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Activated Sterol Regulatory Element-Binding Protein-2 Suppresses Hepatocyte Nuclear Factor-4-Mediated *Cyp3a11* Expression in Mouse Liver[S]

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ABSTRACT

Sterol regulatory element-binding protein-2 (SREBP-2) is a key transcription factor for the cholesterol homeostasis. Recent studies have suggested the association of CYP3A enzymes, major drug-metabolizing enzymes, with cholesterol metabolism. In the present study, we have investigated a possible involvement of SREBP-2 in hepatic *Cyp3a11* expression. Feeding a low-cholesterol diet (LCD) to mice activated hepatic SREBP-2 whereas it attenuated hepatic *Cyp3a11* expression. These phenomena were reversed by cholesterol supplementation to LCD. In reporter assays, the overexpression of constitutively active SREBP-2 reduced *Cyp3a11* reporter activity through the region from −1581 to −1570 of *Cyp3a11*. This region contained a putative hepatocyte nuclear factor-4α (HNF-4α) binding motif, and HNF-4α, but not SREBP-2, bound to the motif in in vitro binding assays. With the mutation or deletion of this motif, the SREBP-2-dependent suppression of *Cyp3a11* expression disappeared in reporter assays. In pull-down assays and coimmunoprecipitation assays, SREBP-2 bound to peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), a major coactivator for HNF-4α, via its transactivation domain and inhibited the interaction between HNF-4α and PGC-1α in vitro. A mutant SREBP-2 lacking the transactivation domain consistently failed to reduce *Cyp3a11* reporter activity. Furthermore, PGC-1α overexpression relieved the SREBP-2-mediated reduction of *Cyp3a11* reporter activity. Finally, chromatin immunoprecipitation assays demonstrated that the extent of PGC-1α binding to the *Cyp3a11* promoter was reduced by LCD-feeding in mouse livers. In conclusion, activated SREBP-2 interacts with PGC-1α in mouse livers at reduced cholesterol intake. This results in the reduced PGC-1α recruitment to HNF-4α on the *Cyp3a11* promoter and the subsequent down-regulation of *Cyp3a11* expression.

Introduction

Sterol regulatory element-binding proteins (SREBPs) are transcription factors that play major roles in the regulation of cholesterol, triglyceride, and fatty acid homeostasis. SREBPs are synthesized as membrane-bound endoplasmic reticulum proteins with two membrane-spanning domains (Brown and Goldstein, 1997; Horton et al., 2002). Newly synthesized premature SREBPs are associated with the SREBP cleavage-activating protein, a sterol-sensing molecule (Brown and Goldstein, 1997; Horton et al., 2002). When cellular sterol levels are low, SREBP cleavage-activating protein escorts SREBPs to the Golgi compartment, in which SREBPs are processed sequentially by two proteases, designated site-1 and site-2 proteases (Brown and Goldstein, 1997; Horton et al., 2002). Proteolytic cleavage releases the transcriptionally active N-terminal portion containing a DNA binding motif, basic helix-loop-helix leucine zipper (Brown and Goldstein, 1997; Horton et al., 2002). It enters the nucleus and binds to specific sterol regulatory elements in the promoters of cholesterogenic and lipogenic genes, thereby activating their transcription (Horton et al., 2002). Among three known SREBP isoforms (SREBP-1a, SREBP-1c, and

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ABBREVIATIONS: SREBP, sterol regulatory element-binding protein; LDL-R, low-density lipoprotein receptor; P450, cytochrome P450; HNF-4α, hepatocyte nuclear factor-4α; DR1, direct repeat separated by mononucleotide; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1α; CAR, constitutive androstane receptor; PXR, pregnane X receptor; BSA, bovine serum albumin; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; CD, conventional laboratory diet; LCD, low-cholesterol regular diet; EMSA, electrophoretic mobility shift assay; GST, glutathione transferase; ChIP, chromatin immunoprecipitation; PEPCK1, phosphoenolpyruvate carboxykinase 1; PCR, polymerase chain reaction; NE, nuclear extract.

SREBP-2), SREBP-2 is constitutively expressed in various tissues, and, when activated, it up-regulates the expression of numbers of genes for cholesterol uptake and synthesis to maintain cellular cholesterol level. Its target genes include those encoding low-density lipoprotein receptor (LDL-R), HMG-CoA synthase, HMG-CoA reductase, and squalene epoxidase (Brown and Goldstein, 1997; Horton et al., 2002).

Excess cellular cholesterol is converted into oxysterols and bile acids by multiple enzymes including cytochromes P450 (P450s). A major human drug-metabolizing enzyme, CYP3A4, and its mouse homolog, CYP3A11, have been reported to be involved in this cholesterol metabolism. They catalyze the 4β-hydroxylation of cholesterol and mediate the formation of various intermediates in the bile acid synthetic pathway such as 5β-cholestane-3α,7α,12α,25-tetrol (Bodin et al., 2001, 2002; Honda et al., 2001; Goodwin et al., 2003; Pikuleva, 2006). These suggest a distinct endogenous role of CYP3As in cholesterol homeostasis. However, the contribution of CYP3As to cholesterol homeostasis remains to be defined.

Hepatocyte nuclear factor-4α (HNF-4α) is a highly conserved member of the nuclear receptor superfamily. HNF-4α binds to a direct repeat with a one- or two-nucleotide spacer (designated DR1 or DR2, respectively) as a homodimer and is involved in the expression of genes associated with a variety of liver functions, including P450 genes (Jiang et al., 1995; Miyata et al., 1995; Gonzalez, 2008). The expression of HNF-4α antisense RNA in primary human hepatocytes dramatically decreased mRNA levels of *CYP3A4*, *CYP3A5*, *CYP2A6*, *CYP2B6*, *CYP2C9*, and *CYP2D6* (Jover et al., 2001). We have also reported that mRNA levels of several P450 genes, including *CYP3A4*, were dramatically decreased in cultured human hepatocytes after the infection of the adenovirus-expressing human HNF-4α-small interfering RNA (Kamiyama et al., 2007). Furthermore, hepatic *Cyp3a* expression is greatly reduced in liver-specific HNF-4α knockout mice (Wiwi et al., 2004). These data indicate that HNF-4α plays a dominant role in the expression of CYP3A genes in the liver.

The HNF-4α-mediated gene transcription is cooperated with coactivators, such as peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) (Yoon et al., 2001; Rhee et al., 2003). PGC-1α recruits chromatin-modifying enzymes having histone acetyltransferase activity, which opens the chromatin and promotes RNA polymerase II occupancy, resulting in the increased transcription of target genes (Puigserver et al., 1999; Monsalve et al., 2000). PGC-1α interacts with numerous nuclear receptors, in addition to HNF-4α, including peroxisome proliferator-activated receptor α, constitutive androstane receptor (CAR), and pregnane X receptor (PXR) (Vega et al., 2000; Shiraki et al., 2003; Li and Chiang, 2005). These nuclear receptors regulate diverse biological functions, such as lipid, glucose, and xenobiotic metabolism. Thus, PGC-1α seems to be a convergence point of cross-talk between xenobiotic and other signaling pathways.

We have observed that hepatic *Cyp3a11* expression is attenuated in mice fed a high triglyceride-containing diet (Yoshinari et al., 2006). Given that the involvement of CYP3A enzymes in cholesterol metabolism is suggested, cellular cholesterol levels may affect CYP3A expression levels. In the present study, we have found for the first time that *Cyp3a11* expression is down-regulated in mouse livers by feeding a low-cholesterol diet, and investigated the role of SREBP-2 in this down-regulation. We here demonstrate that activated SREBP-2 inhibits the interaction of PGC-1α with HNF-4α, suppressing the HNF-4α-mediated *Cyp3a11* expression in mouse liver.

