

The Basic Helix-Loop-Helix Proteins Differentiated Embryo Chondrocyte (DEC) 1 and DEC2 Function as Corepressors of Retinoid X Receptors

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ABSTRACT

The basic helix-loop-helix proteins differentiated embryo chondrocyte 1 (DEC1) and DEC2 are involved in circadian rhythm control. Because the metabolism of dietary nutrients has been linked to circadian regulation, we examined the effect of DEC1 and DEC2 on the function of the metabolite-sensing nuclear receptors, ligand-dependent transcription factors, including retinoid X receptor (RXR) and liver X receptor (LXR). Transfection assays showed that DEC1 and DEC2 repressed ligand-dependent transactivation by RXR. Knockdown of endogenous DEC1 and DEC2 expression with small interfering RNAs augmented ligand-dependent RXR α transactivation. DEC1 and DEC2 interacted directly with RXR α , and ligand addition enhanced their association. DEC1 and DEC2 modified interaction of RXR α with cofactor proteins. Transfection assays using

DEC1 and DEC2 mutants revealed that the C-terminal region of DEC2 is required for repression and that an LXXLL motif in DEC1 and DEC2 is necessary for RXR α repression. DEC1 and DEC2 repressed the induction of LXR target genes, associated with the promoter of an LXR target gene, and dissociated from the promoter with ligand treatment. Knockdown of endogenous DEC1 and DEC2 enhanced the LXR target gene expression in hepatocytes. Expression of *Dec1*, *Dec2*, and *Srebp-1c* showed a circadian rhythm in the liver of mice, whereas that of *Lxr α* , *Lxr β* , and *Rxr α* was not rhythmic. DEC1 and DEC2 also repressed the transactivation of other RXR heterodimers, such as farnesoid X receptor, vitamin D receptor, and retinoic acid receptor. Thus, the repressor function of DEC1 and DEC2 may be extended to other RXR heterodimer nuclear receptors.

Circadian rhythms are important in maintaining the sleep-wake cycle, thermogenesis, feeding behavior, and metabolic homeostasis (Hastings et al., 2003). The CLOCK-BMAL1 heterodimer regulates expression of circadian genes in the hypothalamus and peripheral tissues through binding to E-box elements in the promoters of target genes, including *Per*,

Cry, *Dec1*, and *Dec2* (Honma et al., 2002; Hastings et al., 2003). The PER and CRY proteins repress CLOCK-BMAL1 activity, a mechanism that establishes a 24-h long feedback loop and contributes to maintaining circadian oscillation. Differentiated embryo chondrocyte 1 (DEC1; also called BHLHE40, BHLHB2, Stra13, or Sharp2) and DEC2 (also called BHLHE41, BHLHB3, or Sharp1) are basic helix-loop-helix transcription factors that are rhythmically expressed in the suprachiasmatic nucleus and peripheral tissues (Honma et al., 2002; Noshiro et al., 2005). DEC1s repress CLOCK-BMAL1-dependent transcription by binding to BMAL1 and competing for E-box association (Honma et al., 2002; Li et al.,

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ABBREVIATIONS: DEC, differentiated embryo chondrocyte; RAR, retinoic acid receptor; RXR, retinoid X receptor; LXR, liver X receptor; 9CRA, 9-*cis* retinoic acid; FXR, farnesoid X receptor; VDR, vitamin D receptor; SMRT, silencing mediator of retinoic acid and thyroid hormone receptor; GFP, green fluorescent protein; SRC-1, steroid receptor coactivator 1; DRIP205, vitamin D receptor-interacting protein 205; NCoR, nuclear receptor corepressor; SREBP-1c, sterol regulatory element-binding protein 1c; HEK, human embryonic kidney; siRNA, small interfering RNA; GST, glutathione transferase; ChIP, chromatin immunoprecipitation; ZT, zeitgeber time; AF2, activation function 2; HDAC, histone deacetylase; ABCA1, ATP-binding cassette transporter A1; HIF-1 α , hypoxia-inducing factor 1 α ; PCR, polymerase chain reaction; BMAL1, brain and muscle Arnt-like protein-1; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; T0901317, *N*-(2,2,2-trifluoroethyl)-*N*-[4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]phenyl]-benzenesulfonamide; tk, thymidine kinase.

2004; Sato et al., 2004). The single- or double-mutant mice of *Dec1* and *Dec2* exhibit abnormal rhythm amplitude (Nakashima et al., 2008; Rossner et al., 2008). Therefore, DEC1 and DEC2 are essential components of the molecular clock system.

Nuclear receptors are ligand-inducible transcription factors that are involved in many biological processes, including cellular growth and differentiation, embryonic development, and metabolic homeostasis (Makishima, 2005; Shulman and Mangelsdorf, 2005). Several nuclear receptors play a role in the regulation of circadian rhythm through molecular and functional interactions with the regulatory loop composed of CLOCK, BMAL1, PER, and CRY (Teboul et al., 2008). REV-ERB α (NR1D1) and the retinoid-related orphan receptor α (NR1F1) regulate expression of *BMAL1*. Retinoic acid receptor α (RAR α ; NR1B1) and retinoid X receptor α (RXR α ; NR2B1) repress the transcriptional activity of CLOCK through a ligand-dependent interaction. Peroxisome proliferator-activated receptor α , a regulator of lipid metabolism, maintains circadian expression of *BMAL1*, which induces expression of peroxisome proliferator-activated receptor α gene in a positive feedback loop (Canaple et al., 2006). In addition, several nuclear receptors exhibit circadian expression patterns in metabolic tissues, including muscle, adipose, and liver (Yang et al., 2006). These findings indicate that nuclear receptors play a role in linking hormonal and metabolic signals to circadian rhythm. Compared with the CLOCK-BMAL1 and CRY-PER regulatory loop, data regarding the interaction of DEC1 or DEC2 with nuclear receptors are only now emerging. We previously reported that liver X receptor α (LXR α ; NR1H3) and LXR β (NR1H2), oxysterol receptors that regulate lipid metabolism, induce *Dec1* transcription in the mouse liver, suggesting that hepatic DEC1 mediates the ligand-dependent LXR signal in the hepatic clock system (Noshiro et al., 2009). In the present study, we investigated the effects of DEC1 and DEC2 on nuclear receptor transcriptional activity and found that DEC1 and DEC2 function as corepressors of RXR heterodimers.

Materials and Methods

Chemical Compounds. 9-*cis* Retinoic acid (9CRA), chenodeoxycholic acid, and all-*trans* retinoic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan), T0901317 was from Cayman Chemical Company (Ann Arbor, MI), and 1,25-dihydroxyvitamin D₃ was from Sigma-Aldrich (St. Louis, MO).

Plasmids. Fragments of human RXR α (GenBank accession no. NM_002957), LXR α (GenBank accession no. NM_005693), LXR β (GenBank accession no. NM_007121), farnesoid X receptor (FXR) (GenBank accession no. NM_005123), vitamin D receptor (VDR) (GenBank accession no. NM_00376), RAR α (GenBank accession no. NM_000964), and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) (GenBank accession no. AF113003) were inserted into pCMX vector to make pCMX-RXR α , pCMX-LXR α , pCMX-LXR β , pCMX-FXR, pCMX-VDR, pCMX-RAR α , and pCMX-SMRT, respectively (Chen and Evans, 1995; Makishima et al., 1998; Morita et al., 2005; Ishizawa et al., 2008). The ligand binding domains of human RXR α , RXR β (GenBank accession no. NM_021976), and RXR γ (GenBank accession no. NM_006917) were inserted into pCMX-GAL4 vector to make pCMX-GAL4-RXR α , pCMX-GAL4-RXR β , and pCMX-GAL4-RXR γ , respectively. A full-length RXR α cDNA was inserted into pCMX-VP16 vector to make pCMX-VP16-RXR α . The VP16 fragment in pCMX-VP16-RXR α was replaced with a fragment encoding the green fluorescent protein (GFP) to make

