A Prospero-related Homeodomain Protein Is a Novel Co-regulator of Hepatocyte Nuclear Factor 4α That Regulates the Cholesterol 7α-Hydroxylase Gene*

Kwang-Hoon Song, Tiangang Li, and John Y. L. Chiang 1

From the Department of Microbiology, Immunology and Biochemistry, Northeastern Ohio Universities College of Medicine, Rootstown, Ohio 44272

Prox1, an early specific marker for developing liver and pancreas in foregut endoderm has recently been shown to interact with α-fetotoprotein transcription factor and repress cholesterol 7α-hydroxylase (CYP7A1) gene transcription. Using a yeast two-hybrid assay, we found that Prox1 strongly and specifically interacted with hepatocyte nuclear factor (HNF4)α, an important transactivator of the human CYP7A1 gene in bile acid synthesis and phosphoenolpyruvate carboxykinase (PEPCK) gene in gluconeogenesis. A real time PCR assay detected Prox1 mRNA expression in human primary hepatocytes and HepG2 cells. Reporter assay, GST pull-down, co-immunoprecipitation, and yeast two-hybrid assays identified a specific interaction between the N-terminal LXXLL motif of Prox1 and the activation function 2 domain of HNF4α. Prox1 strongly inhibited HNF4α and peroxisome proliferators-activated receptor γ coactivator-1α co-activation of the CYP7A1 and PEPCK genes. Knock down of the endogenous Prox1 by small interfering RNA resulted in significant increase of CYP7A1 and PEPCK mRNA expression and the rate of bile acid synthesis in HepG2 cells. These results suggest that Prox1 is a novel co-regulator of HNF4α that may play a key role in the regulation of bile acid synthesis and gluconeogenesis in the liver.

CYP7A1 2 catalyzes the first and rate-limiting step in the conversion of cholesterol to bile acids and plays an important role in maintaining whole body lipid homeostasis (1). Bile acids are physiological detergents that facilitate absorption, transport and distribution of sterols and lipid-soluble vitamins, and disposal of toxic metabolites and xenobiotics. Bile acid synthesis and CYP7A1 gene transcription is feedback inhibited by bile acids returning to the liver via enterohepatic circulation of bile (1). Recent studies have identified farneoids X receptor (NR1H4) as a bile acid-activated receptor that induces an atypical nuclear receptor small heterodimer partner (SHP, NR0B2), which interacts with FTF (NR5A2) and HNF4α (NR2A1) bound to an overlapping sequence located in the bile acid response element II (−144/−126) and represses CYP7A1 gene transcription (2). However, the molecular mechanism by which FTF and HNF4α regulate the CYP7A1 gene is not completely understood.

HNF4α is the most abundant nuclear receptor expressed in the liver and is involved in early liver development (3). Conditional knock-out of the HNF4α gene in mouse liver caused accumulation of lipids in the liver, markedly reduced serum cholesterol and triglycerides, and increased serum bile acids (4). CYP7A1, Na +-taurocholate co-transport peptide, organic anion transporter 1, apolipoprotein B100, and scavenger receptor B-1 expression are reduced in these mice (4). It appears that HNF4α is a key regulator of bile acid and lipoprotein metabolism and plays a central role in lipid homeostasis (5). HNF4α is involved in diabetes; mutation of the HNF4α gene causes maturity onset diabetes of the young type 1 (MODY1) (6). HNF4α regulates the HNF1α gene, a MODY 3 gene (7).

The transcriptional activities of nuclear receptors are largely dependent on ligand binding and activation. Nuclear receptors interact with co-regulators and regulate their target genes in a tissue- and gene-specific manner (8). Upon ligand binding, the helix 12 of nuclear receptor is exposed and binds to the co-activators and activates nuclear receptor activity. Recently, PGC-1α has been identified as a co-activator of HNF4α (9). PGC-1α is highly induced during starvation by glucocorticoids and glucagon to induce PEPCK, a rate-limiting enzyme in gluconeogenesis (10). It has been reported that PGC-1α co-activates HNF4α and induces CYP7A1 gene transcription during starvation in mice (11). It has been suggested that bile acid synthesis and gluconeogenesis may be coordinately regulated in fasted-to-fed cycle (12). Our recent study (13) shows that glucagon and cAMP inhibit CYP7A1 by inducing phosphorylation of HNF4α.

