Inducible cAMP Early Repressor Regulates the *Per1* Gene of the Hepatic and Adrenal Clocks*

Received for publication, December 16, 2012, and in revised form, February 23, 2013. Published, JBC Papers in Press, February 26, 2013, DOI 10.1074/jbc.M112.445692

Uršula Prosenc Zmrzljak1‡*1, Anja Korenčič1‡, Rok Košir1‡, Marko Goličnik2, Paolo Sassone-Corsi1‡, and Damjana Rozman1‡2

From the 1Center for Functional Genomics and Bio-Chips, Institute of Biochemistry, Faculty of Medicine, University of Ljubljana, SI-1000 Ljubljana, Slovenia, the 2Department of Laboratory Diagnostics, Institute of Oncology, SI-1000 Ljubljana, Slovenia, and the 1Department of Biological Chemistry, University of California, Irvine, California 02607

**Background:** We investigated the role of cAMP signaling (*Crem* and its splice variant *Icer*) in liver and adrenal clocks.

**Results:** cAMP signaling fine-tunes adrenal clock genes, whereas the hepatic effect is minimal. ICER regulates *Per1* by binding to its promoter.

**Conclusion:** *Icer* fine-tunes clock gene expression in the absence of light and feeding cues.

**Significance:** ICER is a circadian signal mediator.

Light, restricted feeding, and hormonal inputs may operate as time givers (zeitgebers) for the circadian clock within peripheral organs through the activation of tissue-specific signaling cascades. cAMP signaling through CREM (cAMP-responsive element modulator) and its variant ICER (inducible cAMP early repressor) is linked to the circadian regulation of pineal melatonin synthesis, although little is known about its influence in other organs. We performed experiments in the absence of light and feeding-time cues to test which core clock genes are controlled by CREM/ICER in the liver and adrenal gland. *In vivo*, *Crem* loss-of-function mutation resulted in fine-tuning of all measured adrenal clock genes (*Per1/2/3, Cry1/2, Bmal1, and Rev-erba*), whereas only *Per1* and *Cry1* were affected in the liver. *Icer* expression was circadian in the adrenal gland, with peak gene expression at zeitgeber 12 and the highest protein levels at zeitgeber ~20. The expression of both *Icer* and *Per1* genes responded to cAMP stimuli in an immediate-early fashion. In immortal cells, forskolin induced expression of *Per1* after 2 h, and *de novo* protein synthesis led to *Per1* attenuation. We show that the *de novo* synthesized protein responsible for *Per1* attenuation is ICER. Indeed, *Per1* expression is up-regulated in cells ectopically expressing antisense *Icer*, and mobility shift experiments identified ICER binding to cAMP-responsive elements of the *Per1* promoter. We propose that ICER acts as a noise filter in a time-dependent gating mechanism.

The circadian clock is an internal timekeeper that enables organisms to predict and adapt to daily changes in light intensity, food accessibility, predator activity, etc. Its organization in mammals is hierarchical: the central oscillator lies in the suprachiasmatic nucleus (SCN)1 and is reset daily with dawn light. The SCN synchronizes different peripheral oscillators through neuronal and hormonal signals (1).

Circadian expression of core clock genes (*i.e.* repressors *Per1/2/3* and *Cry1/2* and activators *Clock, Bmal1, Rev-erba*, etc.) is assured by robust autoregulatory loops (2). It is not specific to the SCN but takes place in various peripheral tissues (3). This means that on the periphery, tissue-specific modulators such as feeding and hormones and their downstream signaling pathways contribute to regulation of peripheral clocks. The liver clock is preferentially entrained by feeding-associated cues (4). In contrast, the adrenal gland can be entrained by light *in vivo* (5). It is believed that photic signals from the SCN are transduced through the autonomous nervous system to the adrenal gland, which is the organ responsible for synthesis of corticosteroids (6). Corticosteroids and the adrenal gland are considered to be synchronizers of the peripheral circadian clocks (7). Exposure to light pulses during the night (resetting signal) strongly induces transcription in the adrenal gland and results also in elevated corticosterone synthesis (6).

