5′-monophosphorothioate, *Sp*-isomer

2. Materials and methods

2.1. Cell culture

Human hepatoma (HUH-7) cells were cultured in Dulbecco's minimum essential medium (MEM)/10% fetal calf serum (Biowhittacker), penicillin/streptomycin (100 U/ml), and 2 mM L-glutamine at 37°C in a 5% CO_2 atmosphere.

2.2. TaqMan analysis

500 000 cells per well were seeded in 6 well plates, preincubated overnight and stimulated with 5,6-dichlorobenzimidazole-1-β-D-ribofuranosyl-3′,5′-monophosphorothioate, *Sp*-isomer (*Sp*-5,6-DCl-cBiMPS, 100 μM) and PMA (1 μM) in serum-free MEMα (Gibco) for 2 h or left untreated. For RNA isolation and DNase treatment the NucleoSpin RNA II Kit (Macherey-Nagel) was used. 2 μg of RNA was reverse transcribed using SuperScript II (Gibco) with 250 ng random primer (Gibco). TaqMan PCR was carried out in a Perkin-Elmer 7700 system in duplicate in a total volume of 25 μl using TaqMan Universal PCR Master Mix (Applied Biosystems), 1 μl cDNA diluted 1/100, primers and probes (200 nM each). Thermocycling was performed at 50°C for 2 min, 95°C for 10 min and 45 cycles between 95°C for 15 s and 59°C for 1 min. The quantification of *PER1* was done using two primers and a fluorescent probe covering parts of exons 22 and 23. For normalization of template concentration primers and probes for the housekeeping gene porphobilinogen deaminase (*PBGD*) [20] were used. Resulting C_T values of *hPER1* amplification were normalized to C_T values of *PBGD* and analyzed according to the $\Delta\Delta C_T$ method [21].

2.3. Construction of the hPER1 promoter luciferase reporter gene construct

The *hPER1* promoter was derived from the cosmid 39C2 [3]. A 3.7 kb *Hin*dIII fragment was subcloned into pBluescriptII SK(+) and sequenced (GenBank accession number AF284444). A *Hin*dIII/*Nco*I fragment was ligated into the promoterless pGL2 vector (Promega). The fragment comprises the sequence from position −1347 to +1317 relative to the putative transcription start site [18] and will be referred to as *hPER1–luc*.

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2.4. Transfection, stimulation and luciferase reporter gene assays

Transfection was done according to the supplier's instructions (Qiagen). 40 000 cells per well were plated onto 96 whitewell plates (Costar), incubated for 16 h and stimulated for 4 h in 25 μl total volume. Inhibitors were preincubated for 20 min. Then 25 μl Steady Glo solution (Promega) per well was added and the cells were lysed for 20 min by shaking at 250 U/min. Light emission was measured in a luminometer (Lumistar, BMG).

2.5. Immunoblot analysis

The cells were left untreated or treated for 1 h with *Sp*-5,6-DCl-cBiMPS (100 μM), PMA (30 nM) alone or in combination. The cells were lysed, electrophoresed and blotted as described [22]. Membranes were incubated with antisera against pCREB, CREB, pMAPK42/44 or MAPK42/44 (1:2000, New England BioLabs, Beverly, MA, USA) overnight at 4°C. Signals were detected as described [22].

2.6. Statistics

Data were analyzed using MS Excel and GraphPad Prism (3.0 for Windows; GraphPad Prism Software Inc., San Diego, CA, USA). Data are expressed as the means (±S.D.) and were compared using ANOVA with subsequent Bonferroni tests for multiple comparisons or Student's *t*-test, with $P < 0.05$ as the criterion of significance.