Prox1 has recently been identified as a co-repressor of FTF/LRH-1 by yeast two-hybrid screening (14, 15). Prox1 was originally cloned by homology to the Drosophila melanogaster gene prospero (16). Prox1 is expressed in lens, heart, liver, kidney, skeletal muscle, pancreas, and central nervous system (16, 17). Earlier studies have linked Prox1 function to lens and lymphatic system development (18, 19). More recent studies (20, 21) indicate that Prox1 is required for hepatocyte migration in developing liver and pancreas in the mammalian foregut endoderm. Prox1 interacts with the NR5 subfamily of nuclear receptors including Fftb (NR5A4), a zebrasin homologue of nuclear receptor, steroidogenic factor 1 (NR5A1) (22), and FTF (14, 15) and represses their transactivation activity. We hypothesized that Prox1 may interact with HNF4α and suppressed CYP7A1 gene transcription. To test this hypothesis, we used yeast two-hybrid assay to study the interaction between Prox1 and HNF4α and studied the effect of Prox1 on the HNF4α transactivation of the human CYP7A1 gene. Our findings pro-

This is an Open Access article under the CC BY license.
vide a novel molecular mechanism for Prox1 inhibition of bile acid synthesis and glucocorticoid receptor, retinoic X receptor, and retinoic acid receptor, 100 µl of 1 µM stock solution of the appropriate ligands (T3, dexamethasone, all-trans-retinoic acid and 9-cis-retinoic) was added before plating to test the effect of ligand activation on interaction. Assays were repeated at least three times.

**Transient Transfection and Luciferase Reporter Assay**—For luciferase reporter assay, HepG2 cells were plated in 24-well plates 24 h before transfection with reporter or expression plasmids using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. The total DNA used in each transfection was adjusted by adding the appropriate amount of pcDNA3 vector. Luciferase activities are expressed as relative luciferase unit/β-galactosidase activity as described previously (22).

**Small Interfering RNA (siRNA) Experiments**—The SMART pool siRNAs for human Prox1 were purchased from Dharmacon Research (Lafayette, Co) and transfected into HepG2 cells using Lipofectamine 2000 reagent according to the manufacturer’s instructions. Forty-eight hours after transfection, cells were extracted and analyzed. The SMART pool is a mixture of four sequences located at different regions of mRNA. Two of them were tested to be effective in knockdown Prox1 in HepG2 cells. The siRNA sequences are: siRNA#1, nucleotides 1009–1027, GG GCCAAAACTCCTTTAACAC; siRNA#2 nucleotides 2096–2114, GCCAAGATTGTGATCCTTTC. The control siRNA is a scrambled siRNA that was designed to have the same G-C content as the siRNA#2 but did not display sequence identity with Prox1: GATCGTGTTAGTCTAACC.

**RNA Isolation and Real Time Quantitative PCR**—HepG2 cells was transfected with synthesized siRNA of human Prox1 and total RNA was isolated using Tri-reagent (Sigma, St. Louis, MO) according to the manufacturer’s instruction. Reverse-transcription and real time quantitative PCR were performed to detect Prox1, CYP7A1, PEPCK, HNF4α, and cyclophilin B mRNAs as described previously (13).

**Chromatin Immunoprecipitation (ChIP) Assay**—ChIP assays were performed using a ChIP Assay kit (Upstate Cell Signaling Solutions, Lake Placid, NY) according to the manufacturer’s instructions. HepG2 were transfected with pcDNA3 empty vector or Flag-Prox1, and chromatin was cross-linked in 1% formaldehyde and sonicated as reported previously (24). Cell lysate solution (5%) in ChIP dilution buffer was kept aside as “input.” Ten µg of HNF4α antibody (Santa Cruz Biotechnology) or anti-FLAG antibody (Sigma) was added to 200 µg of the DNA as template and analyzed on a 1.5% agarose gel. PCR primers for amplification were as follows: 5’-ATCAC-GTCTTCTCTGGCAAAGCAC-3’; reverse primer, 5’-CCATTAAC-TGAGCTTGTTGGACAAAG-3’.

**Bile Acid Analysis**—The siRNA for human Prox1 and control siRNA were transfected into HepG2 cells using Lipofectamine 2000 reagent according to the manufacturer’s instructions. Forty-eight hours after transfection, cells were washed and then incubated with serum-free medium for a period of time from 3 to 24 h. The medium was collected at indicated time points and frozen at −80 °C for later analysis of bile acids. At the end of the incubation, the cells were harvested and stored at −80 °C until use. A Sep-Pak C18 reversed phase cartridge (Waters Associates, Inc., Milford, MA) was used for bile acid extraction from media as described previously (25). Total bile acid concentration was analyzed by enzymatic 3α-hydroxysteroid dehydrogenase method using total bile acid assay kit (Bio-quant Inc., San Diego, CA) according to the manufacturer’s instruction.
RESULTS

Prox1 Interacts with HNF4α in Vivo and in Vitro—The nuclear receptors that have been identified to interact with Prox1 all belong to the NR5A subfamily (14, 15, 22). To identify other potential interaction partners of Prox1, we performed yeast two-hybrid protein interaction assays using LexA and B42 constructs available in our laboratory. Table 1 shows that Prox1 interacted with HNF4α. This interaction is stronger than Prox1 interaction with steroidogenic factor 1. On the other hand, Prox1 did not interact with thyroid hormone receptor α, thyroid hormone receptor β, retinoid X receptor, retinoic acid receptor, glucocorticoid receptor, SHP, dosage-sensitive sex reversal, AHC critical region on the X chromosome, gene 1 (DAX-1), and Nur77 regardless of the presence or absence of their respective ligands (Table 1). Transcriptional repressors N-CoR and SMRT did not interact with Prox1. These results showed that Prox1 interacts with HNF4α and NR5A family nuclear receptors.