An important signaling pathway in steroid hormone synthesis is cAMP-dependent regulation by the PAR BZip (proline- and acid-rich basic leucine zipper) transcription factors of the CREB (cAMP-responsive element-binding protein)/CREM (cAMP-responsive element modulator)/ATF family. These proteins form homodimers or heterodimers that act as repressors or activators of transcription and have several splice variants (8). The positive loop (activation) of cAMP signaling is usually phosphorylation of CREB or CREM activators and their subsequent binding to cAMP-responsive element (CRE) sites of cAMP-responsive genes. The negative loop (repression) can involve dephosphorylation or binding of ICER (inducible

---

*1 Young Researchers supported by the Slovenian Research Agency.
2 To whom correspondence should be addressed: Center for Functional Genomics and Bio-Chips, Institute of Biochemistry, Faculty of Medicine, University of Ljubljana, Zaloška 4, SI-1000 Ljubljana, Slovenia. Tel.: 386-1-543-7591; Fax: 386-1-543-7588; E-mail: damjana.rozman@mf.uni-lj.si.
3 The abbreviations used are: SCN, suprachiasmatic nucleus; CRE, cAMP-responsive element; KO, knock-out; ZT, zeitgeber time; ANOVA, analysis of variance.
ICER Fine-tunes Per1 Expression

CAMP early repressor) to CRE sites (9). ICER is a product of the Crem gene and arises from the alternative, intronic P2 promoter (8). The difference between CREM and other PAR bZip relatives is inducibility by CAMP: the immediate-early repressor Icer is transcribed in a CAMP-dependent fashion through phospho-CREB binding to CREs, which is the same mechanism that is important for resetting of the circadian clock (10). In the pineal gland, CAMP signaling through ICER regulates the circadian synthesis of melatonin (11, 12), a pineal hormone that is excreted during the night. Night light induces the transcription of Icer, which represses the synthesis of the rate-limiting enzyme of melatonin synthesis (12).

Could ICER be important also in peripheral organs in vivo? Current evidence suggests that ICER in the liver exerts a physiologically relevant function because it is robustly induced after partial hepatectomy (9). In hepatocellular carcinoma, increased intracellular CAMP resulted in elevated levels of Icer mRNA and protein, which were proposed to inhibit hepatocyte mitogenesis (13).

In the adrenal gland, ICER seems to be a marker of (lithium) induced activation of the hypothalamic-pituitary-adrenal gland axis (14). The link between ICER and the adrenal circadian clock arises from the excellent study of Ishida et al. (6). They showed that light pulse during the night induces transcription of a variety of genes in the adrenal gland. The most elevated (4.37-fold) were transcripts of Crem, whereas Per1 was the only elevated core clock gene (1.5-fold). Both Crem (P2 promoter) and Per1 contain CREs in their promoters, suggesting that they can respond to identical CAMP stimuli.

Herein, we propose ICER as a physiologically relevant modulator that can contribute to fine-tuning of peripheral clocks, particularly in the adrenal gland, where it is expressed in a circadian manner. ICER is a suppressor of CAMP-induced immediate-early transcription of Per1 and can diminish Per1 expression by directly binding to CREs in Per1 promoter.

EXPERIMENTAL PROCEDURES

Circadian Collection of Mouse Samples and RNA Isolation—Fifty-four WT and 45 Crem knock-out (KO; Crem−/−) mice (15) were used in the experiment. Animals had free access to food and water and were maintained under a 12/12-h light cycle regime (light on at 7:00 a.m. and light off at 7:00 p.m.). The experiment was approved by the Veterinary Administration of the Republic of Slovenia (license number 34401-9/2008/4) and was conducted in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (ETS 123) as well as in accordance with the National Institutes of Health guidelines for work with laboratory animals. The process of sample preparation and total RNA isolation was described previously (16). In brief, mice were killed under dim red light every 4 h during a 24-h period starting at 7 a.m. (zeitgeber time (ZT) 0). Liver and adrenal glands were excised, snap-frozen in liquid nitrogen, and stored at −80 °C.

