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Biochemical and Biophysical Research Communications 353 (2007) 895–901
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Synergistic regulation of the mouse orphan nuclear receptor SHP gene promoter by CLOCK–BMAL1 and LRH-1

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Received 4 December 2006
Available online 26 December 2006

Abstract

Small heterodimer partner (SHP; NR0B2) is an orphan nuclear receptor and acts as a repressor for wide variety of nuclear hormone receptors. We demonstrated here that mouse *SHP* mRNA showed a circadian expression pattern in the liver. Transient transfection of the m*SHP* promoter demonstrated that CLOCK–BMAL1, core circadian clock components, bound to E-box (CACGTG), and stimulated the promoter activity by 4-fold. Liver receptor homologue-1 (LRH-1; NR5A2) stimulated the m*SHP* promoter, and CLOCK–BMAL1 synergistically enhanced the LRH-1-mediated transactivation. Interestingly, SHP did not affect the CLOCK–BMAL1-mediated promoter activity, but strongly repressed the synergistic activation of CLOCK–BMAL1 and LRH-1. Furthermore, in vitro pull-down assays revealed the existence of direct protein–protein interaction between LRH-1 and CLOCK. In summary, this study shows that CLOCK–BMAL1, LRH-1 and SHP coordinately regulate the m*SHP* gene to generate the circadian oscillation. The cyclic expression of m*SHP* may affect daily activity of other nuclear receptors and contribute to circadian liver functions.

Keywords: SHP; Circadian rhythm; CLOCK–BMAL1; LRH-1; Orphan nuclear receptor

Circadian rhythm is found in many aspects of behavior and physiology such as sleep–wake cycles, blood pressure, energy metabolism, and liver metabolism. Biochemical approaches have identified 'clock genes' that govern circadian rhythm at transcriptional level. In mammals, *Clock*, *Bmal1*, *Period (Per1, 2)*, and *Cryptochrome(Cry1, 2)* comprise positive and negative transcriptional–translational feedback loops, in which CLOCK–BMAL1 heterodimer binds to E-box (CACGTG) of m*Per1* and m*Per2* genes and activates their transcription, and PER and CRY complex suppresses m*Per1* and m*Per2* transcription [1–4]. This feedback loop is believed to be a basic machinery of 24-h circadian rhythm. Recently, the molecular clocks were found in peripheral tissues including liver [5,6]. Gene array techniques enabled us to identify the hundreds of genes controlled by circadian clock [7,8].

Many liver metabolisms are also under circadian control, in which certain nuclear hormone receptors are expressed and regulating the functions. The cholesterol and bile acid synthesis have marked diurnal rhythms that peak at midnight [9]. For example, the expressions of cholesterol 7α-hydroxylase (CYP7A1) and HMG-CoA reductase, the rate-limiting enzyme for cholesterol synthesis, have circadian rhythm [10]. On the other hand, *CYP7A1* gene is under the control of several nuclear hormone receptors, activated by liver X receptor α (LXRα; NR1H3) and repressed by small heterodimer partner (SHP; NR0B2) through inhibition of liver receptor homologue-1 (LRH-1; NR5A2) function. Furthermore, *SHP* gene transcription is stimulated by LRH-1 or farnesoid X receptor α (FXRα; NR1H4), and suppressed by SHP itself [11,12]. SHP is an

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0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.12.131

atypical orphan receptor that lacks a DNA-binding domain and forms heterodimer with nuclear receptors, such as LXR, CARβ, LRH-1, hepatocyte nuclear factor 4 (HNF4), and repress their functions. [11–13]. Thus, SHP has a central role in the liver functions.

In this study, we examined the circadian expression pattern of nuclear receptors expressed in mouse liver, and found the circadian oscillation of *SHP* gene. We also demonstrated that CLOCK–BMAL1 bound to E-box of mouse *SHP* (m*SHP*) promoter and activated the transcription. Furthermore, the m*SHP* promoter activity was synergistically regulated by CLOCK–BMAL1 and LRH-1, and suppressed by SHP itself through LRH-1. Our results demonstrate the novel mechanisms of m*SHP* gene expression, and suggest that SHP is involved in the circadian liver function.

Materials and methods

Animals. All male mice Jcl:ICR (Charles River Laboratory, Japan) 4 weeks of age were maintained under a 12:12-h light–dark cycle (L12/D12) for at least 2 weeks before the day of the experiment. Mice were housed within a temperature-controlled facility (25 °C), and food and drinking water were available freely.

RNA extraction and RT. The four mice were sacrificed at every 4 h, and the livers from them were dissected. Immediately, they were immersed in RNAlater (Sigma Inc.). The total RNA was extracted from 25 to 30 mg of a slice of each liver by using GeneElute Mammalian Total RNA Miniprep Kit (Sigma–Aldrich, Japan). Total RNA concentrations were determined by spectrophotometry at 260 nm, and cDNA was synthesized by oligo $d(T)_{16}$ from the extracted RNA using GeneAmp RNA PCR Core kit (Roche, New Jersey, USA).