To further confirm the results of the yeast two-hybrid assay, we performed in vitro GST pull-down assays to study Prox1 and HNF4α interaction. Consistent with yeast two-hybrid assay results, GST-Prox1 interacted with HNF4α but not retinoid X receptor and retinoic acid receptor (Fig. 1A). The physical interaction between GST-Prox1 and 35S-labeled FTF was used as a positive control (Fig. 1A). To further verify the interaction between Prox1 and HNF4α, we performed a co-immunoprecipitation assay using human primary hepatocyte extracts. Cell extracts were immunoprecipitated with an anti-HNF4α antibody. The immunoprecipitated complexes were then analyzed on an immunoblot.

**TABLE 1** Interaction of Prox1 with nuclear receptors in yeast two-hybrid interaction assay

|        | LEXA | B42 | Interaction |
|--------|------|-----|------------|
| Prox1  | Prox1| Prox1| +          |
| Thyroid hormone receptor α | Prox1| Prox1| +          |
| Thyroid hormone receptor β | Prox1| Prox1| +          |
| Glucocorticoid receptor | Prox1| Prox1| +          |
| Retinoic acid receptor α | Prox1| Prox1| +          |
| Retinoic X receptor α | Prox1| Prox1| +          |
| SHP | Prox1| Prox1| +          |
| DAX-1 | Prox1| Prox1| +          |
| N-COR | Prox1| Prox1| +          |
| SMRT | Prox1| Prox1| +          |
| Prox1 | NUR77| Prox1| +          |
| Prox1 | HNF4α| HNF4α| +          |

We then investigated which region of HNF4α was required for interaction with Prox1. A series of deletion constructs of HNF4α were used to map the HNF4α interaction domain in yeast-two hybrid assay. Fig. 2B shows that the full-length HNF4α (HNF4α-full) and LBD, which contains activation function 2 (AF2) domain (HNF4α-LBD), interacted with Prox1. However, the N-terminal DBD region and a construct without AF2 (HNF4α-AF2) did not interact with Prox1. These results indicate that HNF4α interacts with Prox1 through the AF2 domain of HNF4α.

Prox1 Is Expressed in Human Hepatocytes—Although its expression has been reported to be high in liver and pancreas (14), the expression of Prox1 in human hepatocytes has not been reported before. Using real-time quantitative PCR, we were able to detect the mRNA expressions of CYP7A1, Prox1, PGC-1α, and several nuclear receptors that are known to regulate CYP7A1 in five donor human primary hepatocytes and HepG2 cells. Table 2 shows Ct, the threshold cycle number, for each mRNA transcripts assayed in these hepatocytes. The mRNA expression levels were normalized to internal reference gene UBC and the ΔCt values and S.D. are shown in the Table 2. The expression patterns of these mRNA transcripts are similar in five donor hepatocytes and HepG2 cells. The Ct and ΔCt values of CYP7A1 and PGC-1α are high, reflecting low levels of mRNA expression. Those values for Prox1,

with anti-Prox1 antibody. As shown in Fig. 1B, the anti-Prox1 antibody detected Prox1 in the immunoprecipitates, whereas non-immune IgG did not. These results indicated that Prox1 interacted with HNF4α in primary human hepatocytes, consistent with the results from yeast two-hybrid assay and GST pull-down assay.

Mapping of the Interaction Regions of Prox1 and HNF4α—Co-regulators have conserved LXXL motifs that are known to interact with the ligand binding domain (LBD) of nuclear receptors. Prox1 has two LXXL motifs located in the N terminus and another motif located in the C terminus region. It has been reported that the N-terminal nuclear receptor box 1 (NR1, LRKLL) is critical for interaction with FTF/LRH-1, whereas NR2 (ISQIL) and NR3 are not essential for interaction (14, 15, 22). The yeast two-hybrid assay revealed that the full-length and N-terminal amino acid residues from 1 to 312 (Prox1-NT-WT) interacted with HNF4α (Fig. 2A). However, the C-terminal homeo and prospero domains (Prox1-H-Homeo) did not interact with HNF4α. These results demonstrated that the N-terminal region of Prox1 interacted with HNF4α and the LRKLL motif is critical for Prox1 to interact with HNF4α.