pCMX-GFP-RXR α . Nuclear receptor-interacting domains of steroid receptor coactivator 1 (SRC-1) (amino acids 595–771; GenBank accession no. U90661), vitamin D receptor-interacting protein 205 (DRIP205) (amino acids 578–728; GenBank accession no. Y13467), nuclear receptor corepressor (NCoR) (amino acids 1990–2416; GenBank accession no. U935312), and SMRT (amino acids 2003–2517; GenBank accession no. AF113003) were inserted into pCMX-GAL4 vector to make pCMX-GAL4-SRC-1, pCMX-GAL4-DRIP205, pCMX-GAL4-NCoR, and pCMX-GAL4-SMRT, respectively (Kaneko et al., 2003; Inaba et al., 2007). The expression plasmids for human DEC1 (pcDNA3.1-DEC1 and p3xFLAG-DEC1), DEC2 (pcDNA3.1-DEC2), mouse DEC1 (pcDNA3.1-mDEC1), DEC2 (pcDNA3.1-mDEC2), CLOCK (pcDNA3.1-CLOCK), and BMAL1 (pcDNA3.1-BMAL1) were reported previously (Honma et al., 2002; Kawamoto et al., 2004; Sato et al., 2004). DEC1 and DEC2 fragments shown in Fig. 3A were generated by PCR and were inserted into pcDNA3.1 to make mutant plasmids. A full-length fragment of DEC2 was cloned into the pFLAG-CMV2 vector to make pFLAG-CMV2-DEC2. The LXXAA mutants of DEC1 (p3xFLAG-DEC1-LXXAA) and DEC2 (pFLAG-CMV2-DEC2-LXXAA) were created by the QuikChange Site-Directed Mutagenesis kit (Agilent Technologies, Inc., Santa Clara, CA). RXR-responsive CRBP-II-tk-LUC (Mangelsdorf et al., 1991), GAL4-responsive MH100x4-tk-LUC (Kaneko et al., 2003), LXR-responsive LXREx3-tk-LUC and pGL3-ABCA1 (Uno et al., 2009), FXR-responsive IR1x3-tk-LUC, VDR-responsive hCYP3A4-ER6x3-tk-LUC (Ishizawa et al., 2008), RAR-responsive TREp2-tk-LUC (Makishima et al., 1998), and CLOCK-BMAL1-responsive E54-tk-LUC (Honma et al., 2002) were used in luciferase reporter assay. The sterol regulatory element-binding protein 1c (*Srebp-1c*) promoter-luciferase reporter (pGL3-SREBP-1c) was constructed by inserting 1271 base pairs of the mouse *Srebp-1c* promoter into the luciferase reporter pGL3 basic (Promega, Madison, WI) (Repa et al., 2000). A fragment from pCMX-GAL4-RXR α was inserted into pGEX vector (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) to make pGEX-RXR α . The C-terminal 19 amino acids were deleted from pGEX-RXR α to make pGEX-RXR α - Δ AF2 (Willy and Mangelsdorf, 1997). All plasmids were sequenced before use to verify DNA sequence fidelity.

Cell Culture and Transfection Assays. Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO₂, and HepG2 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Transfections in HEK293 cells were performed by the calcium phosphate coprecipitation technique as described previously (Ishizawa et al., 2008). Eight hours after transfections, ligands were added. Transfection experiments in HEK293 cells used 50 ng of reporter plasmid, 10 ng of pCMX- β -galactosidase, and 15 ng of each expression plasmid in combination with plasmids indicated in the figure legends for each well of a 96-well plate. Transfections in HepG2 cells were performed with Fugene HD (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. Transfection of HepG2 cells used 2 ng of reporter plasmid, 10 ng of pCMX- β -galactosidase, and 50 ng of each expression plasmid in combination with plasmids indicated in the figure legends. Total concentrations of plasmids in each experiment were adjusted by the addition of empty vectors. HEK293 cells and HepG2 cells were harvested 16 to 18 h and 48 h, respectively, and were analyzed for luciferase and β -galactosidase activities. Luciferase data were normalized to internal β -galactosidase control. Small interfering RNAs (siRNAs), directed against DEC1 and DEC2, and control siRNA were purchased from Thermo Fisher Scientific (Waltham, MA). siRNA oligonucleotides were transfected into HEK293 and HepG2 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For endogenous mRNA expression analysis, HepG2 cells were transfected with pFLAG-CMV2, p3xFLAG-DEC1, or pFLAG-CMV2-DEC2 with Lipofectamine 2000 (Invitrogen) according to the manu-

facturer's instructions. Twenty-four hours after transfection, T0901317 was added, and cells were harvested after 24 h for RNA preparation.

Reverse Transcription and Quantitative Real-Time PCR Analysis. Total RNAs from cells were prepared by the acid guanidine thiocyanate-phenol/chloroform method. cDNAs were synthesized with the ImProm-II Reverse Transcription system (Promega Corporation). Quantitative real-time PCR was performed on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) with Power SYBR Green PCR Master Mix (Applied Biosystems) (Uno et al., 2009). Primers were as follows: β -actin, 5'-GAC AGG ATG CAG AAG GAG AT-3' and 5'-GAA GCA TTT GCG GTG GAC GAT-3'; DEC1, 5'-CAG GGT GGT ACC TCC AGG AGG-3' and 5'-CTC ACT GCG CAA GTC GCC CTT-3'; DEC2, 5'-CTG AAA TCG CCC ATT CAG TCC-3' and 5'-ACA CCG CGG CTC CCT GGG TGT-3'; and SREBP-1c, 5'-GCG CCT TGA CAG GTG AAG TC-3' and 5'-GCC AGG GAA GTC ACT GTC TTG-3'. The mRNA values were normalized to the amount of β -actin mRNA.

Coimmunoprecipitation and Immunoblotting. HEK293 cells were transfected with pCMX-GFP-RXR α in combination with pFLAG-CMV2, p3xFLAG-DEC1, or pFLAG-CMV2-DEC2 with Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, cells were treated with ligand for 4 h. Nuclear extracts (0.8 mg of protein per sample) were subjected to immunoprecipitation with FLAG M2 antibody (Sigma-Aldrich). Immunocomplexes were separated by SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, probed with anti-GFP antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-FLAG antibody (Sigma-Aldrich), and visualized with an alkaline phosphatase conjugate substrate system.

Glutathione Transferase Pull Down Assays. HEK293 cells were transfected with pFLAG-CMV2, p3xFLAG-DEC1, or pFLAG-CMV2-DEC2 with Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, cells were harvested with lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 100 mM NaF, 0.3% CHAPS, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 10 μ g/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride). The lysates were mixed with 20 μ g of glutathione transferase (GST) proteins and glutathione Sepharose 4B (GE Healthcare) in the presence or absence of ligand and rotated overnight at 4°C (Inaba et al., 2007). The Sepharose beads were washed three times with ice-cold lysis buffer, and the bound proteins were eluted by boiling in SDS sample buffer, and subjected to immunoblotting with an anti-FLAG antibody (Sigma-Aldrich).

Chromatin Immunoprecipitation Assays. HepG2 cells were transfected with pFLAG-CMV2, p3xFLAG-DEC1, or pFLAG-CMV2-DEC2 with Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, cells were treated with ligand for 2 h and fixed with 1% formaldehyde for 15 min at 37°C. The reaction was stopped by the addition of glycine at a final concentration of 150 mM for 5 min at room temperature. Chromatin immunoprecipitation (ChIP) assays were performed with Acetyl-Histone H4 ChIP Assay Kit (Millipore, Billerica, MA) and anti-FLAG antibody (Sigma-Aldrich) (Uno et al., 2009). DNA was purified with MonoFas DNA Purification Kit (GL Sciences, Inc. Japan, Tokyo, Japan). PCR was performed using GoTaq Master Mix (Promega) with *SREBP-1c* primers 5'-TTT GAC CCA CCC TAT GCT GGG-3' and 5'-ACT GAC TCT TCC TTG ATA CCA-3'. PCR products were detected on agarose gel electrophoresis.