Primer Design and Quantitative PCR Analysis—Intron-spanning primers were designed for the core clock genes (Table 1). Primer specificity and amplification efficiency were also validated empirically with melting curve and standard curve analysis of a 6-fold dilution series. The primers and probe for Icer were designed by Primer Design Ltd. Normalization was done as described (17) using Eq-PCR Wizard. Real-time quantitative PCR was performed in a 384-well format on LightCycler 480 system (Roche Applied Science) using LightCycler 480 SYBR Green I Master for the core clock genes and LightCycler 480 Probes Master (Roche Applied Science) for the Icer gene. PCR consisted of 2.5 μl of Master, 1.15 μl of RNase-free water, 0.6 μl of 300 nm primer mixture, and 0.75 μl of cDNA in a total volume of 5 μl. Three technical replicates were performed for each sample. Cycling conditions were as follows: 10 min at 95 °C, followed by 40 rounds of 10 s at 95 °C, 20 s at 60 °C, and 20 s at 72 °C. Melting curve analysis for determining the dissociation of PCR products was performed from 65 to 95 °C. Two-way analysis of variance (ANOVA) was used for determining statistically significant differences between KO and WT mice.

Fitting Trigonometric Functions to Gene Expression Data—Experimental data were fitted by trigonometric functions as described (18). Briefly, in addition to the 24-h period, 12-h harmonics were included to reproduce variable waveforms and peak width (Equation 1).

\[ x_t = a_1 \sin\left(\frac{2\pi}{24\ h} t\right) + a_2 \cos\left(\frac{2\pi}{24\ h} t\right) + a_3 \sin\left(\frac{2\pi}{12\ h} t\right) + a_4 \cos\left(\frac{2\pi}{12\ h} t\right) + a_5 \quad (\text{Eq. 1}) \]

To evaluate the differences in gene expression in WT and KO mice, we performed bootstrapping on the original gene expression data. Each time, half of the data points were randomly chosen, and phases were recalculated. After 10 repetitions, results were plotted as box plots. To avoid the effect of differential gene expression levels on amplitudes, fits were subsequently normalized (divided by the mean of expression of each gene in 24 h), and box plots for amplitudes are shown for the normalized fits (see Figs. 2 and 3).

Protein Isolation and Western Blotting—Proteins from adrenal glands were isolated from the same samples as RNA with Tri Reagent® (Sigma) according to the manufacturer’s instructions. Samples of animals from the same time points were pooled to obtain enough protein for ICER detection. Anti-CREM antibodies (Santa Cruz Biotechnology) and HRP-conjugated mouse anti-rabbit secondary antibodies (Sigma) were applied. As loading controls, anti-β-actin monoclonal antibodies (Sigma) and HRP-conjugated anti-mouse secondary antibodies (Amersham Biosciences) were used. Detection of CREM was performed with West Femto substrate (Pierce). For detection of actin, we used ECL substrate (Pierce). Detection was done using a Fujifilm LAS 4000 system.

Cell Cultures and Transfections—Mouse adrenocortical cell line Y-1 (ATCC CCL-79) and mouse hepatoma cell line Hepa 1-6 (ATCC CRL-1830) were grown in DMEM (Sigma D7777) supplemented with 10% fetal calf serum (Sigma F9665). When cells were confluent, they were cultured in serum-free DMEM and treated with different chemicals dissolved in dimethyl sulfoxide.
oxide (Sigma), 10 μM forskolin (Sigma), 25 μM cycloheximide (Sigma), and 25 μM H-89 (Sigma).

At the time of sampling, cells were harvested, and RNA was extracted with QuickGene RNA cultured kit S (Fujifilm) on QuickGene-810 (Fujifilm) according to the manufacturer’s instructions. RNA quantity and quality were assessed with NanoDrop and Agilent 2100 Bioanalyzers. DNase treatment was performed using DNase I (Sigma). cDNA synthesis was carried out using a SuperScript VILO cDNA synthesis kit (Invitrogen) with 1 μg of RNA according to the manufacturer’s instructions. The experiment was repeated independently two times in three replicates.

For protein extraction, cells were washed three times with ice-cold PBS and lysed with lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 5 mM EDTA, and Mini mixture protease inhibitors (Roche Applied Science)). The whole procedure was performed on ice. Lysates were transferred to cooled microcentrifuge tubes, rotated for 10 min at 4 °C, and cold-centrifuged at 13,200 rpm.

For transient transfection, Lipofectamine 2000 reagent (Invitrogen) was used together with luciferase reporter constructs containing the Per1 promoter (19) and an Icer antisense plasmid (a kind gift from Dr. M. S. Reitz) (22). The binding reaction of ICER and oligonucleotides was carried out in the presence of binding buffer (200 mM HEPES (pH 7.6), 500 mM KCl, 10 mM EDTA), glycerol, DTT, MgCl₂, and poly(dI/dC) at 37 °C for 20 min. Anti-His tag antibodies (Abcam ab9108) were added to the supershift reaction. Samples were separated on a polyacrylamide gel and scanned with a microarray scanner (Tecan) at 670 nm.