Materials and Methods

Materials. Streptavidin Magnetic Beads, T4 polynucleotide kinase and restriction enzymes were purchased from New England BioLabs (Ipswich, MA). Fetal bovine serum, pepstatin A, and [γ-^{32}P]ATP were purchased from BioWest (Nuaille, France), Peptide Institute (Minoh, Japan) and PerkinElmer Life and Analytical Sciences (Waltham, MA), respectively. Bovine serum albumin (BSA), cholesterol, dithiothreitol (DTT), leupeptin, phenylmethylsulfonyl fluoride (PMSF), spermidine, spermine, protease inhibitor cocktail, and proteinase K were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were purchased from Wako Pure Chemicals (Osaka, Japan). Oligonucleotides were commercially synthesized by Fasmac (Atsugi, Japan).

Animal Treatment. Male C57BL/6N mice (7 weeks old; Charles River Laboratories Japan, Yokohama, Japan) were maintained under a 12-h light/12-h dark cycle and fed a conventional laboratory diet (CD; CE-2, CLEA Japan, Tokyo, Japan) and water ad libitum for a week for acclimatization. Mice were then fed CD, a low-cholesterol regular diet (LCD; Research Diet, New Brunswick, NJ) or 2% cholesterol-supplemented LCD for 6 days. Composition of CD and LCD are shown in Supplemental Tables S1 and S2. CD and LCD contains in 0.1 and 0.0056% of cholesterol, respectively (data obtained from CLEA Japan and Research Diet). The animal experiment was approved by the Animal Care and Use Committee of Tohoku University.

Preparation of Nuclear Extracts. Mouse liver was pooled for each diet group (n = 6 or 7), homogenized in homogenization buffer (10 mM HEPES-KOH, pH 7.6, 2 M sucrose, 25 mM KCl, 0.15 mM spermidine, 0.5 mM spermine, 1 mM EDTA, and 10% glycerol) and centrifuged at 20,000 rpm for 30 min at 4°C with a swing rotor (Hitachi koki, Tokyo, Japan). The precipitate was suspended in 10 mM HEPES-KOH, pH 7.6, 0.1 M KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 1 mM Na_3VO_4, 10% glycerol, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 0.1 mM PMSF, and 1 mM DTT. After addition of NaCl to make final salt concentration of 400 mM, the suspension was incubated at 4°C for 30 min and centrifuged at 20,000g for 20 min at 4°C. The supernatant was dialyzed against 20 mM HEPES-KOH, pH 7.6, 0.1 M KCl, 0.2 mM EDTA, 1 mM Na_2MoO_4, 20% glycerol, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 0.1 mM PMSF, and 1 mM DTT. Nuclear extracts (NEs) were subjected to immunoblotting with anti-SREBP-2 (Cayman Chemical, Ann Arbor, MI) antibody. The band intensities were quantified using NIH Image software (http://rsbweb.nih.gov/nih-image/).

Determination of Protein Concentration. The protein concentration was determined by Bradford method with Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) with BSA as standard.

Quantitative Reverse Transcription-PCR. Total liver RNA was extracted using the acid guanidine thiocyanate-phenol-chloroform method and cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) with Thermal Cycler Dice Real Time System TP800 (Takara Bio, Otsu, Japan). The sequences of the primers used are shown in Supplemental Table S3.

Plasmid Preparation. *Cyp3a11* luciferase reporter constructs were prepared by inserting *Cyp3a11* promoter DNA, which were amplified by PCR using TaKaRa Ex Taq (Takara Bio) or KOD FX (TOYOBO, Osaka, Japan) with mouse genomic DNA as a template, into Acc65I and XhoI sites of pGL4.10 (Promega, Madison, WI). A mutation construct was made by using QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) with the primers, 5′-CACAACATTGCAGGGTAGATGATATCAGCTAATGAGTTACC-

CTTTCTCAGGACTGTAAATATTAGCAATCATTCTGTGA-3′ and 5′-TCACAGAATGATTGCTAATATTTACAGTCCTGAGAAAGGGT-AACTCATTAGCTGATATCATCTACCCTGCAATGTTGTG-3′. cDNAs of nuclear forms of human (h) and mouse (m) SREBP-2, hHNF-4α, and mHNF-4α, dominant-negative forms of hSREBP-2 and mSREBP-2, mSREBP-2DN-2, and nuclear form of mSREBP-1a were amplified by PCR and inserted into pTargeT (Promega). The fragments obtained by digesting the pTargeT expression plasmids with MluI and NotI and hPGC-1α cDNA fragment obtained by digesting pcDNA3/HA-hPGC-1α (gift of Dr. Anastasia Kralli, The Scripps Research Institute, La Jolla, CA) with KpnI and NotI were inserted into the same restriction sites of pTNT (Promega). Primers used for PCR are shown in Supplemental Tables S4 and S5.

Reporter Assay. HepG2 (RIKEN Bio Resource Center, Tsukuba, Japan) were maintained as described previously (Yoshinari et al., 2010). The cells were seeded in 24-well plates (BD Biosciences, Heidelberg, Germany) at 5×10^4 cells/well 24 h before transfection. Reporter construct and pTargeT expression plasmid were cotransfected using calcium phosphate method. pSV-β-galactosidase Control Vector (Promega) was cotransfected to normalize transfection efficiency. Eight hours after transfection, medium was changed, and the cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum for an additional 40 h. Thereafter, the cells were harvested, and luciferase and β-galactosidase activities were determined as described previously (Yoshinari et al., 2010).

Electrophoretic Mobility Shift Assay. Electrophoretic mobility shift assay (EMSA) was performed as described previously (Toriyabe et al., 2009). hHNF-4α was synthesized in vitro with the pTNT plasmid containing hHNF-4α cDNA using TNT SP6 Quick-Coupled Transcription/Translation System (Promega). The probe sequences are shown in Fig. 3B. Supershift reaction was performed with anti-HNF-4α antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Preparation of DR1-Affinity Resin. Oligonucleotides (5′-ACTG-TAATGAGGGCAAAGTTCTCAGG-3′ and 5′-CAGTCCTGAGAACTTT-GCCCTCATTA-3′) corresponding to *Cyp3a11* DR1 were annealed and phosphorylated with T4 polynucleotide kinase. After purification with Wizared SV Gel and PCR Clean-Up System (Promega), they were self-ligated using T4 DNA ligase (Takara Bio), labeled with biotin-14-dATP (Invitrogen, Carlsbad, CA) using Klenow fragment (Takara Bio), and purified as described above. The biotin-labeled oligonucleotides were incubated with Streptavidin Magnetic Beads to obtain DR1-affinity resin.