Animal Studies. Four- to five-month-old male C57BL/6J mice (Crea Japan, Tokyo, Japan) were housed under a 12-h light/dark cycle at constant temperature and given food and water ad libitum before the experiments for at least 2 weeks. All procedures were performed in compliance with standard principles and guidelines for the care and use of laboratory animals in Hiroshima University Graduate School of Biomedical Sciences. Three mice for each time point were decapitated at 6-h intervals beginning at zeitgeber time (ZT) 4 in a normal light/dark cycle. The tissues were quickly dissected and stored in RNAlater (Applied Biosystems) at -80°C until

processing. Total RNAs were prepared, and quantitative real-time PCR was preformed as described previously (Noshiro et al., 2009). The sequences for the primers and TaqMan fluorogenic probes (Applied Biosystems) for *Dec1*, *Dec2*, *Lxr α* , and *Lxr β* were described previously (Noshiro et al., 2005, 2007, 2009). The sequences of the primers and TaqMan fluorogenic probes for *Rxr α* and *Srebp-1c* were designed according to the ProbeFinder software of Roche Universal Probe Library system (Roche Applied Science): 5'-ACA TGC AGA TGG ACA AGA CG-3', 5'-GGG TTT GAG AGC CCC TTA GA-3', and number 26 fluorescent probe (Roche) for *Rxr α* ; and 5'-ACG GAG CCA TGG ATT GCA CA-3', 5'-CGG GAA GTC ACT GTC TTG GT-3', and number 78 fluorescent probe (Roche) for *Srebp-1c*.

Statistics. For cell experiments, values are shown as means \pm one S.D. of triplicate assays. The unpaired two-group Student's *t* test was performed to assess significant differences. For animal experiments, values are shown as means \pm S.E.M.

Results

DEC1 and DEC2 Repress RXR-Dependent Transcription. Because metabolite-sensing nuclear receptors, including the LXRs, function as RXR heterodimers (Shulman and Mangelsdorf, 2005), we examined the effects of DEC1 and DEC2 on RXR transactivation. HEK293 cells were transfected with an RXR α expression vector and an RXR-responsive reporter, CRBP-II-tk-LUC, in the presence or absence of a DEC1 or DEC2 expression vector, and were treated with 9CRA, a natural RXR ligand. The CRBP-II-tk-LUC reporter has five repeats of an RXR-binding half site, which can be bound by RXR homodimers (Mangelsdorf et al., 1991). Expression of the exogenous DEC proteins suppressed 9CRA-induced RXR α activation on the CRBP-II-tk-LUC reporter in a dose-dependent manner, and DEC2 repressed RXR α transactivation more effectively than DEC1 (Fig. 1A). Next, we examined the effects of DEC1 and DEC2 on the activity of GAL4-RXR chimera receptors, consisting of the ligand-binding domain of RXR fused to the DNA binding domain of the yeast transcription factor GAL4 (Kaneko et al., 2003; Morita et al., 2005). DEC2 effectively suppressed 9CRA-induced activity of GAL4-RXR α , with DEC1 demonstrating more modest suppression (Fig. 1B). Although DEC1 was not effective on GAL4-RXR β and weakly suppressed GAL4-RXR γ , DEC2 strongly suppressed 9CRA-dependent transactivation of GAL4-RXR β and GAL4-RXR γ . Thus, DEC2 and, to a lesser extent, DEC1 suppress ligand-dependent activation of RXRs.

To examine whether endogenous DEC1 and DEC2 suppress RXR-dependent transcription, we used siRNAs against *DEC1* and *DEC2*. We observed the expression of endogenous DEC1 and DEC2 in HEK293 cells, and 9CRA treatment did not change their mRNA expression (data not shown). DEC1 and DEC2 siRNAs effectively suppressed endogenous mRNA levels in HEK293 cells (Fig. 1C). Next, we examined the effect of DEC siRNA on ligand-dependent transactivation of RXR α on the CRBP-II-tk-LUC reporter. Cotransfection of DEC1 siRNA and DEC2 siRNA increased 9CRA-induced RXR α transactivation 1.4-fold compared with the control siRNA, although treatment with DEC1 siRNA or DEC2 siRNA alone had no effect (Fig. 1D). The effect of DEC knockdown on GAL4-RXR α transactivation was examined next. DEC1 siRNA transfection increased 9CRA-induced GAL4-RXR α activity 1.3-fold, and DEC2 siRNA had a similar effect (Fig. 1D). Cotransfection of DEC1 siRNA and DEC2 siRNA further enhanced GAL4-RXR α activity (1.4-fold). These find-

ings indicate that endogenous DEC1 and DEC2 suppress ligand-induced RXR α transactivation activity.

DEC1 and DEC2 Interact with RXR. To examine the interaction of DEC1 and DEC2 with RXR α , we prepared GST fusion proteins of RXR α in a bacterial expression system. We incubated GST-RXR α proteins with cell extracts prepared from HEK293 cells transfected with a FLAG-DEC1 or FLAG-DEC2 expression vector and detected interaction by immunoblotting with anti-FLAG antibody. DEC1 and DEC2 bound to GST-RXR α in the absence of ligand, and 9CRA treatment enhanced their interaction (Fig. 2A). Upon ligand binding, nuclear receptors undergo a conformational change in the C-terminal activation function 2 (AF2) domain that induces recruitment of coactivators to the AF2 surface (Rosenfeld et al., 2006). We next examined the interaction of DEC1 and DEC2 with GST-RXR α - Δ AF2, an AF2-deletion mutant of GST-RXR α . DEC1 bound to GST-RXR α - Δ AF2, but 9CRA did not enhance the interaction (Fig. 2A). DEC2 did not bind to

GST-RXR α - Δ AF2 in the presence or absence of 9CRA. Thus, DEC1 and DEC2 bind to RXR α ligand-binding domain in an AF2-dependent manner in vitro.

To examine the interaction between DEC1/2 and RXR in vivo, we transfected HEK293 cells with an expression plasmid of RXR α tagged with GFP and a FLAG-DEC1 or FLAG-DEC2 plasmid. After treatment with 9CRA, nuclear extracts were prepared for immunoprecipitation with anti-FLAG antibody, and the immunoprecipitates were subjected to immunoblotting for GFP-tagged proteins. GFP-RXR α proteins coprecipitated with FLAG-DEC1 or FLAG-DEC2 proteins (Fig. 2B), indicating that DEC1 and DEC2 associate with RXR α in vivo. Because the protein expression levels of FLAG-DEC1 and FLAG-DEC2 were similar, the more potent RXR repression by DEC2 (Fig. 1A) is unlikely to be due to differences in protein expression levels. Protein levels of GFP-RXR α were not altered by cotransfection of DEC1 or DEC2 (Fig. 2B), indicating that suppression of RXR α transactivation by DEC1 and DEC2 is not due to decreased protein expression of RXR α .

DEC1 and DEC2 show high sequence homology of the N-terminal regions [62% identity between human DEC1 (amino acids 1–181) and human DEC2 (amino acids 1–174)] but low homology in the C-terminal regions [35% identity between DEC1 (amino acids 182–412) and DEC2 (amino acids 175–482)] (Fujimoto et al., 2001) (Fig. 3A). To examine the effect of the C-terminal region on isotype-selective activity, we generated domain-swapped expression plasmids that express DEC1-DEC2 (1N2C) and DEC2-DEC1 (2N1C) chimeric proteins (Fig. 3A). We also generated DEC1 and DEC2 deletion mutants. Although the chimeric DEC-2N1C protein had activity similar to that of DEC1, the chimeric DEC-1N2C