**TABLE 1**

<table>
<thead>
<tr>
<th>Genes</th>
<th>NCBI accession no.</th>
<th>Sequence</th>
<th>Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference genes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rplp0</td>
<td>NM_007475.4</td>
<td>CACTGTCCTAGACCAGGAGAAAG</td>
<td>1.98</td>
</tr>
<tr>
<td>Hprt</td>
<td>NM_013556.2</td>
<td>GCCCTGCTGAGATTTTCG</td>
<td>1.89</td>
</tr>
<tr>
<td>Rn18s</td>
<td>NR_003278.1</td>
<td>GGGCCTAGAGTGAATTTTC</td>
<td>1.79</td>
</tr>
<tr>
<td>Core circadian clock</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Per1</td>
<td>NM_011065.3</td>
<td>TCCCTCTCTACTACCTGCTCT</td>
<td>1.90</td>
</tr>
<tr>
<td>Per2</td>
<td>NM_011066.3</td>
<td>CAACAGACGACGACGCTCA</td>
<td>1.94</td>
</tr>
<tr>
<td>Per3</td>
<td>NM_011067.1</td>
<td>CTGGTCTCTCTCTGACACAC</td>
<td>2.00</td>
</tr>
<tr>
<td>CRY1</td>
<td>NM_007771</td>
<td>CCCAGCTCTTTCAAGATGGAACCA</td>
<td>2.00</td>
</tr>
<tr>
<td>CRY2</td>
<td>NM_009963</td>
<td>AGGCTGCAGAAGTGGCAT</td>
<td>1.94</td>
</tr>
<tr>
<td>Bmal1</td>
<td>NM_007489</td>
<td>GGTACGCCACTGACTCCAAGGA</td>
<td>2.00</td>
</tr>
<tr>
<td>Rev-erha</td>
<td>NM_145434.3</td>
<td>AGACCCTGACTCCCAATAA</td>
<td>2.00</td>
</tr>
</tbody>
</table>

**TABLE 2**

<table>
<thead>
<tr>
<th>Oligonucleotide sequences used for EMSA</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Name</td>
<td>Oligonucleotide sequence (5’−3’)</td>
</tr>
<tr>
<td>mPer1_wt_fw_Cy5</td>
<td>TCCCTCTCTGAGCACCCTCCT</td>
</tr>
<tr>
<td>mPer1_wt_rev_Cy5</td>
<td>AGGGAGGTGAGCACCTAAGGA</td>
</tr>
<tr>
<td>mPer1_mut-fw_Cy5</td>
<td>TCCCTCTCTCAGAGCACCCTC</td>
</tr>
<tr>
<td>mPer1_mut_rev_Cy5</td>
<td>AGGGAGGTGAGCACCTAAGGA</td>
</tr>
<tr>
<td>mPer1_wt_fw</td>
<td>TCCCTCTCTGAGCACCCTCCT</td>
</tr>
<tr>
<td>mPer1_wt_rev</td>
<td>AGGGAGGTGAGCACCTAAGGA</td>
</tr>
</tbody>
</table>

**Electron Mobility Shift Assay**—5′-Cy5-labeled oligonucleotides (Eurogentec) for WT and mutant CREs in the Per1 promoter were used (Table 2). Single-stranded oligonucleotides were annealed for 5 min at 95 °C and then left to cool slowly to room temperature for 3 h. The ICER protein was prepared with the TnT system (Promega) following the manufacturer’s instructions with an Icer-His tag expression plasmid (a kind gift from Dr. M. S. Reitz) (22). The binding reaction of ICER and oligonucleotides was carried out in the presence of binding buffer (200 mM HEPES (pH 7.6), 500 mM KCl, 10 mM EDTA), glycerol, DTT, MgCl₂, and poly(dI/dC) at 37 °C for 20 min. Anti-His tag antibodies (Abcam ab9108) were added to the supershift reaction. Samples were separated on a polyacrylamide gel and scanned with a microarray scanner (Tecan) at 670 nm.
RESULTS