**FIGURE 1.** GST pull-down and co-immunoprecipitation assays of Prox1 interaction with HNF4α. A. GST pull-down assay. Purified GST alone (negative control) or GST-Prox1 bound to glutathione-Sepharose beads were incubated with 35S-labeled HNF4α, retinoid X receptor (RXR), retinoic acid receptor (RAR), and FTF (positive control). The reactions were analyzed by SDS-polyacrylamide gel electrophoresis, and bound proteins were visualized by autoradiography. The input represents 10% of the labeled proteins used for the pull-down assay. B. Co-immunoprecipitation assay. Protein extracts were prepared from human primary hepatocytes and immunoprecipitated (IP) with anti-HNF4α antibody or non-immune serum (IgG, as control). Immunoprecipitated proteins were resolved on SDS-polyacrylamide gel and analyzed by immunoblotting with anti-Prox1 and anti-HNF4α antibodies. Data represent one of three separate experiments. WB, Western blot.
HNF4α and SHP are low, indicating a relatively abundant expression of these three mRNA transcripts.

Prox1 Is a Transcriptional Repressor of HNF4α—We then studied the transcriptional activity of Prox1 in reporter assays in HepG2 cells. As shown in Fig. 3A, ectopic expression of HNF4α increased a heterologous HNF4α-tk-luciferase reporter activity. Addition of Prox1 substantially repressed HNF4α transactivation activity in a dose-dependent manner. As a negative control, a reporter construct containing 3 copies of Nur77 response element (NurRE-Luc) was not affected by Prox1 (Fig. 3B). Because the N-terminal domain that contains an NR1 motif is important for Prox1 to interact with HNF4α, we performed transfection assays to test the effect of wild-type and mutant Prox1 constructs on HNF4α reporter activity. Fig. 3C shows that wild-type Prox1-Full and Prox1-NT-WT repress HNF4α-mediated transactivation but Prox1-NT-MT failed to repress the activity suggesting that the N-terminal region of the LXXLL motif of Prox1 is involved in the interaction and repression of HNF4α transactivation activity.

Prox1 Suppresses HNF4α Transactivation of the Human CYP7A1 Gene—Recent studies have provided substantial evidence that HNF4α is an important transcription factor that regulates liver-specific expres-

### TABLE 2
Quantitative real time PCR analysis of mRNA expression levels of CYP7A1, nuclear receptors, and co-regulators in human primary hepatocytes and HepG2 cells

| Gene     | HH1201 | HH1205 | HH1209 | HH1247 | HH1248 |
|----------|--------|--------|--------|--------|--------|
| UBC      | 20.6 ± 0.3 | 19.8 ± 0.3 | 19.9 ± 0.05 | 22.7 ± 0.03 | 21.6 ± 0.08 | 20.9 ± 0.07 |
| CYP7A1   | 30.4 ± 0.09 | 25.0 ± 0.19 | 29.2 ± 0.05 | 35.9 ± 0.49 | 35.5 ± 0.31 | 31.2 ± 0.03 |
| ΔCt      | 9.7 ± 0.31 | 4.9 ± 0.36 | 9.3 ± 0.70 | 13.1 ± 0.49 | 13.4 ± 0.32 | 10.2 ± 0.76 |
| PGC-1α   | 27.7 ± 0.09 | 24.4 ± 0.01 | 27.4 ± 0.02 | 28.2 ± 0.02 | 28.3 ± 0.03 | 27.8 ± 0.09 |
| ΔCt      | 7.0 ± 0.31 | 4.3 ± 0.31 | 7.5 ± 0.05 | 5.5 ± 0.04 | 6.7 ± 0.09 | 6.8 ± 0.11 |
| FXR      | 26.1 ± 0.4 | 22.8 ± 0.4 | 23.3 ± 0.07 | 26.1 ± 0.05 | 24.8 ± 0.04 | 27.8 ± 0.3 |
| ΔCt      | 5.5 ± 0.5 | 2.7 ± 0.5 | 3.4 ± 0.07 | 3.4 ± 0.06 | 3.2 ± 0.09 | 6.7 ± 0.3 |
| FTF      | 24.4 ± 0.03 | 24.2 ± 0.05 | 24.2 ± 0.03 | 27.2 ± 0.03 | 26.3 ± 0.08 | 25.8 ± 0.08 |
| ΔCt      | 3.7 ± 0.3 | 4.1 ± 0.58 | 4.3 ± 0.06 | 4.5 ± 0.04 | 4.7 ± 0.11 | 4.9 ± 0.11 |
| Prox1    | 24.8 ± 0.23 | 23.9 ± 0.2 | 24.2 ± 0.08 | 26.1 ± 0.08 | 25.3 ± 0.08 | 24.1 ± 0.03 |
| ΔCt      | 4.2 ± 0.38 | 3.8 ± 0.36 | 4.3 ± 0.09 | 3.4 ± 0.09 | 3.7 ± 0.11 | 3.1 ± 0.08 |
| HNF4α    | 24.6 ± 0.01 | 21.4 ± 0.08 | 21.9 ± 0.01 | 25.2 ± 0.02 | 23.4 ± 0.04 | 23.8 ± 0.05 |
| ΔCt      | 3.9 ± 0.3 | 1.3 ± 0.31 | 2.0 ± 0.05 | 2.5 ± 0.04 | 1.8 ± 0.09 | 2.8 ± 0.08 |
| SHP      | 24.4 ± 0.03 | 21.7 ± 0.4 | 23.4 ± 0.1 | 25.7 ± 0.07 | 23.9 ± 0.14 | 21.8 ± 0.07 |
| ΔCt      | 3.7 ± 0.3 | 1.6 ± 0.5 | 3.5 ± 0.11 | 3.0 ± 0.08 | 2.3 ± 0.16 | 0.9 ± 0.10 |
Prox1 Regulates CYP7A1