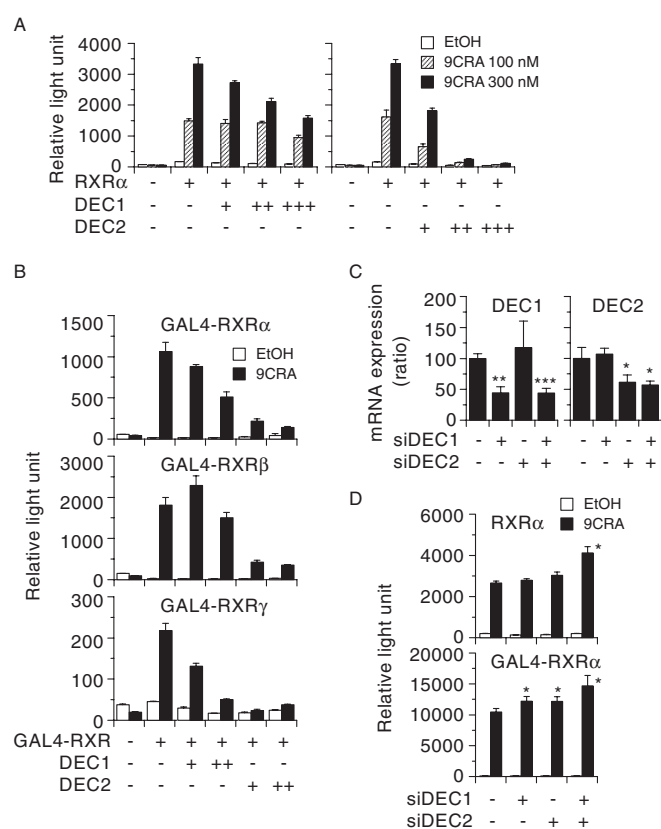


Fig. 1. DEC1 and DEC2 repress RXR transactivation. A, effects of DEC1 and DEC2 on RXR α . HEK293 cells were transfected with pCMX control (–) or pCMX-RXR α (+) and CRBP11-tk-LUC in the presence of pcDNA3.1 control (–), 0.5 ng (+), 5 ng (++), or 10 ng (+++) of pcDNA3.1-DEC1 or pcDNA3.1-DEC2 and treated with ethanol (EtOH) or 100 or 300 nM 9CRA. B, effects of DEC1 and DEC2 on GAL4-RXR chimera receptors. HEK293 cells were transfected with pCMX-GAL4-RXR α , pCMX-GAL4-RXR β , or pCMX-GAL4-RXR γ and MH100x4-tk-LUC in the presence of pcDNA3.1 control (–) or 5 (+) or 15 ng (++) of pcDNA3.1-DEC1 or pcDNA3.1-DEC2, and treated with ethanol (EtOH) or 100 nM 9CRA. C, knockdown of endogenous DEC1 and DEC2 by siRNA. HEK293 cells were transfected with DEC1 and/or DEC2 siRNA and examined for mRNA expression of endogenous DEC1 and DEC2. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with control siRNA. D, enhancement of RXR α transactivation by knockdown of DEC1 and DEC2. HEK293 cells were transfected with pCMX-RXR α and CRBP11-tk-LUC or pCMX-GAL4-RXR α and MH100x4-tk-LUC in combination with control siRNA, DEC1 siRNA, and/or DEC2 siRNA and treated with 100 nM 9CRA.

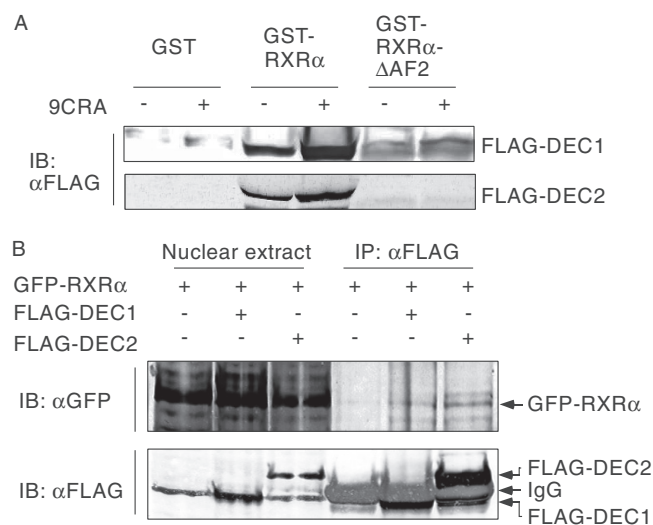


Fig. 2. DEC1 and DEC2 interact with RXR α . A, in vitro interaction of DEC1 and DEC2 with RXR α . Control GST, GST-RXR α , or GST-RXR α - Δ AF2 was expressed in *Escherichia coli*, and purified proteins were incubated with HEK293 cell lysates that contains FLAG-DEC1 or FLAG-DEC2 in the presence or absence of 1 μ M 9CRA. The protein complexes were pulled down with glutathione-coupled agarose beads and analyzed by immunoblotting (IB) with anti-FLAG antibody. B, in vivo interaction of DEC1 and DEC2 with RXR α in cells. HEK293 cells were transfected with pCMX-GFP-RXR α in combination with control pFLAG-CMV2, p3xFLAG-DEC1 (FLAG-DEC1), or pFLAG-CMV2-DEC2 (FLAG-DEC2). Protein complexes in nuclear extracts were immunoprecipitated with anti-FLAG antibody and then immunoblotted with anti-GFP antibody or anti-FLAG antibody. IP, immunoprecipitation.

repressed RXR α transactivation as effectively as DEC2 (Fig. 3B), suggesting that the C-terminal region of DEC2 mediates a strong repressive activity. Because DEC1 and DEC2 have been shown to repress transactivation by the CLOCK-BMAL1 heterodimer (Kawamoto et al., 2004), we examined the effects of DEC mutants on CLOCK-BMAL1-dependent transcription. DEC2 and DEC-1N2C suppressed CLOCK-BMAL1 transactivation activity more effectively than DEC1 and DEC-2N1C (Fig. 3C). We next examined the effects of DEC1 and DEC2 deletion mutants on RXR α transactivation. Deletion of the C-terminal region reduced DEC2-mediated repression (DEC2 > DEC2-309 > DEC2-258 > DEC2-N) (Fig. 3D). DEC2-C, which consists of the DEC2 C terminus