Expression Profiles of Core Clock Genes in Livers and Adrenal Glands of Crem<sup>−/−</sup> Mice—To evaluate the role of CREM in the circadian clock, we compared expression profiles of selected core clock genes (Per1/2/3, Cry1/2, Bmal1, and Rev-erbα) between WT and KO mice (Fig. 1). The Period genes were chosen because of their inducibility by cAMP. We measured Cryptochrome because deletion of Cry1 and Cry2 in mouse embryonic fibroblasts results in modified expression of genes from cholesterol synthesis (23). Bmal1 and Rev-erbα have also been linked previously to cholesterol metabolism, which is relevant for liver and adrenal glands (24). In the liver, we observed only subtle changes in clock gene expression between wild-type and Crem KO mice, as shown in Fig. 1A with an overlay of fitted trigonometric functions. Two-way ANOVA between WT and KO samples suggested a statistically significant role for Crem in fine-tuning the circadian expression of Cry1 (p < 0.0001) and Per1 (p = 0.0405). Bootstrapping of experimental data (Fig. 2) suggested that deletion of Crem results in the phase advance of Cry1 and its lower amplitude in the liver, whereas it failed to detect changes in amplitude or phase for Per1.

A more prominent role of Crem was observed in the adrenal gland (Fig. 1B); tuning of all measured clock genes could be observed. Bootstrapping analysis (Fig. 3) suggested that the absence of Crem influences the circadian phase: Per1 was phase-delayed, and Cry1 was phase-advanced. The effect of Crem KO on the Per1 and Cry1 amplitudes seemed less obvious.

Overall, Crem influenced the phases and amplitudes of other circadian clock genes as well. The effect was stronger in the
adrenal gland, where we observed changes in phases (Per and Cry) and amplitudes (Bmal1 and Rev-erba) (Figs. 1–3).

Icer mRNA and Protein Are Circadian in the Adrenal Gland—The Crem gene has at least two functional promoters, leading to several transcripts that have not yet been investigated in a circadian context in the periphery. The internal P2 promoter of Crem is cAMP-inducible and leads to Icer transcription (Fig. 4A). Due to very low expression levels of Icer in the liver, it is difficult to assess the potential circadian profile (data not shown). On the other hand, Icer showed a circadian pattern with an expression peak at ZT 12 in the adrenal gland (Fig. 4B). Differences between the subjective day and the subjective night were detected also at the protein level, where more ICER was present in adrenal glands in the subjective night (Fig. 4C). Unfortunately, the Western blots could not be statistically evaluated because we pooled protein samples of several animals for each time point to obtain sufficient amounts of proteins.

cAMP Signaling and ICER Control Per1 Immediate-early and Circadian Expression—In the core clock gene screen (Figs. 1–3), Per1 was among the most affected genes in Crem KO samples. Both Icer (25) and Per1 (19) are inducible by cAMP in an immediate-early fashion. The mouse Period1 gene (Per1) promoter contains CREs that bind CREB from SCN protein extracts. CREB acts as a pivotal end point of signaling pathways for the regulation of mouse Per genes in the SCN. However, in the brain, the signaling-dependent activation of mouse Per genes is distinct from the CLOCK/BMAL1-driven transcription required within the clock feedback loop (19). To evaluate the response of Icer transcription to cAMP signaling, we tested the hepatic Hepa 1-6 and adrenal Y-1 cells. To our surprise, a 4-h forskolin treatment of Hepa 1-6 cells induced ICER protein expression more intensely than treatment of Y-1 cells (Fig. 5A). We used Hepa 1-6 cells in further experiments. Fig. 5B shows that forskolin induced Icer mRNA expression after 2 h, which is in line with the detected ICER protein (Fig. 5A and B). The addition of cycloheximide, an inhibitor of protein synthesis, resulted in elevated levels of Icer mRNA, whereas the addition of H-89, a protein kinase A inhibitor, repressed Icer mRNA synthesis (Fig. 5B). Similar conclusions could be reached for Per1: cycloheximide elevated expression of Per1, whereas H-89 led to diminishment (Fig. 5D). This suggests that de novo protein synthesis and phosphorylation by PKA contribute to cAMP-dependent immediate-early expression of Icer and Per1.
in Hepa 1-6 cells. After this early wave of expression, forskolin provoked also the circadian expression of Per1 (Fig. 5E). According to current knowledge, ICER is the only cAMP-inducible transcription factor from the CREB/CREM/ATF family. It is a repressor of the cAMP-mediated early response that binds to CREs in promoter regions of cAMP-responsive genes (25).
ICER Represses Transcription of Per1 through Binding to the CRE in the Per1 Promoter—The Per1 promoter contains several CREs that can bind CREB (19). CREB is a constitutively expressed transcription factor that is activated by phosphorylation upon cAMP stimuli and is involved in Per1 regulation in the pineal gland. We tested the hypothesis that fine-tuning Per1 on the periphery can be caused by the cAMP-inducible repressor ICER. Hepa 1-6 and Y-1 cells cotransfected with the Icer antisense plasmid and the Per1 promoter-reporter construct revealed an increased reporter activity in the presence of antisense Icer (Fig. 6A). Gel shift analysis with supershift and competition assay identified ICER as the protein that specifically binds the CRE of the Per1 promoter (Fig. 6B). Together, the data indicate that ICER is likely an important repressor of cAMP-dependent Per1 transcription. We could not show the direct inhibition of Per1 with ICER transfections (data not shown). According to recent literature, ICER introduced into immortal cells by transient transfection is immediately phosphorylated, which is a signal for its transport to the cytoplasm (26). To evaluate this result for Hepa 1-6 cells, we measured ICER proteins after transfection (Fig. 6C). Indeed, a large proportion of the overexpressed ICER protein also in Hepa 1-6 cells ended in the cytoplasm, where it cannot influence transcription.