DR1-Affinity Assay. mHNF-4α, mSREBP-2, and hPGC-1α were synthesized in vitro as described above. Reaction mixture (200 μl), containing DR1-affinity resin, 20 mM HEPES-KOH, pH 7.6, 0.8 μg of salmon sperm DNA (BioDynamics Laboratory, Tokyo, Japan), 0.1% Nonidet P-40 (Nacalai Tesque, Kyoto, Japan), 0.1 M KCl, 0.2 mM EDTA, 20% glycerol, and 10 μl of in vitro synthesized proteins, was incubated at 4°C for 30 min. The supernatant after centrifugation was kept as an unbound fraction. The resin was washed with 20 mM HEPES-KOH, pH 7.6, 0.1 M KCl, 0.2 mM EDTA, 1 mM Na_2MoO_4, 20% glycerol, and bound proteins were eluted successively with 25 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 0.5 mM DTT, 10% glycerol, 0.05% Nonidet P-40 containing 0.1, 0.5, or 1 M NaCl. Eluates and unbound fractions were subjected to immunoblotting with anti-SREBP-2 (Cayman Chemical), anti-HNF-4α (Santa Cruz Biotechnology), or anti-PGC-1α (Santa Cruz Biotechnology) antibody.

GST Pull-Down Assay. A series of the cDNA fragments of mHNF-4α or hSREBP-2 were inserted into pGEX-4T-1 (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Primers used are shown in Supplemental Table S6. Glutathione transferase (GST), a series of GST-HNF-4α and GST-SREBP-2 were expressed in BL21(DE3)pLysS (Novagen, Darmstadt, Germany), Origami2(DE3) (Novagen) or Rosetta-gami(DE3)pLysS (Novagen) and purified with glutathione-Sepharose 4B (GE Healthcare). mSREBP-2, mSREBP-2DN, mSREBP-2DN-2, and mSREBP-1a were synthesized in vitro as described above. Glutathione-Sepharose 4B was incubated with 20 μg of GST, GST-HNF-4α, or GST-SREBP-2 in binding buffer (50 mM Tris-HCl, pH 8.0, 34 mM NaCl, and 1 mM EDTA) at 4°C for 1 h and washed three times with the same buffer. Protein-bound beads were incubated with 10 μl of each in vitro-synthesized protein at room temperature for 1 h in binding buffer. The supernatant after centrifugation was kept as an unbound fraction. In competition assays using oligonucleotides, various amounts (4, 133, or 400 pmol) of oligonucleotides (Fig. 3B) were added to the reaction before the addition of mSREBP-2. In competition assays using in vitro-synthesized proteins, hSREBP-2- or hSREBP-2DN-containing lysate (10 or 30 μl), synthesized in vitro as described above, was added to the reaction before the addition of PGC-1α lysate. The beads were washed five times with binding buffer, and bound proteins were eluted with sample buffer (31 mM Tris-HCl, pH 6.8, 1% SDS, 5% sucrose, 0.1 μg/μl bromphenol blue, and 5% 2-mercaptoethanol) and subjected to immunoblotting with anti-SREBP-2 (Cayman Chemical), anti-PGC-1α (Santa Cruz Biotechnology), or anti-SREBP-1a (Novus Biologicals, Littleton, CO) antibody.

Coimmunoprecipitation Assay. NEs of mouse livers (0.6 mg) were precleared by incubation with 50 μl of protein G-coupled Dynabeads (Invitrogen) at 4°C for 3 h. The precleared samples were divided into two tubes (0.3 mg each) and subjected to immunoprecipitation at 4°C overnight with 5 μg of normal rabbit IgG (Millipore Corporation, Billerica, MA) or anti-SREBP-2 antibody (Cayman Chemical). The immune complexes were incubated with protein G-coupled Dynabeads (50 μl) at 4°C for 2 h. The beads were washed five times in 20 mM HEPES-KOH, pH 7.6, 100 mM NaCl, and 1 mM EDTA. The immune complexes were eluted with sample buffer and subjected to immunoblotting with anti-SREBP-2 (one third of the eluates; Cayman Chemical) and anti-PGC-1α antibody (two thirds of the eluates; Santa Cruz Biotechnology).

Chromatin Immunoprecipitation Assay. Pooled mouse livers (0.2 g; $n = 6$ or 7) were minced and cross-linked in 1% formaldehyde at room temperature for 5 min. The cross-linking was stopped by adding glycine at a final concentration of 125 mM. After 5-min incubation at room temperature, the liver was collected by centrifugation, homogenized in homogenization buffer used for NE preparation containing protease inhibitor cocktail, and centrifuged at 20,000 rpm for 30 min at 4°C with a swing rotor. The precipitate was suspended in chromatin immunoprecipitation (ChIP) lysis buffer (50 mM Tris-HCl, pH 8.0, 1% SDS, and 10 mM EDTA) and sonicated (12 times for 15 s each with a 45-s interval) with Bioruptor (Cosmo Bio, Tokyo, Japan) at the middle power output setting. An aliquot was saved as input sample. After centrifugation, the supernatant was diluted 10 times with 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, and 0.1% sodium deoxycholate. After the addition of BSA (100 μg), salmon sperm DNA (10 μg), and 50 μl of protein G-coupled Dynabeads, samples were incubated at 4°C for 3 h. The precleared samples were divided into five tubes and subjected to immunoprecipitation at 4°C overnight with 6 μg of normal goat IgG (Santa Cruz Biotechnology), normal rabbit IgG (Merck, Darmstadt, Germany), anti-HNF-4α (Santa Cruz Biotechnology), anti-SREBP-2 (Cayman Chemical), or anti-PGC-1α (Merck) antibody. The immune complexes were incubated with protein G-coupled Dynabeads (50 μl) at 4°C for 2 h. The beads were washed five times with 50 mM HEPES-KOH, pH 7.55, 500 mM LiCl, 1 mM EDTA, 1% Nonidet P-40, and 0.7% sodium deoxycholate and once with 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 50 mM NaCl. The immune complexes were eluted by incubation in ChIP lysis buffer at 65°C for 30 min. The supernatant after centrifugation was incubated at 65°C for 6 h to de–cross-link. After the addition of 80 μg of RNase A (Nacalai Tesque), samples were incubated at 37°C for 30 min. Proteins were then digested with proteinase K (80 μg) at 55°C for 1 h. DNA samples were purified with Wizard SV Gel and PCR Clean-Up System and used as a template for PCR with Takara Ex Taq Hot Start Version (Takara Bio) and primers (Supplemental Table S7).

Statistical Analysis. One-way analysis of variance with Dunnett's post hoc test was performed using Prism software (ver. 4.0; GraphPad Software Inc., San Diego, CA).

Results

Influence of Dietary Cholesterol Levels on *Cyp3a11* Expression and SREBP-2 Function in Mouse Liver. To verify the effects of dietary cholesterol on hepatic *Cyp3a11* expression, mice were fed CD, LCD, or 2% cholesterol-supplemented LCD for 6 days, and hepatic *Cyp3a11* mRNA and CYP3A11 protein levels were determined. Hepatic *Cyp3a11* mRNA levels in LCD-fed mice were significantly lower than those in CD-fed mice, and the supplementation of LCD with 2% cholesterol significantly reversed the mRNA levels (Fig. 1A). Likewise, hepatic CYP3A11 protein levels were substantially lower in LCD-fed mice than in CD-fed mice and increased by the cholesterol supplementation to LCD (Supplemental Fig. S1). These results suggest a possible association of the reduced nutritional intake of cholesterol with the down-regulation of *Cyp3a11* expression in mouse livers.

SREBP-2 is activated under conditions of low cellular cholesterol to maintain cholesterol levels. As expected, hepatic nuclear SREBP-2 levels determined by immunoblotting markedly higher in LCD-fed mice than in CD-fed mice and mice fed cholesterol-supplemented LCD (Fig. 1B). The protein levels in LCD-fed mice and 2% cholesterol-supplemented LCD-fed mice were approximately 271 and 71% of that of CD-fed mice, respectively. Hepatic mRNA levels of SREBP-2-responsive genes, *Srebf2* (SREBP-2), *Ldlr* (LDL-R), *Hmgcs1* (HMG-CoA synthase 1), and *Sqle* (squalene epoxidase) were consistently also significantly higher in LCD-fed mice than in CD-fed mice, and the cholesterol supplementation to LCD reduced the mRNA levels (Fig. 1A).