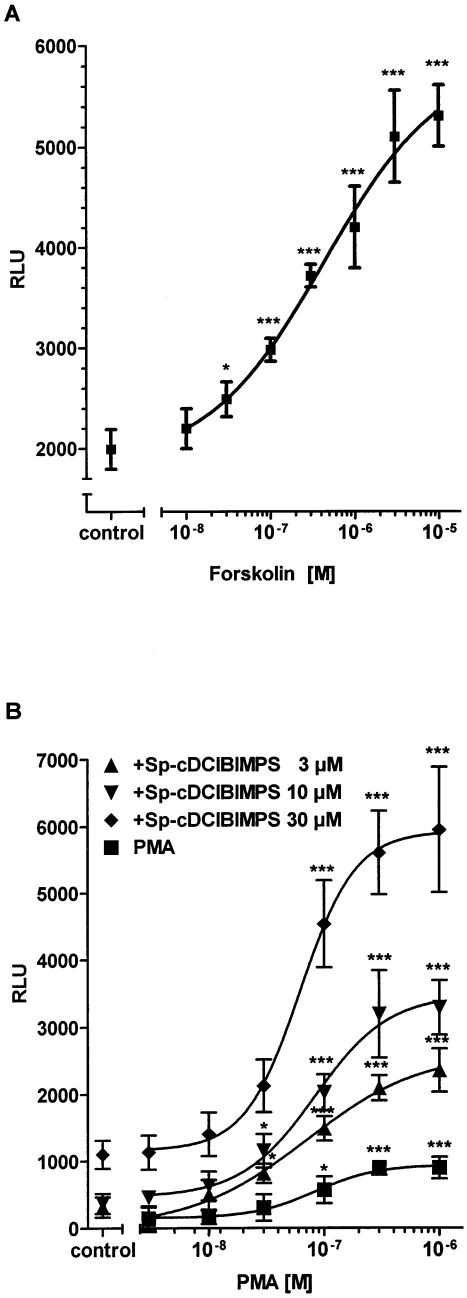

Fig. 1. Induction of *hPER1–luc* in HUH-7 human hepatoma cells. A: Dose-dependent stimulation of *hPER1–luc* by forskolin ($n = 8$). B: Dose–response curve of *hPER1–luc* activation by the phorbol ester PMA alone or in combination with increasing amounts of the protein kinase A (PKA) activator *Sp*-5,6-DCl-cBiMPS (*Sp*-5,6-DCl-cBiMPS alone = control) ($n = 6$). Statistical significance was calculated by ANOVA with Bonferroni post test relative to the control values with $*P < 0.05$, $***P < 0.001$.

3. Results

We first tested the inducibility of the *hPER1–luc* reporter construct by forskolin, a known inducer of circadian *Per1* expression [13]. Forskolin led to a concentration-dependent induction of the *hPER1* reporter gene activity in HUH-7 human hepatoma cells (Fig. 1A). This was comparable to the induction observed in rat-1 fibroblasts [13] indicating that the human *PER1* promoter responds in a similar fashion to forskolin as the rat *Per1* promoter.

Forskolin elevates cAMP levels in the cell. Therefore we tested whether the cell-permeable and PKA-specific cAMP analog *Sp*-5,6-DCl-cBiMPS [23] could activate the human *PER1* promoter. Fig. 1B (control) shows that this is the case. Also the protein kinase C (PKC) inducer PMA led to a significant increase of *hPER1–luc* activity (Fig. 1B). When PMA was applied in increasing concentrations together with different amounts of *Sp*-5,6-DCl-cBiMPS (3, 10 and 30 μM) the *hPER1–luc* activity was potentiated (Fig. 1B). To test whether the endogenous human *PER1* promoter is stimulated in a similar manner, we performed TaqMan PCR analysis for relative quantification of endogenous *hPER1* expression. The mRNA level of *hPER1* was elevated 2.25 (±0.25 S.D.) fold after stimulation with *Sp*-5,6-DCl-cBiMPS/PMA (Fig. 2), which is comparable to the induction of the *hPER1–luc* re-