DISCUSSION

The organization of peripheral circadian clocks in mammals is not well understood. It is known that all tissues have some
kind of a circadian clock (3). The circadian clock in the liver is important for synchronization of liver processes (27, 28), and the corticosteroid-producing adrenal gland has an important role in circadian clock resetting and synchronization of other peripheral clocks (29). Besides light, the time of feeding is the most important zeitgeber in peripheral organs such as the adrenal gland and liver (30). In our experiment, we tested which core clock genes are cAMP-controlled in the absence of both zeitgebers. We performed the experiment in dark/dark conditions, and animals had free access to food ad libitum. From the literature, CREB-controlled circadian transcription seems to predominate, whereas CREM and ICER are linked mainly to the SCN (31) or the pineal gland (32).

Using the Crem KO mice, we have shown that Crem is not crucial for the free running circadian oscillations of the core clock genes in the liver and adrenal glands (Fig. 1). We identified Crem as important for fine-tuning clock genes, especially in the adrenal gland (Per1/2 and Cry1/2 exhibit obvious phase shifts in Crem KO mice). Regulation of Per transcription through cAMP signaling is widely discussed in the literature, but for Cry genes, no clear answer has been provided. Microarray analysis of ACTH-treated chickens identified Icer and CRY as differentially expressed genes (33). Both are up-regulated because they respond to cAMP signaling due to ACTH receptor MC2 (34). Cry1 and Cry2 can regulate the CREB-mediated expression of hepatic enzymes in gluconeogenesis (35), but cAMP-dependent transcription factors were so far not directly associated with regulation of Cry genes. We analyzed promoters of Cry1 and Cry2 in silico and identified some well-conserved CRE sequences in proximal promoters −1000 bp upstream from the transcription start site of both genes (data not shown). Providing experimental proof of cAMP-dependent expression of Cry genes remains a challenge for the future.

What about Crem circadian expression? Of all Crem isoforms, only Icer has a short specific exon and can be specifically measured by quantitative PCR. In vivo, Icer expression is higher in adrenal glands compared with the liver, where measurements were at the border of the detection limit. This is probably the reason why the absence of Crem leads only to minor modifications of the hepatic circadian expression patterns (Fig. 1A). It was suggested previously that repressors such as ICER and Fra-2 might appear in insufficient amounts to exert a significant effect on gene expression in vivo (36). As shown in this study, Icer is expressed in sufficient amounts in the adrenal glands at mRNA and protein levels. The expression is circadian, with the maximum at ZT 12 (Fig. 4B), whereas ICER protein is more abundant in the subjective night, at ZT ~20 (Fig. 4C). This is in line with the literature showing that mRNA-to-protein delay intervals for the core circadian genes are estimated at 4–8 h (37, 38).