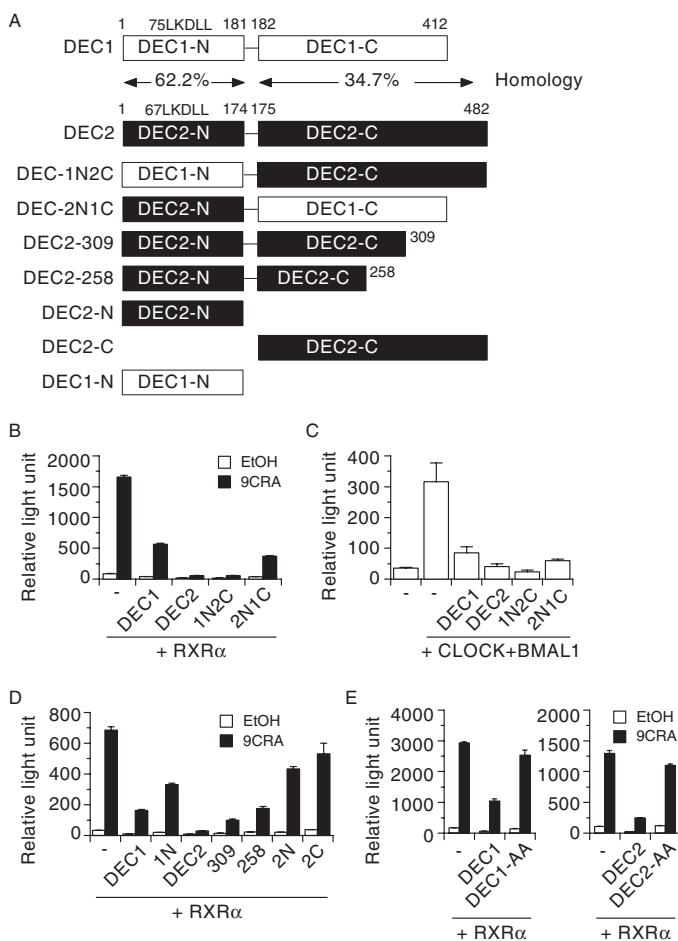


Fig. 3. Effects of DEC1 and DEC2 mutants on RXR α transactivation. **A**, chimeric and deletion mutants of DEC1 and DEC2. Wild-type DEC1 and DEC2 are presented at the top. Numbers indicate amino acids. **B**, effects of chimeric mutants of DEC1 and DEC2 on RXR α transactivation. HEK293 cells were transfected with pCMX-RXR α and CRBP11-tk-LUC in combination with pcDNA3.1 control (-), pcDNA3.1-DEC1, pcDNA3.1-DEC2, pcDNA3.1-DEC-1N2C (1N2C), or pcDNA3.1-DEC-2N1C (2N1C) and treated with ethanol (EtOH) or 100 nM 9CRA. **C**, effects of chimeric mutants of DEC1 and DEC2 on CLOCK-BMAL1 transactivation. HepG2 cells were transfected with pcDNA3.1-CLOCK, pcDNA3.1-BMAL1, and E54-TK-LUC in combination with pcDNA3.1 control (-), pcDNA3.1-DEC1, pcDNA3.1-DEC2, pcDNA3.1-DEC-1N2C (1N2C), or pcDNA3.1-DEC-2N1C (2N1C). **D**, effects of deletion mutants of DEC1 and DEC2 on RXR α transactivation. HEK293 cells were transfected with deletion mutants shown in **A** as in **B**. **E**, LXXLL motif is necessary for RXR α repression by DEC1 and DEC2. HEK293 cells were transfected with pCMX-RXR α and CRBP11-tk-LUC in combination with 5 ng of p3xFLAG-DEC1 or p3xFLAG-DEC1-LXXAA (DEC1-AA), or in combination with 1.5 ng of pFLAG-CMV2-DEC2 or pFLAG-CMV2-DEC2-LXXAA (DEC2-AA), and treated with ethanol (EtOH) or 100 nM 9CRA.

alone, was not effective in repressing RXR α activity. These findings indicate that both N- and C-terminal regions of DEC2 are required for effective RXR α suppression. DEC1-N and DEC2-N, which have only the N-terminal regions of DEC1 and DEC2, showed similar repression activity, although these activities were weaker than those of full-length DEC1 and DEC2. These results indicate that the N-terminal regions of DEC1 and DEC2 are required for repression and that more potent repression by DEC2 is due to its unique C-terminal region. Both DEC1 and DEC2 possess a conserved N-terminal region, containing an LKDLL sequence (Fig. 3A), consistent with the conserved LXXLL nuclear receptor-interacting motif (where L is a leucine and X is any amino acid) (Rosenfeld et al., 2006). Fifty-two amino acid sequences around LKDLL are identical among human and mouse DEC1 and DEC2 proteins (Fujimoto et al., 2001; Hamaguchi et al., 2004). To examine whether this motif is necessary for repressive activity, we generated DEC1 and DEC2 mutants that replaced the LKDLL motif with an inactive form (LKDA). The LXXAA mutants of DEC1 and DEC2 abolished RXR α repression (Fig. 3E), indicating that the LXXLL motif in the conserved N-terminal region is necessary for the inhibition of RXR α . Unlike siRNAs against DEC1 and DEC2 (Fig. 1D), the LXXLL mutants did not increase RXR α transactivation (Fig. 3E). The LXXLL mutants of DEC1 and DEC2 lost the ligand-dependent interaction with GST-RXR α (data not shown). These findings indicate that the LXXLL motif is important for RXR α interaction and suppression.

DEC1 and DEC2 Repress RXR α Transactivation through Modification of Histone Acetylation and Coactivator Recruitment. Because DEC1 and DEC2 have been reported to associate with histone deacetylases (HDACs) (Sun and Taneja, 2000; Fujimoto et al., 2007), we examined the effect of an HDAC inhibitor, trichostatin A, on DEC-induced RXR α repression. Treatment with trichostatin A reversed DEC1-mediated RXR α repression and the repressive activity of SMRT, a corepressor that represses nuclear receptor activity by recruiting HDACs (Rosenfeld et al., 2006) (Fig. 4A). In contrast, DEC2-induced RXR α repression was resistant to trichostatin A treatment. These findings indicate that HDAC is involved in the repression of RXR α activity by DEC1 but not by DEC2. The effects of DEC1 and DEC2 on coactivator recruitment to RXR α were examined in a mammalian two-hybrid assay. The receptor-interacting domains of SRC-1 and DRIP205 were fused to the GAL4 DNA-binding domain. Cotransfection of GAL4 cofactors with RXR α fused with the transactivation domain of herpesvirus VP16 protein allows for the detection of ligand-dependent cofactor recruitment (Kaneko et al., 2003). 9CRA treatment effectively induced the interaction between SRC-1 and RXR α . Although DEC1 slightly enhanced the interaction of RXR α with SRC-1, the effect of DEC2 on SRC-1-RXR α interaction was modest (Fig. 4B). DEC2, but not DEC1, interfered with the 9CRA-induced association of DRIP205 and RXR α (Fig. 4C). We next examined the interactions of RXR α with the corepressors NCoR and SMRT in a mammalian two-hybrid assay. In the absence of ligand, NCoR and SMRT bound to RXR α , and treatment with 9CRA reduced these interactions (Fig. 4, D and E). Cotransfection of DEC1 and DEC2 decreased the interactions of RXR α with NCoR and SMRT, indicating that DEC1 and DEC2 do not induce recruitment of these corepres-

sors to RXR α . These findings suggest that DEC1 and DEC2 act as corepressors by modulating the cofactor complex associated with RXR α .

DEC1 and DEC2 Repress RXR/LXR-Dependent Transcription. RXR plays a role in metabolic regulation through heterodimerization with other nuclear receptors, such as LXR (Shulman and Mangelsdorf, 2005), and *Dec1* is a target gene of the RXR-LXR heterodimer in hepatocytes (Noshiro et al., 2009). To examine the effects of DEC1 and DEC2 on the RXR-LXR heterodimer, we transfected HEK293 cells with expression plasmids for RXR α and LXR α and an LXR-responsive reporter LXREx3-tk-LUC in the presence or absence of a DEC1 or DEC2 expression plasmid and then treated the cells with a synthetic LXR ligand, T0901317. As reported previously (Willy and Mangelsdorf, 1997), the LXR-responsive reporter was activated in the presence of RXR α and LXR α transfection in the absence of ligand, and T0901317 treatment further increased the reporter activity (Fig. 5A). Although the effect of DEC1 was weak, DEC2 effectively suppressed ligand-independent and -dependent

activation of the RXR α -LXR α heterodimer. Compared with GAL4-RXR α (Fig. 1B), DEC1 and DEC2 were less effective in suppressing the ligand-dependent activation of GAL4-LXR α (Fig. 5B). In the presence of RXR α cotransfection, DEC1 enhanced GAL4-LXR α transactivation, whereas DEC2 was not effective. Although this DEC1 effect is similar to the effect on RXR α -SRC-1 interaction (Fig. 4B), further studies are required to elucidate the mechanism. Thus, DEC1 and DEC2 effectively suppress the RXR α heterodimer of full-length LXR α but not GAL4-LXR α . Next, we examined the effects of DEC1 and DEC2 on the activation of LXR target genes, *Srebp-1c* and ATP-binding cassette transporter A1 (*ABCA1*). These target gene promoters were slightly activated by T0901317 treatment in the absence of cotransfection of LXR α or RXR α in HEK293 cells (Fig. 5C), consistent with activation of endogenous receptors. Cotransfection of LXR α and RXR α increased the basal activities of these promoters, and T0901317 treatment further enhanced promoter induction. DEC1 weakly and DEC2 strongly repressed the *Srebp-1c* and *ABCA1* promoter activities elicited by the RXR α -LXR α heterodimer and T0901317 treatment (Fig. 5C).