SREBP-2-Dependent Suppression of *Cyp3a11* Transcription. To examine the influence of SREBP-2 on *Cyp3a11* transcription, reporter assays were performed using the constructs containing various *Cyp3a11* 5′-flanking regions and expression plasmid of SREBP-2 nuclear form. The insertion of 8.9, 3.5, 2, or 1 kilobase of the *Cyp3a11* promoter increased

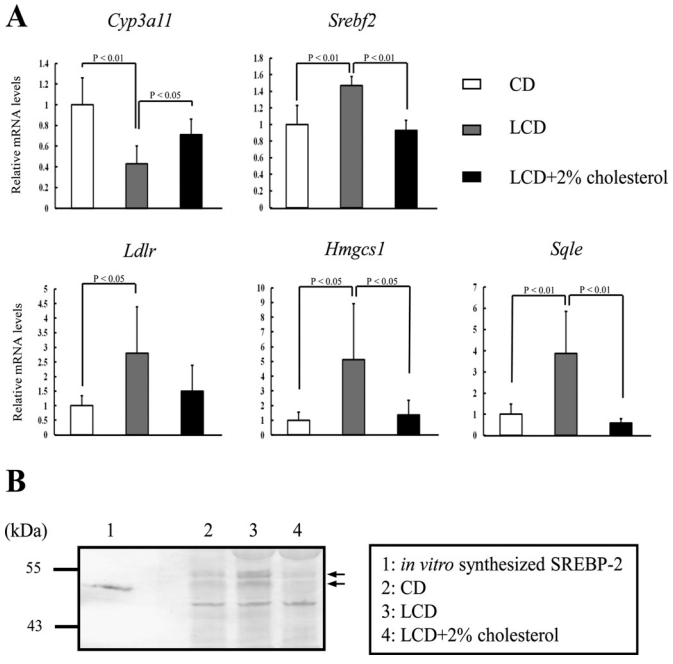

Fig. 1. Influence of dietary cholesterol level on the hepatic expression of *Cyp3a11* and SREBP-2-responsive genes. Mice were fed CD, LCD, or 2% cholesterol-supplemented LCD for 6 days. A, hepatic mRNA levels were determined by quantitative reverse transcription-PCR. mRNA levels were normalized by those of *β-actin*, and those in CD-fed mice are set at 1. Data are the mean ± S.D. (n = 6–7). *Srebf2*, *Ldlr*, *Hmgcs1*, and *Sqle* encode SREBP-2, LDL-R, HMG-CoA synthase 1, and squalene epoxidase, respectively. B, mouse liver NEs (100 μg) and in vitro-synthesized nuclear form of SREBP-2 were subjected to immunoblotting with 8% SDS-polyacrylamide gel and anti-SREBP-2 antibody. Molecular mass markers are shown on the left. The arrows indicate the bands corresponding to SREBP-2 for which intensity was quantified by using NIH Image software.

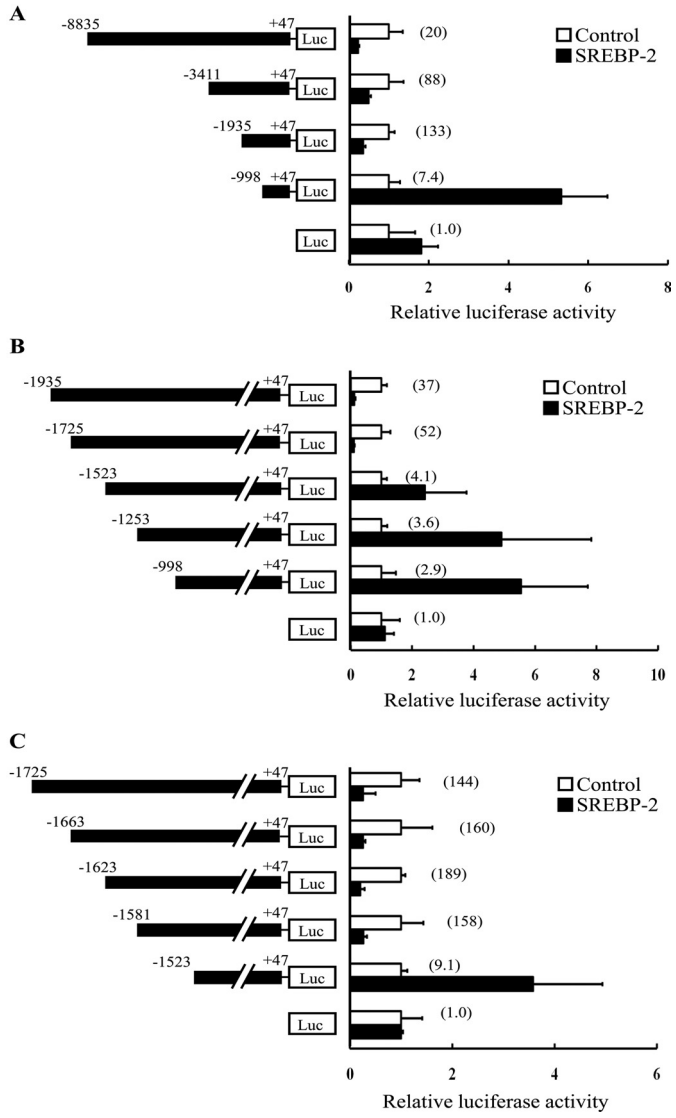

Fig. 2. Identification of a region responsible for the SREBP-2-dependent suppression of *Cyp3a11* expression. HepG2 cells were transfected with pSV-β-galactosidase control vector (1 μg) and each reporter construct (1 μg) shown on the left in the presence of either empty plasmid (□) or expression plasmid of hSREBP-2 nuclear form (■) (0.5 μg each), and reporter activities were determined as described under *Materials and Methods*. Luciferase activities were normalized with β-galactosidase activities and are shown as ratios to those in control cells for each construct. The numbers in parentheses represent ratios of the activity to those in the cells transfected with pGL4.10 and empty expression plasmid. The numbers above the constructs represent the positions from the *Cyp3a11* transcriptional starting point. Data are the mean ± S.D. (n = 4). Results shown are representative of three independent assays.

reporter activities, and the overexpression of active SREBP-2 reduced their reporter activities except for the construct containing the region from −998 to +47 (Fig. 2A). To identify the region responsible for the SREBP-2-dependent suppression of *Cyp3a11* expression, detailed assays were performed with another set of reporter constructs. The deletion of the region from −1581 to −1524 resulted in complete loss of the SREBP-2-mediated suppression of *Cyp3a11* expression (Fig. 2, B and C).