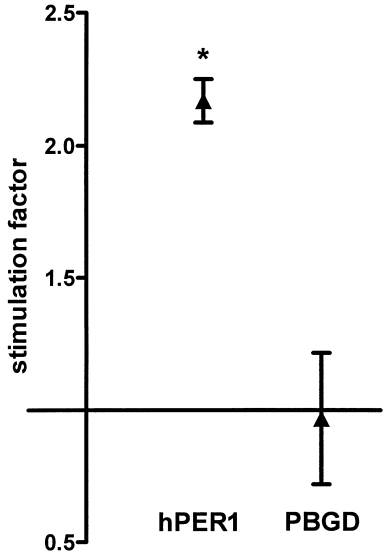

Fig. 2. Relative quantification of *hPER1* and *PBGD* after stimulation with *Sp*-5,6-DCl-cBiMPS (100 μM) and PMA (1 μM) for 2 h. Endogenous *hPER1* expression in HUH-7 cells is induced 2.25±0.25 fold whereas *PBGD* is not induced. Results show the mean of five independent experiments ±S.D. Statistical significance was calculated by Student's *t*-test, $*P < 0.001$.

porter in our assay. This indicates that the endogenous promoter responds in a similar fashion as the reporter construct.

The next question we addressed was whether this induction of *hPER1–luc* activity via the PKA/PKC pathway is inhibited by 8-(4-chlorophenylthio)adenosine-3′,5′-cyclic monophosphorothioate, *Rp*-isomer (*Rp*-8-CPT-cAMPS), a PKA inhibitor [24], and chelerythrin, a PKC inhibitor. The cells were treated with either one of the inhibitors alone, or in combination. Then the cells were stimulated with *Sp*-5,6-DCl-cBiMPS/PMA (Fig. 3). The PKA inhibitor completely blocked the *Sp*-5,6-DCl-cBiMPS/PMA-mediated induction at 1 mM. Chelerythrin, however, inhibited 40% of the *hPER1–luc* activity at 1 μM. There was no additional reduction if *Rp*-8-CPT-cAMPS and chelerythrin were added simultaneously compared to *Rp*-8-CPT-cAMPS treatment alone (Fig. 3). Both chelerythrin and *Rp*-8-CPT-cAMPS inhibited the *hPER1–luc* activity in a dose-dependent fashion (data not shown). Next we studied *hPER1–luc* induction in the presence of killer-CREB (K-CREB); [25] and A-CREB [26], two dominant-negative mutated forms of the transcription factor cAMP responsive element binding protein (CREB). Both A-CREB and K-CREB inhibited the *Sp*-5,6-DCl-cBiMPS-mediated effect, but did not influence the PMA-mediated *hPER1–luc* induction (Fig. 4). However, A-CREB also considerably reduced the basal expression of *hPER1–luc* in comparison to control and K-CREB-cotransfected cells (Fig. 4).

Next we investigated whether PKA and PKC pathways can induce CREB phosphorylation in HUH-7 cells. When treated with *Sp*-5,6-DCl-cBiMPS or *Sp*-5,6-DCl-cBiMPS/PMA an increased amount of pCREB in comparison to control or PMA-stimulated cells was observed (Fig. 5). As expected PMA (30 nM) also increased the levels of pMAPK42/44 (Fig. 5). In contrast, *Sp*-5,6-DCl-cBiMPS treatment inhibited both basal and PMA-induced pMAPK42/44 activity.

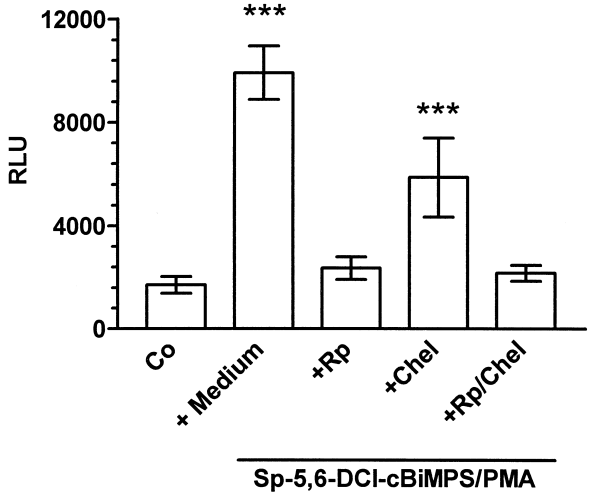

Fig. 3. Effect of PKA and PKC inhibitors on *hPER1–luc* activity in HUH-7 cells treated with PKA (*Sp*-5,6-DCl-cBiMPS) and PKC (PMA) activators. Cells were left untreated (Co) or treated with *Sp*-5,6-DCl-cBiMPS/PMA (Sp+PMA; 30 μM+300 nM) alone or together with either PKC inhibitor chelerythrin (1 μM), the PKA inhibitor *Rp*-8-CPT-cAMPS (1 mM) or both. Statistical significance was calculated by ANOVA with Bonferroni post test relative to the control value (Co) with $***P < 0.001$ and $n = 6$.