Figure 3. Prox1 abrogates HNF4α-mediated transactivation. A, HepG2 cells were co-transfected with the pHNF4α-tk-Luc reporter (200 ng) along with the HNF4α expression plasmid (200 ng) and increasing amount of Prox1 expression plasmid (10, 50, and 100 ng). B, HepG2 cells were co-transfected with the pHNF4α-tk-Luc reporter (200 ng) along with the Nur77 response element-Luc reporter (NurRE-Luc reporter, 200 ng) along with the Nur77 plasmid (100 ng) and increasing amount of Prox1 plasmid (10, 50, and 100 ng). C, HepG2 cells were co-transfected with the pHNF4α-tk-Luc reporter (200 ng) along with the HNF4α plasmid (200 ng) and increasing amounts of Prox1-Full, Prox1-NT-WT (amino acids 1–312), and Prox1-NT-MT (amino acids 1–312), mutations that convert LRKLL (amino acids 70–74) to ARKAL) expression plasmids (10, 50, and 100 ng). Luciferase activity was normalized to β-galactosidase activity. All experiments were done in duplicate, and data represent the mean ± S.D. of three individual experiments. RLU, relative luciferase units.

Prox1 Blocks HNF4α Recruitment of PGC-1α to CYP7A1 Chromatin—

To investigate the molecular mechanism of Prox1 inhibition of the HNF4α transactivation of CYP7A1, we first performed an in vivo ChIP assay to study the effect of Prox1 on the HNF4α binding to CYP7A1 chromatin. HepG2 cells were transfected with pcDNA3 or Flag-Prox1 plasmid, and the DNA-protein complex was immunoprecipitated with an anti-FLAG or anti-HNF4α antibody as indicated for PCR amplification of the CYP7A1 promoter sequence from −432 to −41, which contains an HNF4α binding site (lane 4 versus lane 3). The negative control sequence from +860 to +1160 of hCYP7A1 was not amplified by immunoprecipitation with anti-HNF4α antibody (data not shown). These results suggested that Prox1 interacts with HNF4α and represses HNF4α-mediated human CYP7A1 gene expression.

Previously we have reported that HNF4α recruits PGC-1α to CYP7A1 chromatin (24). We thus studied the effect of Prox1 on HNF4α recruitment of PGC-1α to CYP7A1 chromatin. HepG2 cells were co-transfected with
Prox1 Regulation of CYP7A1

HA-PGC-1α and Flag-Prox1 or pcDNA3 empty vector (negative control) for ChIP assay using an antibody against HA. Fig. 5B shows that HA-PGC-1α was recruited to CYP7A1 chromatin (lane 2) via interaction with HNF4α, because PGC-1α does not bind to DNA. Co-expression of Flag-Prox1 together with HA-PGC-1α markedly reduced the DNA fragment immunoprecipitated with anti-HA antibody (lane 3) indicating that Flag-Prox1 blocked HNF4α recruitment of HA-PGC-1α to CYP7A1 chromatin. These data suggested that Prox-1 and PGC-1α might compete for binding to HNF4α in CYP7A1 chromatin.

Prox1 and PGC-1α Compete for Interaction with HNF4α and Regulate CYP7A1 and PEPCK Genes—We then performed transfection assay to study the effect of Prox1 on HNF4α and PGC-1α co-activation of a heterologous reporter, 5X UAS-Luc and native human CYP7A1-Luc and PEPCK-Luc reporters. As shown in Fig. 6A, Gal4-HNF4α alone slightly stimulates Gal4 reporter activity. Addition of PGC-1α drastically stimulated Gal4 reporter activity. Addition of Prox1 dose-dependently inhibited Gal4 reporter activity. These results suggest that Prox1 strongly inhibited HNF4α and PGC-1α transactivation activity. Fig. 6B shows that the Prox1 inhibition of HNF4α-mediated hCYP7A1-Luc reporter activity could be reversed by expression of PGC-1α in a dose-dependent manner. The PEPCK gene is known to be induced by HNF4α and PGC-1α (9). We thus performed similar experiments to study the effect of Prox1 on PEPCK-Luc reporter activity. Fig. 6C shows that HNF4α stimulation of PEPCK-Luc reporter activity was repressed by Prox1 and increasing amounts of PGC-1α reversed the repressive effect of Prox1. These results further support the idea that direct interaction between Prox1 and HNF4α prevented the recruitment of co-activator, PGC-1α to stimulate HNF4α transactivation of the CYP7A1 and PEPCK genes.