Binding of HNF-4α to the DR1 Motif in *Cyp3a11*. To identify a transcription factor(s) that binds to the region from −1581 to −1524 of *Cyp3a11*, the web-based program Transcription Element Search System (http://www.cbil.upenn.edu/tess) was used. The analysis revealed that there was no sterol regulatory element in this region. Instead, a putative HNF-4α binding motif, the direct repeat separated by a nucleotide or DR1, was found (Fig. 3A). To examine the direct binding of HNF-4α to the DR1 motif, we performed EMSAs with radiolabeled oligonucleotides (Fig. 3B) and in vitro-synthesized proteins. CYP7A1 and phosphoenolpyruvate carboxykinase 1 (PEPCK1) probes containing known HNF-4α binding motifs (Yamamoto et al., 2004; Miao et al., 2006) were used as positive controls. HNF-4α bound to DR1 probe and CYP7A1 and PEPCK1 probes but not mutated probe DR1mt, and the addition of anti-HNF-4α antibody supershifted the HNF-4α-DR1 complex (Fig. 3C). The binding specificity of HNF-4α to DR1 probe was confirmed by competition with unlabeled DR1 or PEPCK1 probe but not with DR1mt probe (Supplemental Fig. S2A). In contrast, SREBP-2 bound to LDL-R probe, containing a known SREBP-2 binding motif (Yamamoto et al., 2004), but not to DR1 probe (Supplemental Fig. S2B).

The binding of HNF-4α to the DR1 motif was further confirmed with DR1 affinity assays. NEs prepared from mouse livers or Hepa1–6 cells, or in vitro-synthesized proteins were incubated with DR1 oligonucleotides immobilized on streptavidin beads, and bound proteins were analyzed with immunoblotting. HNF-4α synthesized in vitro or that in the NEs strongly bound to the resin, whereas SREBP-2 did not (Supplemental Fig. S2C). These results further indicate that HNF-4α but not SREBP-2 binds to the DR1 motif. The cotransfection of HNF-4α-expressing plasmid consistently increased the reporter activity of the *Cyp3a11* reporter construct containing the DR1 motif in HepG2 cells (Supplemental Fig. S2D).

Contribution of the DR1 Motif to the SREBP-2-Dependent Suppression of *Cyp3a11* Expression. The contribution of the DR1 motif to the SREBP-2-mediated suppression of *Cyp3a11* expression was examined in reporter assays. The partial deletion of the DR1 motif or the introduction of mutations into the motif resulted in the complete loss of the SREBP-2-mediated suppression in HepG2 (Fig. 4) and Hepa1–6 cells (data not shown). These results suggest that the DR1 motif is necessary for the SREBP-2-mediated suppression of *Cyp3a11* expression.

Interaction of SREBP-2 with HNF-4α In Vitro. The obtained data suggested that SREBP-2 suppressed the HNF-4α-mediated transcription of *Cyp3a11*. To test this possibility, we first performed GST pull-down assays to investigate the direct binding of SREBP-2 to HNF-4α. In vitro-synthesized SREBP-2, SREBP-2DN, SREBP-2DN-2, or SREBP-1a (Fig. 5A) was incubated with GST-fused HNF-4α fragments (Fig. 5B) bound to beads. SREBP-2, SREBP-2DN, and SREBP-2DN-2 interacted with GST-HNF-4α (Fig. 5B, a) and the fragment (Fig. 5B, b) containing the DNA binding domain but not with GST and other fragments (Figs. 5B, c, d, and e, and C). These results suggest that SREBP-2 interacts with the N terminus of HNF-4α (amino acids 1–129) containing DNA binding domain through the region containing basic helix-loop-helix domain (amino acids 314–473). SREBP-1a interacted with HNF-4α through the ligand binding domain as reported previously (Fig. 5C) (Yamamoto et al., 2004).

To examine whether SREBP-2 could interact with HNF-4α bound to the DR1 motif, DR1-affinity assays were performed. As shown in Fig. 5D, when HNF-4α and SREBP-2 were mixed with the DR1 resin, only HNF-4α (lane 3) but not SREBP-2 (lane 8) bound to the resin. The addition of SREBP-2 consistently had no affect on the binding of HNF-4α to DR1 probe in EMSAs (data not shown). These results suggest no formation of the ternary complex of SREBP-2-HNF-4α-DR1. The results were further confirmed with GST pull-down assay. The binding of in vitro-synthesized SREBP-2 to GST-HNF-4α was abolished by the addition of DR1 oligonucleotides but not mutated oligonucleotides (Fig. 5, E and F). These results suggest that SREBP-2 is unable to associate with HNF-4α bound to the DR1 motif.

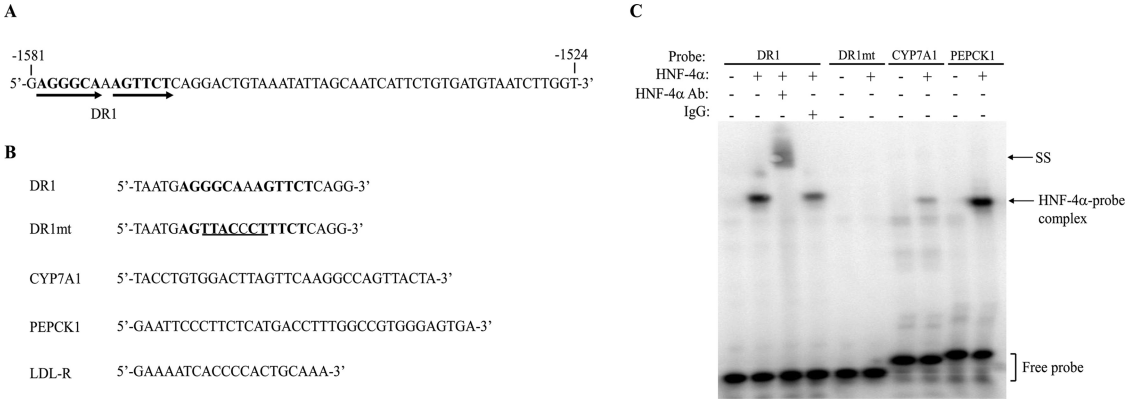

Fig. 3. Binding of HNF-4α to the DR1 motif in *Cyp3a11*. A, nucleotide sequence of the region from −1581 to −1524 of *Cyp3a11* is shown. Putative HNF-4α binding motif DR1 is shown in boldface type with arrows. B, the sequences of oligonucleotides used in EMSAs are shown. DR1 and mutated DR1 are shown in boldface type. Mutated nucleotides are underlined. C, EMSAs were performed as described under *Materials and Methods* with radiolabeled oligonucleotides and in vitro-synthesized HNF-4α. SS, supershifted complex with anti-HNF-4α antibody.

SREBP-2 Inhibition of the Interaction between HNF-4α and PGC-1α. Because SREBP-2 bound to free HNF-4α (Fig. 5C) but not that bound to the DR1 motif (Fig. 5, D–F), SREBP-2 could inhibit the HNF-4α-mediated *Cyp3a11* transcription through a mechanism other than its direct binding to HNF-4α. Therefore, we hypothesized that SREBP-2 might affect the interaction between HNF-4α and coactivators. To test this possibility, we performed GST pull-down assays with GST-HNF-4α, in vitro-synthesized SREBP-2, and PGC-1α, a well known coactivator for HNF-4α. It was confirmed that PGC-1α interacted with HNF-4α in this system (Fig. 6A). As expected, the addition of SREBP-2 attenuated the interaction between PGC-1α and HNF-4α (Fig. 6A). We then performed DR1-affinity assays using in vitro synthesized HNF-4α and PGC-1α with or without SREBP-2. PGC-1α was associated with HNF-4α bound to the DR1 resin (Fig. 6B and Supplemental Fig. S3), and the interaction was attenuated in the presence of SREBP-2 (Fig. 6B, top). On the other hand, SREBP-2 had no influence on the HNF-4α binding to the resin (Fig. 6B, bottom). These results suggest that SREBP-2 inhibits the interaction of PGC-1α with HNF-4α on the DR1 motif.