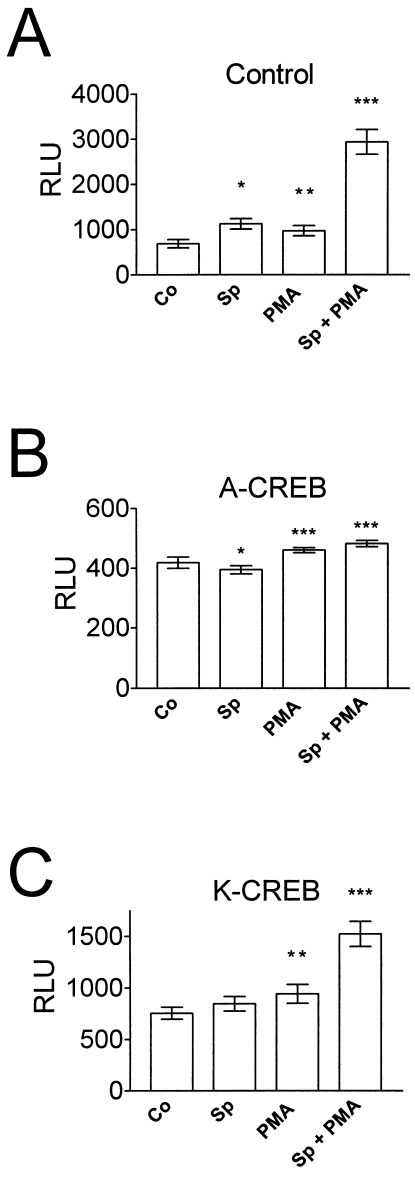

Fig. 4. Reduction of *Sp*-5,6-DCl-cBiMPS/PMA-stimulated *hPER1–luc* activity in cells cotransfected with CREB inhibitors. A: Cotransfection of the *hPER1–luc* construct with pGL2-basic. *Sp*-5,6-DCl-cBiMPS (30 μM), PMA (100 nM) and the combination of both showed increased *hPER1–luc* activity. B: Cotransfection of the *hPER1–luc* construct with A-CREB. C: Cotransfection of the *hPER1–luc* construct with K-CREB. *hPER1–luc* activity in A-CREB- and K-CREB-cotransfected cells was no longer inducible by *Sp*-5,6-DCl-cBiMPS. The PMA response was still present in the A-CREB- and K-CREB-transfected cells (B,C). Shown are the means ± S.D. of four experiments; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Statistical significance was calculated by ANOVA with Bonferroni post test relative to the control values.

4. Discussion

Recent reports [17–19] imply a high organizational complexity of *Per1* expression, e.g. murine *Per1* possesses two alternative promoters. Our data show that endogenous *hPER1* mRNA in HUH-7 cells is significantly elevated by stimulation with *Sp*-5,6-DCl-cBiMPS/PMA. To investigate the signal transduction pathway leading to *hPER1* expression a *hPER1*–luciferase promoter construct was designed which

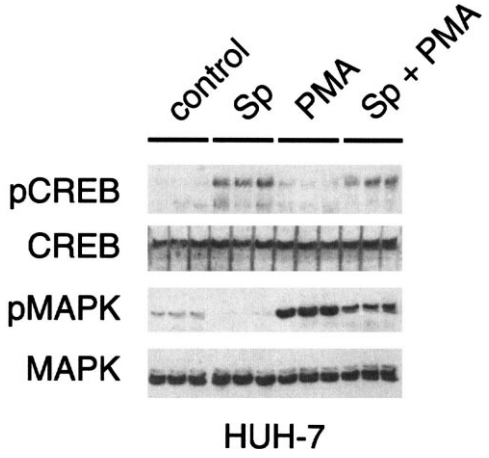

Fig. 5. Induction of CREB phosphorylation in human HUH-7 hepatoma cells treated for 1 h with the indicated agents. The antigen detected is shown on the left. As illustrated by the horizontal black bars on top, each experiment was carried out in triplicate.

reflects the endogenous stimulation in this particular system, the HUH-7 human hepatoma cell line.