Knock Down of Prox1 Increases CYP7A1 and PEPCK Expression in HepG2 Cells—To further confirm the role of endogenous Prox1 in HNF4α function, we examined the miRNA expression of HNF4α target genes (CYP7A1 and PEPCK) in HepG2 cells upon knock down of Prox1 using Prox1 siRNA. We transfected Prox1 oligonucleotide siRNA in HepG2 cells and analyzed protein and miRNA expression of the Prox1 and the effect on CYP7A1 and PEPCK mRNA expression levels with real time PCR. Among four different regions of human Prox1 siRNA we have tested, siRNA against the N-terminal region (nucleotides 1009–1027; GGGCCAAACTCCTTACAAC) and the C-terminal region (nucleotides 2096–2114; GCAAAAGATGTGTACCCCTC) were found to efficiently knock down Prox1 protein expression in HepG2 cells as shown by Western blot analysis (Fig. 7A). A control siRNA, which has the same G-C content but different sequence from the siRNA#2 did not affect Prox1 protein expression. Real time PCR assay shows that the siRNA#1 and #2 decreased Prox1 mRNA expression by ~50% but increased CYP7A1 expression by 2.53- and 2.59-fold, and PEPCK expression by 1.75- and 2.28-fold, respectively (Fig. 7B). Prox1 siRNA had no effect on HNF4α and cyclophilin B mRNA expression levels.
These data support the idea that Prox1 siRNA specifically knock down Prox1 expression, and this results in induction of the HNF4α target genes CYP7A1 and PEPCK in HepG2 cells.

**Knock Down of Prox1 Increases the Rate of Bile Acid Synthesis**—To confirm whether knock down of Prox1 expression affects the rate of bile acid synthesis, we analyzed the total bile acid synthesized in HepG2 cells. Knock down of Prox1 mRNA expression levels resulted in increasing total bile acid synthesis by ~50% in Prox1 knock-down cells compared with control cells (Fig. 8), indicating that Prox1 negatively regulated bile acid synthesis in hepatocytes.

**DISCUSSION**

Bile acid synthesis is tightly regulated under physiological conditions to protect the liver from accumulation of highly toxic bile acids. Under normal physiological conditions, expression of CYP7A1 must be suppressed by various factors including insulin, glucagon, bile acids, and cholesterol in the human (13). Nuclear receptors and co-regulators apparently play important roles in regulation of CYP7A1 gene transcription (27). Prox1 is constitutively expressed at high levels in adult livers and may be the major repressor of CYP7A1 gene transcription in human hepatocytes.

This study revealed a strong and specific interaction between Prox1 and HNF4α by yeast two-hybrid assay, in vivo communoprecipitation assay using human primary hepatocytes, and in vitro GST pull-down assay. Our results indicate that Prox1 directly interacts with HNF4α via the N-terminal LXXLL motif of Prox1 and C-terminal AF2 domain of HNF4α. Co-regulators are known to interact with nuclear receptors via interaction of the LXXLL motif of co-regulators and the AF2 domain of nuclear receptor (8, 28). However, the interaction of Prox1 with FTF also requires helices 2 and 10 of the LBD of FTF (15). Thus, it is likely that the interaction domain of Prox1 might be quite diverse compared with other nuclear receptors to allow interaction with various co-regulators such as SHP (29, 30). It is noted that Prox1 may inhibit HNF4α and FTF by somewhat different mechanisms. Prox1 impairs the FTF binding to DNA (15), whereas our data show that Prox1 does not affect HNF4α binding to CYP7A1 chromatin as demonstrated by ChIP assays (Fig. 5). Our results suggest that Prox1 competes with PGC-1α for interaction with HNF4α and thus counteracts PGC-1α co-activating activity. This is because both Prox1 and PGC-1α interact with the AF2 domain of HNF4α. Competition for binding and squelching of the limited co-activators could be a common mechanism for the negative regulation of gene transcription by nuclear receptors. In accordance with this scenario, increasing the amounts of PGC-1α could counteract Prox1-mediated repression of HNF4α activity. Conversely, increasing the amounts of Prox1 may interfere with PGC-1α co-activation of HNF4α activity. Thus, our finding that Prox1 interferes with the co-activator recruitment of HNF4α provides a novel mechanism for Prox1 repression of CYP7A1 gene transcription. Moreover, knock down of Prox1 expression in a hepatocyte cell line increased CYP7A1 mRNA expression and a corresponding increase in bile acid synthesis (Figs. 7 and 8). This study reveals a new biological function of Prox1, which has previously been shown to play an essential role for lymphatic system (19, 31) and lens (18) development in hepatocytes.