To examine the repression of endogenous LXR target gene expression by DEC proteins, we overexpressed DEC1 or DEC2 in hepatocyte-derived HepG2 cells. T0901317 treatment effectively induced *SREBP-1c* mRNA expression, and basal and T0901317-dependent expression of *SREBP-1c* was repressed in HepG2 cells overexpressing DEC1 or DEC2, although the *SREBP-1c* remained inducible in response to T0901317 (Fig. 5D). We performed ChIP assay to examine the recruitment of DEC1 and DEC2 to the *SREBP-1c* promoter. Chromatin fragments in HepG2 cells expressing FLAG-DEC1 or FLAG-DEC2 were subjected to immunoprecipitation with an anti-FLAG antibody, and *SREBP-1c* promoter sequences including an LXR-binding element were amplified by PCR. DEC1 and DEC2 were present on *SREBP-1c* promoter in the absence of T0901317 or 9CRA (Fig. 5E). It is noteworthy that the association of DEC1 and DEC2 to *SREBP-1c* promoter was decreased by treatment with T0901317 or 9CRA. These findings suggest that ligand binding to the RXR-LXR heterodimer inhibits stable complex formation of DEC1 and DEC2 on the *SREBP-1c* promoter. We examined the effect of DEC siRNA on endogenous *SREBP-1c* mRNA expression in HepG2 cells. Treatment with siRNAs against DEC1 and DEC2 increased basal and ligand-induced *SREBP-1c* expression 2.3- and 1.7-fold, respectively, compared with the control siRNA (Fig. 5F). Thus, DEC proteins suppress endogenous LXR target gene expression.

Mouse DEC1 and DEC2 Also Repress Transactivation of RXR Heterodimers. Mouse DEC1 and DEC2 suppress CLOCK-BMAL-dependent transcription as effectively as human DEC1 and DEC2 (Kawamoto et al., 2004). We examined the effects of mouse DEC1 and DEC2 on RXR α transactivation. Mouse DEC2 effectively suppressed 9CRA-induced activity of RXR α , whereas mouse DEC1 had modest suppression (Fig. 6). We next examined the effects of mouse DEC1 and DEC2 on nuclear receptors that form RXR heterodimers, LXR α , LXR β , FXR, VDR, and RAR α (Makishima, 2005). Like human DEC2 (Fig. 5A), mouse DEC2 effectively suppressed ligand-dependent activation of LXR α (Fig. 6). Mouse DEC2 inhibited ligand-independent and -dependent activity of LXR β (Fig. 6) and human LXR β (data not shown). Mouse DEC2 also suppressed ligand-induced activation of

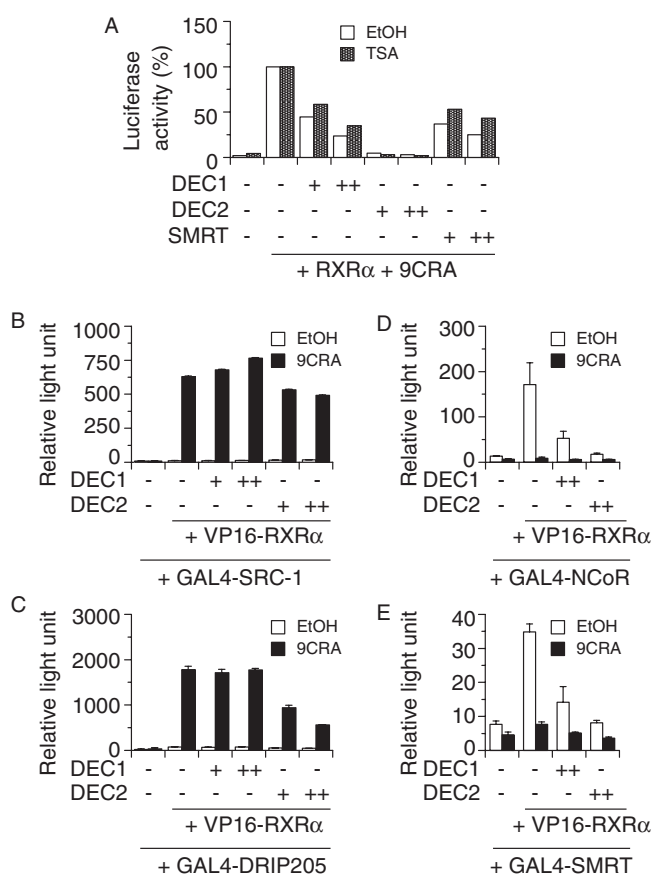


Fig. 4. DEC1 and DEC2 modulate histone deacetylation and RXR α -coactivator interaction. A, effects of HDAC inhibitor treatment on DEC-mediated repression of RXR α . HEK293 cells were transfected with pCMX-RXR α and CRBP11-tk-LUC in the presence of pcDNA3.1 control (–), 5 ng (+) or 15 ng (++) of pcDNA3.1-DEC1 or pcDNA3.1-DEC2, and treated with ethanol (EtOH) or 100 nM 9CRA in combination with 100 nM trichostatin A (TSA). B and C, effects of DEC1 and DEC2 on the interaction of RXR α with coactivators SRC-1 and DRIP205. D and E, effects of DEC1 and DEC2 on the interaction of RXR α with corepressors NCoR and SMRT. HEK293 cells were transfected with pCMX-VP16-RXR α , pCMX-GAL4-SRC-1, pCMX-GAL4-DRIP205, pCMX-GAL4-NCoR, or pCMX-GAL4-SMRT, and MH100x4-tk-LUC in the presence of pcDNA3.1 control (–) or 5 ng (+) or 15 ng (++) of pcDNA3.1-DEC1 or pcDNA3.1-DEC2 and treated with ethanol (EtOH) or 100 nM 9CRA.

FXR, VDR, and RAR α (Fig. 6). Compared with DEC2, mouse DEC1 was less effective in suppressing these RXR heterodimers. Thus, DEC2 and, to a lesser extent, DEC1 suppress transactivation of RXR heterodimers.

Finally, we examined mRNA expression of *Dec1*, *Dec2*, *Lxr α* , *Lxr β* , *Rxr α* , and *Srebp-1c* in the liver of mice. Hepatic expression of *Dec1* and *Dec2* showed a circadian rhythm with a peak at ZT10 (Fig. 7). Increased expression of *Dec1* and *Dec2* in the light phase is consistent with previous reports (Noshiro et al., 2005, 2007, 2009). Expression of *Lxr α* , *Lxr β* , and *Rxr α* was not rhythmic. It is noteworthy that expression of *Srebp-1c* showed a circadian rhythm with a peak at ZT16.

Discussion

In this study, we found that DEC1 and DEC2 repress the transactivation of RXR and the RXR-LXR heterodimer through interaction with RXR. DEC1 and DEC2 play a role in the regulation of circadian rhythms by interacting with BMAL1 and repressing BMAL1-induced transcriptional activity (Honma et al., 2002; Hamaguchi et al., 2004; Kawamoto et al., 2004; Sato et al., 2004). DEC proteins, especially DEC2, also interact with and inhibit transactivation of other transcription factors, including hypoxia-inducing factor 1 α (HIF-1 α) and MyoD (Azmi et al., 2004; Sato et