Forskolin induces circadian gene expression of *Per1* in rat-1 fibroblasts [13] and also in ovine pars tuberalis cells [12]. Our data show that application of forskolin to *hPER1–luc*-transfected HUH-7 cells results in an induction of luciferase activity. Forskolin activates adenylate cyclase, enhances the synthesis of cAMP and results in activation of PKA. This is in agreement with the notion that a cAMP-mediated mechanism is important for the phase resetting of the central circadian rhythm in the SCN [27–29]. Accordingly the cAMP analog *Sp*-5,6-DCl-cBiMPS induces *hPER1–luc* activity in a dose-dependent manner. However, the *hPER1* promoter is not exclusively activated through the PKA pathway. The PKC activator PMA leads to an induction of *hPER1–luc* in a dose-dependent manner in HUH-7 cells, whereas in sheep pars tuberalis cells PMA did not induce *Per1* gene expression [12]. This indicates that the PKC signaling pathway can contribute to the activation of the *hPER1* promoter in hepatoma cells. Interestingly, coapplication of PMA and *Sp*-5,6-DCl-cBiMPS elicits a potentiation of the *hPER1* promoter activity. This suggests that coincidental extracellular events can potentiate *hPER1* induction which in turn might influence the strength of behavioral responses of an organism to environmental changes [29].

To dissect the contributions of the PKA and PKC pathways we looked at phosphorylation events. In HUH-7 cells CREB phosphorylation is primarily stimulated via the PKA pathway whereas MAPK phosphorylation is stimulated via the PKC pathway.

Although coapplication of *Sp*-5,6-DCl-cBiMPS and PMA leads to a lower elevation of pMAPK in comparison to PMA alone, both the reporter gene activity and the endogenous expression of *hPER1* were potentiated. Therefore, we conclude that MAPK phosphorylation seems to be of minor importance. The potentiation of reporter gene activation is probably assisted by additional signaling components as suggested by others [29–31].

As expected, pharmacological inhibitors specific for blocking the PKA and the PKC pathways prevented induction of *hPER1–luc*. Similarly, cotransfected mutated forms of CREB (A-CREB and K-CREB) inhibited stimulated *hPER1–luc* reporter activity. However, both CREB antagonists did not inhibit the PMA-stimulated reporter gene activity. This suggests that the PMA stimulation of *hPER1–luc* activity is not mediated by CREB phosphorylation. This is in agreement with the immunoblot results (Fig. 5).

The basal expression of *hPER1–luc* was considerably reduced by A-CREB cotransfection. This could be due to the fact that A-CREB irreversibly binds most of the endogenous CREB present in the cell [26]. The consequence would be that even housekeeping functions are affected causing a reduced responsiveness of the cell to various stimuli. Thus PKA/PKC coactivation is partly mediated via pCREB but probably needs additional unknown regulators, such as protein phosphatases [32] or transcription factors [30]. Our data suggest that the *hPER1–luc* is context-sensitive reminiscent of the LAP/C/EBP promoter in HUH-7 cells and liver [33].

In conclusion, we show here that extracellular stimulators acting through the PKA and PKC pathways regulate the human *PER1* promoter in HUH-7 cells. These pathways have previously been shown to reset the circadian clock [14,27,29,34–36]. Most importantly, rapid circadian induction of *Per* genes in cell cultures is independent of protein synthesis [10]. We provide evidence that rapid *Per* gene activation is probably achieved at least in part by phosphorylation of components of classical signaling pathways. It might be speculated that the well established autoregulatory feedback mechanism for *Per1* is modulated by second messenger pathways. Thus the *Per1* promoter may serve as a sensor and integrator of physiological changes in an organism in order to adjust the clock to environmental challenges.

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