It is believed that FTF is an activator of gene transcription. A more recent study supports the idea that FTF is a negative transcription factor in vivo because ablation of one FTF allele strongly induced CYP7A1 and CYP8B1 mRNA expression in mouse liver (32). Reporter assays in HepG2 cells also suggest that FTF is a repressor of the human CYP7A1 gene (26). The repressor function of FTF can now be explained by the presence of high levels of Prox1 and low levels of PGC-1α in hepatocytes. It is noteworthy that the tissue expression patterns of Prox1 (14), FTF (14), and HNF4α (5) are similar. They all express very early in embryogenesis, and expression in liver and pancreas is conserved throughout the vertebrates suggesting the spatial and temporal correlation of Prox1, FTF, and HNF4α in regulation of development and function of the liver and pancreas. Prox1, PGC-1α, FTF, and HNF4α play central roles in regulation of the CYP7A1 and CYP8B1 in bile acid synthesis and PEPCK in gluconeogenesis. These factors play critical roles in regulating a variety of metabolic pathways that are involved in pathogenesis of diabetes (33). It is interesting that the putative endogenous ligands for FTF and HNF4α are phospholipids and fatty acids, respectively (34–36). The relative expression levels of these nuclear receptors as well as their co-regulators Prox1 and PGC-1α may regulate lipid homeostasis. A recent report suggests that Prox1 may play a role in adult-onset obesity (37). PGC-1α is greatly induced in the liver of strep-
Prox1 Regulation of CYP7A1

tozotocin-injected mice, a model of type 1 diabetes of insulin deficiency (9), and in ob/ob mice, a model of type 2 diabetes of insulin resistance (38). PGC-1α and HNF4α induce PEPCK, the rate-limiting enzyme in gluconeogenesis, to prevent hypoglycemia during starvation (9). On the other hand, Prox1 interaction with HNF4α may down-regulate PEPCK gene expression to prevent hyperglycemia during the postprandial period. Thus, regulation of PEPCK gene expression by HNF4α and its co-activators and co-repressors may play a critical role in obesity and diabetes in humans.

In conclusion, here we provide direct experimental evidences that Prox1 acts as a novel co-repressor of nuclear receptor HNF4α. Prox1-mediated repression of HNF4α transactivation may play an important role in the regulation of HNF4α target genes. The intricate regulatory circuitry of Prox1, PGC-1α, HNF4α, SHP, and FTF may maintain lipid and glucose homeostasis and prevent diabetes and obesity.