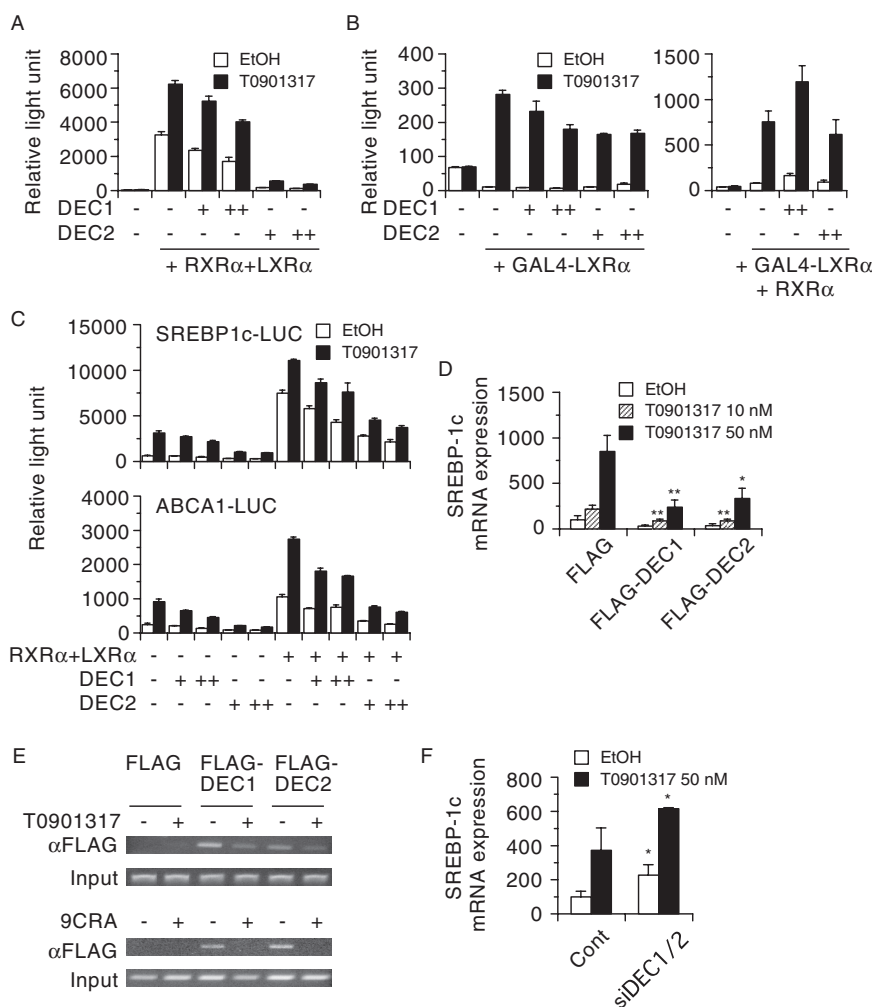


Fig. 5. Effects of DEC1 and DEC2 on the transactivation of the RXR-LXR heterodimer. **A**, effects of DEC1 and DEC2 on the transactivation of the RXR α -LXR α heterodimer. HEK293 cells were transfected with pCMX-RXR α , pCMX-LXR α , and LXREx3-tk-LUC in the presence of pcDNA3.1 control (-) or 5 ng (+) or 15 ng (++) of pcDNA3.1-DEC1 or pcDNA3.1-DEC2 and treated with ethanol (EtOH) or 100 nM T0901317. **B**, DEC1 and DEC2 are not effective repressors of the GAL4-LXR α chimera receptor. HEK293 cells were transfected with pCMX-GAL4-LXR α or pCMX-GAL4-LXR α plus pCMX-RXR α and MH100x4-tk-LUC in the presence of pcDNA3.1 control (-) or 5 ng (+) or 15 ng (++) of pcDNA3.1-DEC1 or pcDNA3.1-DEC2 and treated with ethanol (EtOH) or 100 nM T0901317. **C**, effects of DEC1 and DEC2 on *Srebp-1c* and *ABCA1* promoter activities induced by RXR-LXR heterodimer. HEK293 cells were transfected with pCMX-RXR α , pCMX-LXR α , and pGL3-SREBP-1c or pGL3-ABCA1 in the presence of pcDNA3.1 control (-) or 5 ng (+) or 15 ng (++) of pcDNA3.1-DEC1 or pcDNA3.1-DEC2 and treated with ethanol (EtOH) or 100 nM T0901317. **D**, effects of DEC overexpression on endogenous *SREBP-1c* mRNA expression. HepG2 cells were transfected with pFLAG-CMV2 control, p3xFLAG-DEC1, or pFLAG-CMV2-DEC2, and treated with ethanol (EtOH) or 10 or 50 nM T0901317. Endogenous *SREBP-1c* mRNA levels were determined by quantitative real-time PCR analysis. *, $P < 0.05$; **, $P < 0.01$ compared with FLAG control. **E**, recruitment of DEC1 and DEC2 to the SERBP-1c promoter. HepG2 cells were transfected with pFLAG-CMV2 control, p3xFLAG-DEC1, or pFLAG-CMV2-DEC2, and treated with ethanol (EtOH) or 100 nM T0901317. The ChIP assay was performed using anti-FLAG antibody. Promoter sequences containing an LXR-binding element were detected by PCR. The experiments were repeated twice with similar results. **F**, knockdown of endogenous DEC1 and DEC2 enhances *SREBP-1c* mRNA expression in HepG2 cells. HepG2 cells were transfected with control siRNA (Cont) or siRNAs against DEC1 and DEC2 (siDEC1/2) and treated with ethanol (EtOH) or 50 nM T0901317. Endogenous *SREBP-1c* mRNA levels were determined by quantitative real-time PCR analysis. *, $P < 0.05$ compared with control siRNA.

al., 2008). DEC1 and DEC2 bind to E-box elements in their own promoters and repress transcriptional expression (Sun and Taneja, 2000; Kawamoto et al., 2004). Therefore, DEC1

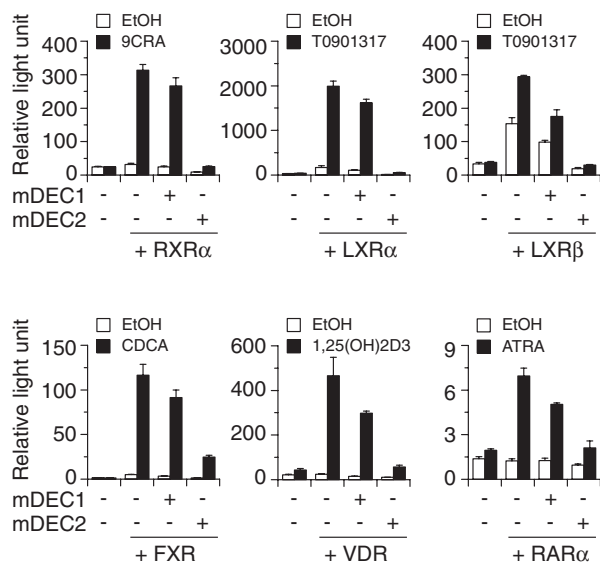


Fig. 6. Effects of mouse DEC1 and DEC2 on transactivation of RXR α , LXR α , LXR β , FXR, VDR, and RAR α . HEK293 cells were transfected with pCMX control (–), pCMX-RXR α , pCMX-LXR α , pCMX-LXR β , pCMX-FXR, pCMX-VDR, or pCMX-RAR α , and CRBP11-tk-LUC (for RXR α), LXREx3-tk-LUC (for LXR α and LXR β), IR1x3-tk-LUC (for FXR), hCYP3A4-ER6x3-tk-LUC (for VDR), or TREp2-tk-LUC (for RAR α) in the presence of pcDNA3.1 control (–), pcDNA-mDEC1, or pcDNA-mDEC2 and treated with ethanol (EtOH), 100 nM T0901317, 30 μ M chenodeoxycholic acid (CDCA), 30 nM 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] or 100 nM all-*trans* retinoic acid (ATRA).

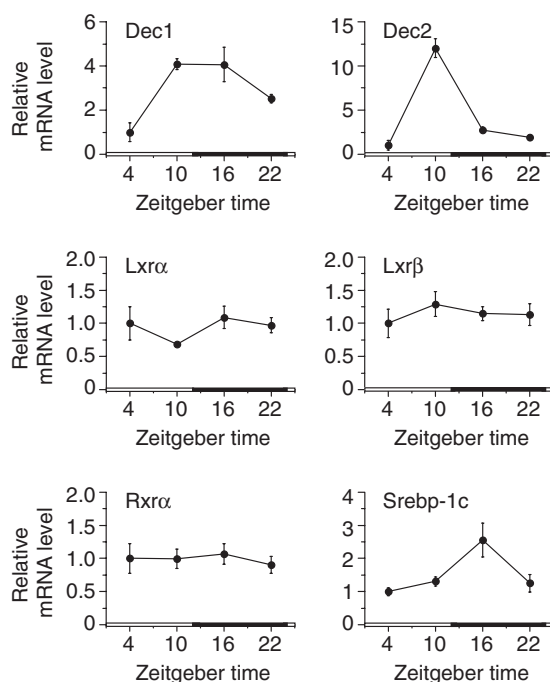


Fig. 7. Circadian expression of *Dec1*, *Dec2*, *Lxrα*, *Lxrβ*, *Rxrα*, and *Srebp-1c* in the liver of mice. Mice were housed under a 12-h light/dark cycle and were sacrificed at the indicated time points. Open and closed bars indicated light and dark phases, respectively. The mRNA levels were determined by quantitative real-time PCR analysis. Data are plotted as mean \pm S.E.M. ($n = 3$). Time series data were analyzed by one-way analysis of variance for rhythmicity, and significant differences were observed in *Dec1* ($P < 0.05$), *Dec2* ($P < 0.001$), and *Srebp-1c* ($P < 0.05$).