Quantitative real-time RT-PCR. We performed Quantitative real-time RT-PCR using ABI PRISM 7300 (Applied Biosystems). Each reaction volume of 40 μl contained 20 μl of SYBR GREEN PCR Master Mix (Applied Biosystems), 2 μl of each primer (10 pmol/μl), 0.5 μl of sample cDNA (20 ng/μl), and 15.5 μl of H_2O. Forward primer, reverse primer were as follows:

m*Gapdh* forward 5′-CATGGCCTTCCGTGTTCCTA-3′, m*Gapdh* reverse 5′-CCTGCTTCACCACCTTCTTGA-3′; m*Clock* forward 5′-AAGTTAGGGCTGAAAGACGGCG-3′, m*Clock* reverse 5′-ATGACTTTCGTGAGCTTCTA-3′; m*Bmal1* forward 5′-CTCCAGACATTCCTTCCACT-3′, m*Bmal1* reverse 5′-CCTCATCGTGGTGTCCGTCAC-3′; m*Per1* forward 5′-GAGAGCGTGGTGTCCGTCAC-3′, m*Per1* reverse 5′-AGATCGGCAGTGGTGTCGGC-3′; m*Rev-erbα* forward 5′-CCAACAGTCTACGGCAAGGC-3′, m*Rev-erbα* reverse 5′-TGTAGGTTGTGCGGCTCAGG-3′; m*SHP* forward 5′-GTACCTGAAGGGCACGATCC-3′, m*SHP* reverse 5′-GTGAAGTCTTGGAGCCCTGGT-3′; m*LRH-1* forward 5′-GCGGACCAGACCCTGTTCTC-3′, m*LRH-1* reverse 5′-ACGGAGCCTCACCACCAGCT-3′; m*HNF4α* forward 5′-GACTCTCGGGGCCGCTTT-3′, m*HNF4α* reverse 5′-TCAGATCCCGAGCCACTTG-3′; m*FXRα* forward 5′-GCTGAAAGGGTCCGCAGTGG-3′, m*FXRα* reverse 5′-CCACCGCCTCTCTGTCCTTG-3′. Each reaction was carried out in triplicate experiments. Data represent means ± SD.

Plasmids constructions. Expression vector for mouse SHP was constructed using cDNAs obtained from RT-PCR of mouse liver RNA. PCR-amplified product was cloned into the pcDNA 3.1/HisC vectors (Invitrogen, Carlsbad, CA). Expression vector for human LRH-1 (hCPF) was kindly provided by Dr. B. Shan (Tularik Inc., CA, USA) [14]. Expression vector for mouse CLOCK was provided by Dr. J.S. Takahashi (Howard Hughes Medical Institute, IL, USA) [1], and human BMAL1 was provided by Dr. M. Ikeda (Saitama Medical School, Saitama, Japan) [2]. The m*SHP* promoter (GenBank Accession No. AY366410) luciferase reporter plasmid was generated by PCR amplification of promoter fragments corresponding to sequences located between −720 and +15 bp of the m*SHP* gene. The PCR product was ligated into the pGL3-basic plasmid (Promega, Madison, WI). Serial deletion constructs were made by fusing various lengths of the m*SHP* promoter to the luciferase gene (−362 bp/Luc, −225 bp/Luc, −200 bp/Luc). The reporter plasmid m*SHP* (−720 bp) E-box mutant-Luc was generated as indicated in Fig. 2A. GST-fused full-length m*SHP* and h*LRH-1* were constructed by inserting *Eco*RI–*Xho*I fragments of each full-length cDNAs into pGEX6P-1 vectors (Amersham Pharmacia Biotech). Correct sequences were confirmed by dideoxy sequencing using ABI 310 Genetic Analyzer (Applied Biosystems).

Cell culture and transient transfection and reporter assays. COS1 and HepG2 cells were maintained with Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum and antibiotics. Transient transfection assays were performed by using FuGENE 6 transfection reagent (0.4 μg/well) according to the manufacturer's recommendation (Roche Molecular Biochemicals). Cells were transfected with 150 ng/well of each of the m*SHP* promoter plasmids and 25, 50, and 100 ng/well of expression vectors with 30 ng of a β-galactosidase expression plasmid pCMV-*lac*Z (Promega) that served as an internal control to normalize transfection efficiencies. In each of transfection assays, the amount of total DNA was adjusted to 400 ng/well by adding pcDNA empty vectors. Luciferase activities of different transfections were normalized by β-galactosidase activities. Each transfection was performed in triplicate dishes and repeated at least three times.

Electrophoretic mobility shift assays. CLOCK, BMAL1, and LRH-1 proteins were synthesized using T7 TNT in vitro transcription/translation

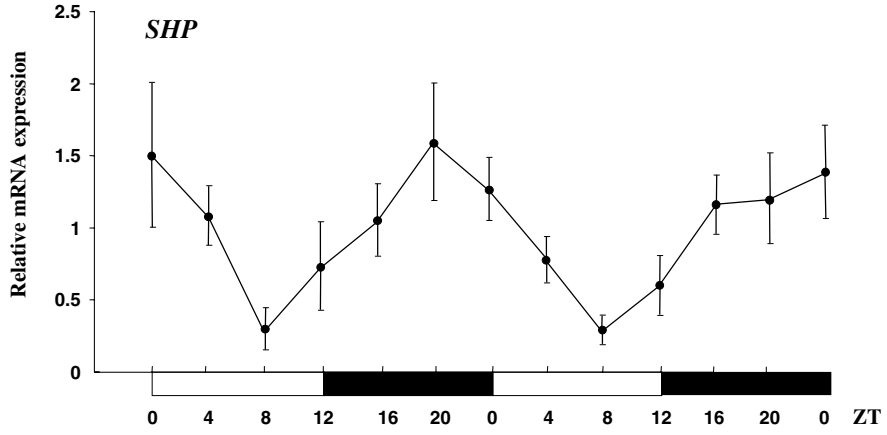

Fig. 1. Circadian gene expression of m*SHP* in mouse liver. Quantitative real-time RT-PCR was performed to determine individual mRNA levels. The values plotted in the diagrams represent arbitrary units of target mRNA normalized by *Gapdh* mRNA. Data are expressed as means ± SEM.