Next, the direct binding of SREBP-2 to PGC-1α was tested in GST pull-down assays. As shown in Fig. 6C, SREBP-2 directly interacted with PGC-1α in solution. The binding of GST-SREBP-2 to PGC-1α was competed out with SREBP-2 but not SREBP-2DN (Fig. 6D). Finally, we performed coimmunoprecipitation assays with the liver NEs of LCD-fed mice using anti-SREBP-2 antibody. As shown in Fig. 6E, PGC-1α was coimmunoprecipitated with SREBP-2. These results suggest that SREBP-2 directly interacts with PGC-1α through its transactivation domain in mouse livers.

Role of PGC-1α in the SREBP-2-Mediated Suppression of *Cyp3a11* Expression. Influence of PGC-1α overexpression on the SREBP-2-mediated suppression of *Cyp3a11* expression was examined in reporter assays (Fig. 7A). The cotransfection of PGC-1α expression plasmid increased reporter activity (lane 4 versus lane 1), and SREBP-2 overexpression reduced reporter activities both in the absence (lane 2 versus lane 1) and presence (lane 5 versus lane 4) of coexpressed PGC-1α. It is noteworthy that the transfection of increasing amounts of PGC-1α expression plasmid rescued the SREBP-2-mediated suppression (lanes 3 and 6 versus lanes 2 and 5, respectively). To investigate the role of transactivation domain of SREBP-2 in the suppression of *Cyp3a11* expression, reporter assays were performed with SREBP-2 and SREBP-2DN expression plasmids. As expected from the results obtained in Fig. 6D, the deletion of the transactivation domain of SREBP-2 completely eliminated its suppressive effect on the *Cyp3a11* expression (Fig. 7B). Taken together, these results suggest that the interaction with PGC-1α is necessary for the suppressive effect of SREBP-2 on the *Cyp3a11* expression.

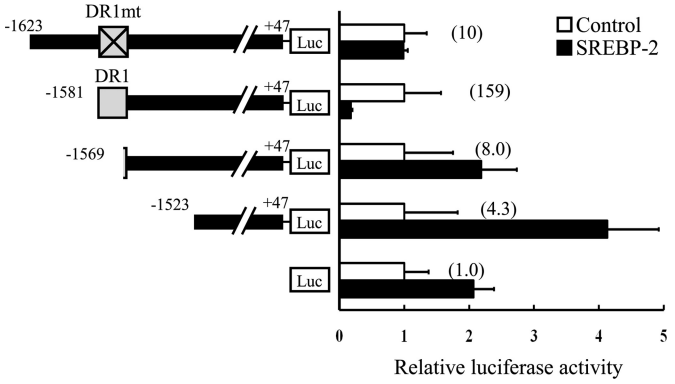

Fig. 4. Contribution of the DR1 motif to the SREBP-2-dependent suppression of *Cyp3a11* expression. Schematic structures of luciferase reporter gene constructs are shown on the left, and a shaded box represents the DR1 motif. In the top construct, the motif was mutated as in Fig. 3B. Reporter assays were performed with HepG2 cells, and results are shown as in Fig. 2.

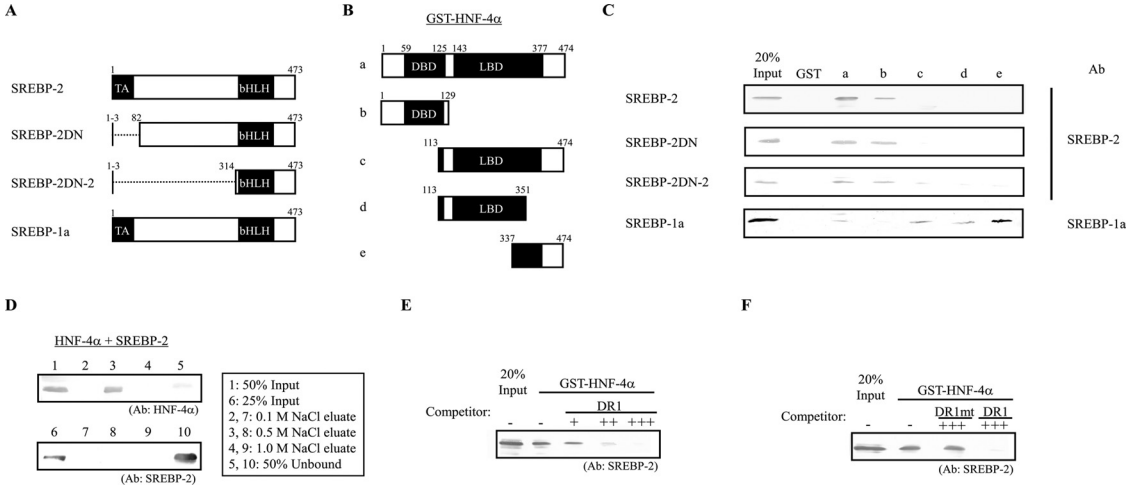

Fig. 5. Interaction of SREBP-2 with HNF-4α. Schematic structures of SREBP-2, SREBP-2 mutants, and SREBP-1a (A) and of HNF-4α and its deletion mutants (B) are shown. TA, transactivation domain. C, GST pull-down assays were performed as described under *Materials and Methods*. GST or GST-HNF-4α bound to beads was incubated with in vitro-synthesized SREBP-2, SREBP-2DN, SREBP-2DN-2, or SREBP-1a. Proteins bound to the resin were subjected to immunoblotting with 8% (SREBP-2 and SREBP-1a), 10% (SREBP-2DN), or 15% (SREBP-2DN-2) SDS-polyacrylamide gels and anti-SREBP-2 or anti-SREBP-1a antibody. D, DR1-affinity assays were performed as described under *Materials and Methods* with in vitro-synthesized HNF-4α and SREBP-2. Eluates, unbound fractions, and input samples were subjected to immunoblotting with 8% SDS-polyacrylamide gel and anti-HNF-4α or anti-SREBP-2 antibody. E and F, GST pull-down assays were performed. GST or GST-HNF-4α bound to beads were incubated with in vitro-synthesized SREBP-2 and various amounts of double-stranded DR1 or DR1mt oligonucleotides (+, 40 pmol; ++, 133 pmol; +++, 400 pmol) shown in Fig. 3B. SREBP-2 bound to GST-HNF-4α was subjected to immunoblotting with 8% SDS-polyacrylamide gel and anti-SREBP-2 antibody.