and DEC2 are transcriptional repressors that bind directly to promoters as E-box transcription factors and inhibit other transcription factors as corepressors. DEC1 and DEC2 bound to the ligand-binding domain of RXR α , and ligand treatment enhanced the interaction in an AF2-dependent manner (Fig. 2). An LXXLL motif (LKDLL), which is conserved in human and mouse DEC1 and DEC2, was necessary for the ligand-dependent RXR α repression by DEC1 and DEC2 (Fig. 3). The LXXLL motif is typically found in coactivators, such as SRC-1 and DRIP205, and mediates the interaction with the AF2 surface of ligand-bound nuclear receptors (Rosenfeld et al., 2006). In the absence of ligand, corepressors, including SMRT, bind to the AF2 surface composed of portions of helix 3, loop 3–4, helices 4/5, and helix 11. Ligand binding reduces the receptor affinity for corepressors and recruits coactivators to the altered AF2 surface formed through the repositioning of helix 12. Thus, DEC1 and DEC2 bind to RXR α in a coactivator-like manner. Like DEC1 and DEC2, a small heterodimer partner (NR0B2) and receptor interaction protein 140 bind to nuclear receptors through LXXLL motifs in a ligand-dependent manner and repress transcriptional activity of nuclear receptors (Lee and Wei, 1999; Johansson et al., 2000). These ligand-dependent corepressors may regulate the ligand activation of nuclear receptors by competing with coactivators. DEC2 interacts with HIF-1 α and MyoD, which are basic helix-loop-helix transcription factors like DEC1 (Azmi et al., 2004; Sato et al., 2008). The interactions of DEC1 with RXR α may be different from those with HIF-1 α and MyoD.

Acetylation and deacetylation of histone tails control transcription, and HDAC activity is associated with transcriptional repression (Rosenfeld et al., 2006). DEC1 and DEC2 have been demonstrated to suppress both HDAC-dependent and -independent transcription (Sun and Taneja, 2000; Fujimoto et al., 2007; Nakamura et al., 2008). Treatment with an HDAC inhibitor reversed RXR α repression by DEC1 but not by DEC2 (Fig. 4), indicating that DEC1 suppresses RXR α transactivation in an HDAC-dependent mechanism. Although DEC2 interacts with HDAC1 and represses an E-box-containing promoter, a DEC2 C-terminal deletion mutant represses transcription in an HDAC-independent manner (Fujimoto et al., 2007). DEC2 was more effective than DEC1 in RXR α repression in luciferase reporter assays (Fig. 1), and experiments using chimeric and deletion mutants of DEC1 and DEC2 demonstrated that the distinct repressive activities of DEC1 and DEC2 are due to their C-terminal regions (Fig. 3). These findings suggest that the C-terminal region of DEC2 is involved in HDAC-independent repression. DEC2, but not DEC1, suppresses the transcription of hepatic cytochrome P450 genes, such as CYP7A, CYP8B, and CYP51 (Noshiro et al., 2004). DEC2 binds to HIF-1 α and suppresses HIF-1 α -dependent transcription of the vascular endothelial growth factor gene, and DEC1 does not interact with HIF-1 α (Sato et al., 2008). DEC2 represses the transactivation of MyoD in an HDAC-independent manner (Fujimoto et al., 2007). Mammalian two-hybrid assays showed that DEC1 slightly enhanced SRC-1 recruitment to RXR α and that DEC2 inhibited ligand-dependent interaction of RXR α with DRIP205 (Fig. 4). Thus, DEC1 and DEC2 may interact with distinct cofactors through unique C-terminal regions and induce selective repression activity. DEC1 and DEC2 decreased the interactions of RXR α with the corepressors NCoR

and SMRT. Other corepressors may mediate HDAC-dependent inhibition by DEC1. ChIP analysis showed that association of DEC1 and DEC2 with the *SREBP-1c* promoter was decreased after ligand treatment (Fig. 5). GST pull-down assays showed that DEC1 and DEC2 bind to RXR α in a ligand-dependent manner (Fig. 2). A ligand-induced conformational change of the RXR heterodimer-DEC complexes may decrease the stability of the complexes on target gene promoters. Further investigation will be required to elucidate the role of cofactor recruitment and protein modification in complexes of DEC1/2 and the RXR heterodimer.

Dec1 is a target gene of the RXR-LXR heterodimer in mouse hepatocytes (Noshiro et al., 2009). Fasting of mice decreases *Dec1* mRNA expression in the liver, and refeeding restores the decreased *Dec1* mRNA (Kawamoto et al., 2006). Because LXR α plays an important role in sensing dietary sterols and regulating the expression of genes involved in cholesterol, fatty acid, and glucose metabolisms in the liver (Shulman and Mangelsdorf, 2005), LXR activation by dietary ligands may be involved in the induction of *Dec1* expression. The *Dec1* and *Dec2* genes are positively regulated by CLOCK and BMAL1 and are negatively autoregulated (Honma et al., 2002; Hamaguchi et al., 2004; Kawamoto et al., 2004). We show in this study that DEC1 and DEC2 act as corepressors of RXR and the RXR-LXR heterodimer. The regulatory loop involving DEC and RXR heterodimers is suggested to play a role in the hepatic clock system and metabolism. Hepatic circadian rhythms in mRNA expression of *Dec1* and *Dec2* is disturbed in *Clock* mutant mice (Noshiro et al., 200, 2009), and *Clock* mutant mice exhibit abnormal fat metabolism (Turek et al., 2005). Moreover, expressions of *SREBP-1c* protein and *Cyp7a1* transcript, which is an LXR target gene, exhibit circadian rhythm variation in the liver of mice (Brewer et al., 2005; Noshiro et al., 2007). Hepatic expression of *Dec1/2* and *Srebp-1c* showed a circadian rhythm with peaks at ZT10 and ZT16, respectively (Fig. 7), and the rhythmicity of *Srebp-1c* disappeared in *Dec1* knockout mice (M. Noshiro, unpublished results). These findings suggest that clock gene products, including DEC1, regulate the transcription of hepatic *Srebp-1c*. A peak of *Cyp7a1* mRNA expression in BALB/c mice is at ZT6 (Noshiro et al., 2007), whereas that in C57BL/6J mice was at ZT16 (M. Noshiro, unpublished results). The mechanisms for the strain difference are unknown. Transcription of *Cyp7a1* is regulated by multiple transcription factors, including D-site binding protein, REV-ERB α/β , LXR α , hepatic nuclear factor 4 α , liver receptor homolog-1, DEC2, and E4BP4. The sum of the contribution of these positive and negative factors seems to constitute the circadian expression profiles of *Cyp7a1* transcript. The mechanisms of the circadian *Srebp-1c* expression have not been investigated. Transcription of hepatic *Srebp-1c* is also regulated by insulin, glucagon, and dietary sterols that activate LXR (Goldstein et al., 2006). These factors may modify circadian expression of *Srebp-1c* regulated by DEC1 and DEC2 may mediate circadian clock signals to metabolic gene expression by participating in the multiple feedback loops for circadian rhythm regulation and interacting with RXR heterodimers and confer circadian control to nuclear receptor metabolic regulation. Further studies using *Dec1/2* double-knockout mice should clarify the role of DEC1 in peripheral circadian clocks and metabolic regulation. RXR plays a role in many physiological

processes by forming heterodimers with nuclear receptors, such as FXR, VDR, and RAR α , as well as LXR (Makishima, 2005; Shulman and Mangelsdorf, 2005), and DEC1 and DEC2, also inhibited transactivation of these RXR heterodimers (Fig. 6). Repression of RXR activity by DEC1 and DEC2 will provide the impetus for future investigation.

In conclusion, DEC1 and DEC2 serve as corepressors for RXR and repress target gene expression mediated by the RXR-LXR heterodimer. These findings provide implications for understanding the reciprocal regulation of circadian rhythms and lipid metabolism.

Acknowledgments

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