REFERENCES

1. Chiang, J. Y. (1998) Front Biosci. 3, d176–193
2. Stroup, D., and Chiang, J. Y. (2000) J. Lipid Res. 41, 1–11
3. Li, J., Ning, G., and Duncan, S. A. (2000) Genes Dev. 14, 464–474
4. Hayhurst, G. P., Lee, Y. H., Lambert, G., Ward, J. M., and Gonzalez, F. J. (2001) Mol. Cell. Biol. 21, 1393–1403
5. Sladek, R., and Giguere, V. (2000) Adv. Pharmacol. 47, 23–87
6. Navas, M. A., Munoz-Elias, E. J., Kim, J., Shih, D., and Stoffel, M. (1999) Diabetes 48, 1459–1465
7. Kuo, C. J., Conley, P. B., Chen, L., Sladek, F. M., Darnell, J. E., Jr., and Crabtree, G. R. (1992) Nature 355, 457–461
8. Glass, C. K., and Rosenfeld, M. G. (2000) Genes Dev. 14, 121–141
9. Yoon, J. C., Puigserver, P., Chen, G., Donovan, J., Wu, Z., Bhee, J., Adelman, G., Stafford, J., Kahn, C. R., Granner, D. K., Newgard, C. B., and Spiegelman, B. M. (2001) Nature 413, 131–138
10. Chakravarty, K., Cassuto, H., Reshef, L., and Hanson, R. W. (2005) Crit. Rev. Biochem. Mol. Biol. 40, 129–154
11. Shin, D. J., Campos, J. A., Gil, G., and Osborne, T. F. (2003) J. Biol. Chem. 278, 50047–50052
12. De Fabiani, E., Mitro, N., Giliardi, F., Caruso, D., Galli, G., and Crestani, M. (2003) J. Biol. Chem. 278, 39124–39132
13. Song, K. H., and Chiang, J. Y. (2006) Hepatology 43, 117–125
14. Steffensen, K. R., Holter, E., Bannen, A., Nilsson, M., Pelto-Huikko, M., Tomarev, S., and Treuter, E. (2004) EMBO Rep. 5, 613–619
15. Qin, J., Gao, D. M., Jiang, Q. F., Zhou, Q., Kong, Y. Y., Wang, Y., and Xie, Y. H. (2004) Mol. Endocrinol. 18, 2424–2439
16. Oliver, G., Sosa-Pineda, B., Geisendorf, S., Spana, E. P., Doe, C. Q., and Gruss, P. (1993) Mech. Dev. 44, 3–16
17. Zinovieva, R. D., Duncan, M. K., Johnson, T. R., Torres, R., Polymeropoulos, M. H., and Tomarev, S. I. (1996) Genomics 35, 517–522
18. Wigle, J. T., Chowdhury, K., Gruss, P., and Oliver, G. (1999) Nat. Genet. 21, 318–322
19. Wigle, J. T., and Oliver, G. (1999) Cell 98, 769–778
20. Sosa-Pineda, B., Wigle, J. T., and Oliver, G. (2000) Nat. Genet. 25, 254–255
21. Burke, Z., and Oliver, G. (2002) Mech. Dev. 118, 147–155
22. Liu, Y. W., Gao, W., Teh, H. L., Tan, J. H., and Chan, W. K. (2003) Mol. Cell. Biol. 23, 7243–7255
23. Song, K. H., Park, Y. Y., Park, K. C., Hong, C. Y., Park, J. H., Shong, M., Lee, K., and Choi, H. S. (2004) Mol. Endocrinol. 18, 1929–1940
24. Li, T., and Chiang, J. Y. (2005) Am. J. Physiol. 288, G74–G84
25. Feldman, D., Fenech, C., and Cuer, J. F. (1983) Clin. Chem. 29, 1694
26. Chen, W., Owsley, E., Yang, Y., Stroup, D., and Chiang, J. Y. (2001) J. Lipid Res. 42, 1402–1412
27. Chiang, J. Y. (2002) Endocr. Rev. 23, 443–463
28. McKenna, N. J., and O’Malley, B. W. (2002) Endocrinology 143, 2461–2465
29. Lee, Y. K., Dell, H., Dowhan, D. H., Hadzopoulou-Cladaras, M., and Moore, D. D. (2000) Mol. Cell. Biol. 20, 187–193
30. Lee, Y. K., and Moore, D. D. (2002) J. Biol. Chem. 277, 2463–2467
31. Petrova, T. V., Makinen, T., Makela, T. P., Saarela, J., Virtanen, I., Ferrell, R. E., Finegold, D. N., Kerjaschki, D., Yla-Herttuala, S., and Alitalo, K. (2002) EMBO J. 21, 4593–4599
32. del Castillo-Olivares, A., Campos, J. A., Pandak, W. M., and Gil, G. (2004) J. Biol. Chem. 279, 16813–16821
33. Silander, K., Mohlke, K. L., Scott, J. I., Peck, E. C., Hollstein, P., Skol, A. D., Jackson, A. U., Deloukas, P., Hunt, S., Stavrides, G., Chines, P. S., Erdos, M. R., Narisu, N., Conneely, K. N., Li, C., Fingerlin, T. E., Dhanjal, S. K., Valle, T. T., Bergman, R. N., Tuomilehto, J., Watanabe, R. M., Boehinke, M., and Collins, F. S. (2004) Diabetes 53, 1141–1149
34. Krylova, I. N., Sablin, E. P., Moore, J., Xu, R. X., Waite, G. M., MacKay, J. A., Juzumiene, D., Bynum, J. M., Madaus, K., Montanoa, V., Lebedeva, L., Suzawa, M., Williams, J. D., Williams, S. P., Guy, R. K., Thornton, J. W., Fletterick, R. J., Willson, T. M., and Ingraham, H. A. (2005) Cell 120, 343–355
35. Ortlund, E. A., Lee, Y., Solomon, I. H., Hager, J. M., Safi, R., Choi, Y., Guan, Z., Tripathy, A., Raetz, C. R., McDonnell, D. P., Moore, D. D., and Redinbo, M. R. (2005) Nat. Struct. Mol. Biol. 12, 357–363
36. Petrescu, A. D., Hertz, R., Bar-Tana, J., Schroeder, F., and Kier, A. B. (2002) J. Biol. Chem. 277, 23988–23999
37. Harvey, N. L., Srivastava, R. S., Dillard, M. E., Johnson, N. C., Witte, M. H., Boyd, K., Sleeman, M. W., and Oliver, G. (2005) Nat. Genet. 37, 1072–1081
38. Sakuma, T., Wang, Z. W., Pan, W., Unger, R. H., and Zhou, Y. T. (2000) Endocrinology 141, 4576–4582