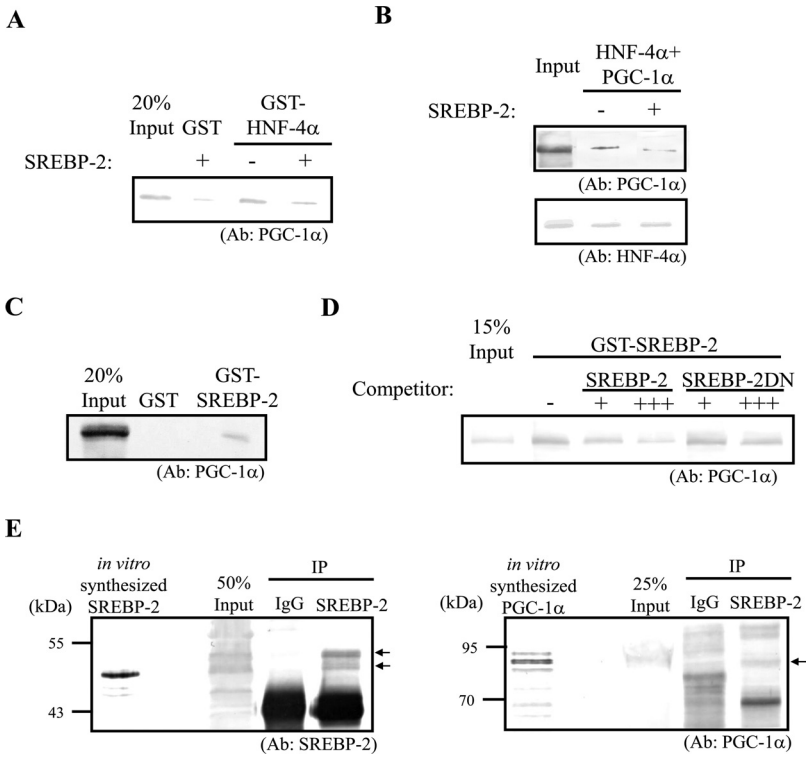

Fig. 6. Influence of SREBP-2 on the interaction between HNF-4α and PGC-1α. A, C, and D, GST pull-down assays were performed as described under *Materials and Methods*. GST or GST-HNF-4α bound to beads was incubated with in vitro-synthesized PGC-1α in the absence or presence of SREBP-2 (A). GST or GST-SREBP-2 bound to beads was incubated with in vitro-synthesized PGC-1α (C and D). In D, various amounts of in vitro-synthesized SREBP-2 or SREBP-2DN were mixed with GST-SREBP-2 before the addition of PGC-1α. PGC-1α bound to GST-HNF-4α or GST-SREBP-2 was subjected to immunoblotting with 8% SDS-polyacrylamide gel and anti-PGC-1α antibody. B, DR1-affinity assays were performed as described under *Materials and Methods* with in vitro-synthesized HNF-4α and PGC-1α with or without SREBP-2. A portion of input proteins (top, 37.5%; bottom, 50%) were also subjected. The eluates with 0.5 M NaCl solution were subjected to immunoblotting with 8% SDS-polyacrylamide gel and anti-HNF-4α or anti-PGC-1α antibody. E, coimmunoprecipitation assays were performed with liver NEs prepared from mice fed LCD for 6 days using control IgG or anti-SREBP-2 antibody as described under *Materials and Methods*. The immunoprecipitated samples, NEs (50 μg) and in vitro-synthesized proteins were subjected to immunoblotting with 8% SDS-polyacrylamide gel and anti-SREBP-2 and anti-PGC-1α antibody. Molecular mass markers are shown on the left, and the arrows indicate the bands corresponding to SREBP-2 or PGC-1α.

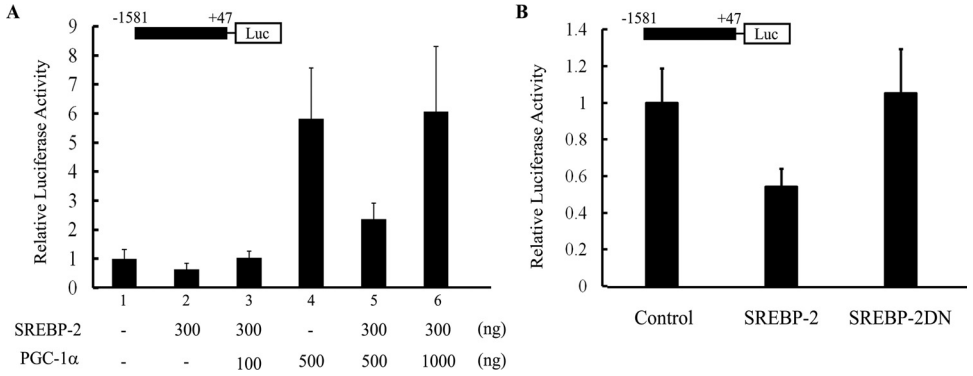

Fig. 7. Role of the interaction of SREBP-2 with PGC-1α in the suppression of *Cyp3a11* expression. Reporter assays were performed with pSV-β-galactosidase control vector (1 μg), the reporter construct shown (1 μg) in combination with hSREBP-2 expression plasmid (0.3 μg), and various amounts (0.1, 0.5, and 1 μg) of PGC-1α expression plasmid (A), or empty (Control, 0.5 μg), hSREBP-2 (0.5 μg), or hSREBP-2DN (0.5 μg) expression plasmid (B) in HepG2 cells. Luciferase activities were normalized with β-galactosidase activities, and the normalized activities in the cells transfected with *Cyp3a11* reporter construct were divided with those in the corresponding cells transfected with pGL4.10. The reporter activities in the cells transfected with empty plasmid are set at 1. Data are the mean ± S.D. (n = 4). Results shown are representative of three independent assays.

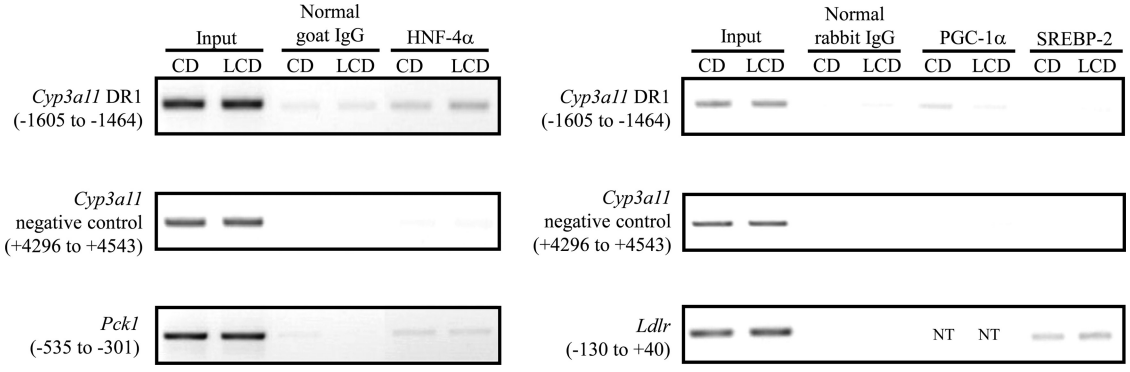

Fig. 8. Influence of dietary cholesterol levels on HNF-4α and PGC-1α binding to *Cyp3a11* promoter. ChIP assays were performed with livers of mice fed CD or LCD for 6 days using antibodies indicated as described under *Materials and Methods*. The immunoprecipitated DNA, along with the DNA isolated before immunoprecipitation (Input), were analyzed by PCR using specific primers (Supplemental Table S7) for the indicated regions. Results shown are representative of three independent assays. NT, not tested.