Icer and Per1 share similar cAMP response characteristics: it was shown previously that ICER regulation of melatonin synthesis is set via the immediate-early cAMP response (11, 12) and that the immediate-early response of Per1 is important in resetting the circadian clock. To test the hypothesis of direct ICER involvement in the attenuation phase of Per1, we designed in vitro experiments in mouse immortal cell lines originating from the liver (Hepa 1-6) and adrenal glands (Y-1). In contrast to our expectation, ICER protein was faintly up-regulated at 4 h after application of forskolin in the adrenal cell line, whereas this was more intense in the Hepa 1-6 liver cells (Fig. 5A). The Icer mRNA response to forskolin follows the patterns described in the literature (Fig. 5B) (25). Because the addition of cycloheximide results in larger quantities of Icer mRNA, we propose that de novo synthesized ICER attenuates its own transcription in mouse immortal hepatocytes. This is in line with the measured expression of ICER protein, which is higher than basal levels already 1 h after forskolin treatment (Fig. 5C). In addition to autoregulation, ICER can at the same time attenuate transcription of other concordantly expressed cAMP-dependent genes such as Per1. After stimulation of Hepa 1-6 cells with forskolin, Per1 shows an immediate-early increase in expression (0–4 h) that transforms into the circadian expression pattern after 12 h (Fig. 5E). The promoter region of Per1 contains CREs, and the cAMP-mediated early response is believed to be the primary mechanism of circadian clock resetting (10). We have shown that a de novo synthesized protein is crucial for inhibition of the immediate-early response of Per1 (Fig. 5D).

We believe that this protein is ICER because ICER repression results in higher Per1 promoter activity (Fig. 6A), and ICER can bind to the CRE of Per1 (Fig. 6B). We could not show the direct inhibition of Per1 expression as a result of ICER transfection (data not shown). According to recent literature, this failure is easily explained. ICER was first described as a small protein that does not contain the P-box domain responsible for phosphorylation (25). The initially described post-transcriptional regulation was polyubiquitination, which guided ICER to proteasome degradation (39). Recently, phosphorylation with the mitotic kinase CDK1 was also described, resulting in ICER export to the cytoplasm (26). When ICER is overexpressed in cell lines, it is usually phosphorylated and therefore not active as a transcription factor. Cyclin D1 was not successfully silenced when intact ICER was used for the transfections, but when lysines at the phosphorylation sites were changed to arginines, repression was achieved (26). In accordance with this, we have shown that the majority of the overexpressed ICER in Hepa 1-6 cells ends in the cytoplasm and not in the nucleus (Fig. 6C).

The expression pattern of Icer is well described in different sections of the brain and in the pituitary gland (40, 41). It is believed that it has an important role in suppressing bursts of different neuronal signals that could be damaging for the nervous system (42). ICER acts as a kind of noise filter that maintains the normal nervous activity. Our study uncovers the roles of ICER in peripheral tissues. At least in adrenal glands, the Icer mRNA and protein are measurable. We postulate that ICER might also play a role as a noise filter in the adrenal gland. It can transduce signals for the synthesis of the adrenal hormones by regulating the expression of suitable receptors (34). This would also explain the importance of circadian expression of Icer: corticosteroid concentrations show a circadian pattern (5), and ICER might act as a gating mechanism. At peak expression, this noise filter is stricter, whereas at times of low expression, the filter is not so effective. The latter theory should be experimentally validated. With the evidence that ICER can directly affect and fine-tune Per1 expression, the basic principles of the theory are explained.
Acknowledgments—We thank Dr. Gregor Majdicer (Center for Animal Genomics, Veterinary Faculty, University of Ljubljana) for help with breeding and genotyping mice, Dr. Martina Persé and Ksenija Kodra (Medical Experimental Centre, Institute of Pathology, University of Ljubljana) for help with setting and performing animal experiments. We thank Dr. Martina Pfeffer (Institute for Anatomy II, Johann Wolfgang Goethe-Universität Frankfurt am Main) for the Icer antisense plasmid and Dr. Marvin S. Reitz (University of Maryland School of Medicine, Baltimore, Maryland) for the Icer expression plasmid with a His tag. We thank Dr. Hanspeter Herzl (Institute for Theoretical Biology, Charite and Humboldt University Berlin) for ideas for analysis of the gene expression data.

REFERENCES