Influence of Dietary Cholesterol Levels on PGC-1α Binding to HNF-4α on the DR1 Motif. To verify whether dietary cholesterol levels affected PGC-1α binding to HNF-4α in a genomic context, ChIP assays were performed with livers of CD- or LCD-fed mice (Fig. 8). *Pck1* (PEPCK1) and *Ldlr* promoters, containing known HNF-4α and SREBP-2 binding motifs, respectively (Miao et al., 2006; Bennett et al., 2008), were also monitored as positive controls. The specific binding of HNF-4α to the DR1-containing region (−1605 to −1464) but not to the negative control region (+4296 to +4543) of *Cyp3a11* was detected in the liver of both mice, and the intensity was comparable (Fig. 8, left). Similar results were obtained for the *Pck1* promoter. In contrast, the specific binding of PGC-1α to the *Cyp3a11* was clearly detected in the liver of CD-fed mice but was barely detected in the liver of LCD-fed mice (Fig. 8, right) despite that PGC-1α protein levels in the liver nucleus of LCD-fed mice were 3.5 times higher than those of CD-fed mice (data not shown). As expected from the results shown above, no specific binding of SREBP-2 to the DR1-containing region of *Cyp3a11* was detected, whereas it bound to the *Ldlr* promoter, and the extent of the binding was higher in the liver of LCD-fed mice than in that of CD-fed mice. These results suggest that under conditions with reduced nutritional intake of cholesterol, the binding of PGC-1α, but not HNF-4α, to the DR1 motif in the *Cyp3a11* promoter is reduced in mouse livers.

Discussion

In this study, we found that limited supply of cholesterol resulted in the down-regulation of *Cyp3a11* expression in mouse livers. To understand the molecular mechanism of this phenomenon, we have investigated the role of SREBP-2 in the hepatic *Cyp3a11* expression. Present results demonstrate that the activation of SREBP-2 in mouse livers at limited cholesterol intake results in the down-regulation of *Cyp3a11* expression through a novel cross-talk of SREBP-2 and HNF-4α involving PGC-1α. SREBP-2 interacted with PGC-1α in vitro (Fig. 6, C–E) through its transactivation domain (Fig. 6D), which was necessary for the suppression of *Cyp3a11* expression in reporter assays (Fig. 7B). This SREBP-2 binding was suggested to inhibit the interaction of PGC-1α with HNF-4α in vitro (Fig. 6, A and B). DR1, the newly identified HNF-4α binding motif of *Cyp3a11*, was consistently necessary for the SREBP-2-mediated suppression of *Cyp3a11* expression in reporter assays (Fig. 4). Moreover, the binding of PGC-1α to the DR1 motif in the *Cyp3a11* promoter was reduced under an SREBP-2-activated condition, although activated SREBP-2 had no influence on the HNF-4α binding to the motif both in vitro and in vivo (Figs. 6B and 8). Finally, the overexpression of PGC-1α, but not HNF-4α, completely rescued the SREBP-2-mediated suppression of *Cyp3a11* expression (Fig. 7A and unpublished results). Taken together, it is suggested that activated SREBP-2 inhibits the interaction between HNF-4α and PGC-1α, reducing the HNF-4α-mediated transactivation of *Cyp3a11*.

We have found for the first time that SREBP-2 suppresses the HNF-4α-mediated gene expression. Although SREBP-1 has been reported to inhibit the HNF-4α-mediated gene expression (Yamamoto et al., 2004; Ponugoti et al., 2007), the mechanism of SREBP2-mediated transcriptional suppression is likely to differ from that by SREBP-1 in two ways. First, SREBP-2 did not interact with HNF-4α bound to DNA (Fig. 5, D–F), whereas SREBP-1 directly interacted with HNF-4α bound to the promoters of the target genes such as *Cyp7a1* and *Pck1* (Yamamoto et al., 2004; Ponugoti et al., 2007). Second, SREBP-2 directly interacted with PGC-1α in vitro (Fig. 6, C and D), whereas SREBP-1 did not interact with PGC-1α (Yamamoto et al., 2004). Instead, SREBP-1 competed with PGC-1α for the binding to HNF-4α (Yamamoto et al., 2004; Ponugoti et al., 2007). Thus, our data clearly indicate that SREBP-2 down-regulates the HNF-4α-mediated gene expression through a mechanism different from that for SREBP-1, involving the direct interaction with PGC-1α.

PGC-1α is reported to be crucial for the transcription mediated by other nuclear receptors, including CAR and PXR (Shiraki et al., 2003; Li and Chiang, 2005), and by HNF-4α. Indeed, ligand-activated CAR and PXR have been shown to suppress the HNF-4α-mediated transactivation by sequestering the common coactivator PGC-1α (Bhalla et al., 2004; Li and Chiang, 2005; Miao et al., 2006). The increase in CAR/PGC-1α or PXR/PGC-1α complex formation and the concomitant decrease in HNF-4α/PGC-1α formation lead to the suppression of *Cyp7a1* and *Pck1* expression (Bhalla et al., 2004; Li and Chiang, 2005; Miao et al., 2006). Therefore, it is of great interest to investigate in future studies whether hepatic gene transcriptions involving PGC-1α, in addition to that of *Cyp3a11*, are also down-regulated under the condition with reduced cholesterol intake in which SREBP-2 is activated.

Recent studies have demonstrated that transactivation by HNF-4α requires the formation of a complex with coactivators, such as PGC-1α, glucocorticoid receptor interacting protein-1, and steroid receptor coactivator-1 (Wang et al., 1998; Rhee et al., 2003; Miao et al., 2006). At present, it remains unknown whether SREBP-2 interacts with coactivators other than PGC-1α. Future studies may help to understand the specificity for the interaction of SREBP-2 with coactivators.

HNF-4α is reported to be crucial for the constitutive expression of several hepatic genes involved in lipid homeostasis and drug metabolism (Jiang et al., 1995; Gonzalez, 2008). In the present study, we have found that HNF-4α transactivates *Cyp3a11* through the DR1 motif. Reporter assays demonstrated that *Cyp3a11* reporter constructs lacking the motif or having a mutation in the motif showed drastically reduced constitutive expression (Fig. 4). EMSAs, DR1-affinity assays, and ChIP assays demonstrated that HNF-4α bound to the DR1 motif in *Cyp3a11* both in vitro and in vivo (Figs. 3C and 8, and Supplemental Figs. S2A and S2C). Recent studies using HNF-4α antisense RNA or the recombinant adenovirus that expresses HNF-4α-small interfering RNA have demonstrated that HNF-4α is critical for the *CYP3A4* expression in human hepatocytes (Jover et al., 2001; Kamiyama et al., 2007), which is consistent with our present results showing that HNF-4α is involved in the constitutive expression of *Cyp3a11*.

CYP3A4 plays a crucial role in the xenobiotic metabolism, mediating the oxidation of more than 50% of clinically used therapeutic drugs (Gonzalez, 1992; Eichelbaum and Burk, 2001). In addition to xenobiotic metabolism, it also catalyzes the oxidation of steroid hormones such as testosterone and estradiol (Mäenpää et al., 1993; Stresser and Kupfer, 1997). CYP3A4 has been reported to mediate the conversion of cholesterol into 4β-hydroxycholesterol, an endogenous oxys-

terol found in the human circulation (Bodin et al., 2001, 2002), and to be involved in the bile acid synthesis (Honda et al., 2001; Goodwin et al., 2003; Pikuleva, 2006). These findings imply that CYP3A enzymes are of importance in cholesterol metabolism and xenobiotic metabolism. In the present study, we have demonstrated that hepatic *Cyp3a11* expression is reduced under the condition with reduced nutritional intake of cholesterol through an SREBP-2-mediated mechanism. This decreased expression of *Cyp3a11* may be a physiological response to maintain hepatic cholesterol level, corroborating the role of CYP3A enzymes in cholesterol homeostasis.

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Authorship Contributions

Participated in research design: Inoue, Yoshinari, and Yamazoe.

Conducted experiments: Inoue, Yoshinari, and Sugawara.

Performed data analysis: Inoue, Yoshinari, Sugawara, and Yamazoe.

Wrote or contributed to the writing of the manuscript: Inoue, Yoshinari, and Yamazoe.

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