SREBP-1 Interacts with Hepatocyte Nuclear Factor-4α and Interferes with PGC-1 Recruitment to Suppress Hepatic Gluconeogenic Genes*

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The hepatocyte nuclear factor-4α (HNF-4α)/PGC-1 pathway plays a crucial role in the transcriptional regulation of hepatic gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase (PEPCK) and Glc-6-Pase, genes that are activated at fasting and suppressed in a fed state. SREBP-1c dominates the nutritional regulation of lipogenic genes inverse to gluconeogenesis. Here we show the mechanism by which SREBP-1 suppresses expression of gluconeogenic genes. A series of luciferase reporter assays demonstrated that SREBP-1a and -1c effectively inhibited the PEPCK promoter activity that was induced by HNF-4α. The HNF-4α-binding site in the glucocorticoid-response unit was responsible for the SREBP-1 inhibition, although SREBP-1 did not bind to the PEPCK promoter as demonstrated by electrophoretic mobility shift assays. The inhibitory effect was more potent in the isoform of SREBP-1a than SREBP-1c and was eliminated by deletion of the amino-terminal transactivation domain of SREBP-1. Coimmunoprecipitation experiments demonstrated that these two transcription factors directly interact through the transactivation domain of SREBP-1 and the ligand binding/AF2 domains of HNF-4α. Estimation of coactivator recruitment using HNF-4α-Gal4DBD fusion assay showed that SREBP-1 competitively inhibited PGC-1 recruitment, a requirement for HNF-4α activation. Consistent with these results, hepatic PEPCK and Glc-6-Pase mRNA levels are suppressed by overexpression of SREBP-1a and -1c in the transgenic mice. Our data indicate that SREBP-1 has a novel role as negative regulator of gluconeogenic genes through a cross-talk with HNF-4α interference with PGC-1 recruitment.

Regulation of gluconeogenesis is crucial to maintain glucose homeostasis. Gluconeogenic genes such as phosphoenolpyruvate carboxykinase (PEPCK)[1] and glucose-6-phosphatase (Glc-6-Pase) are nutritionally regulated in the liver at the transcriptional level, are highly activated during fasting or starvation, and are suppressed in a fed state. PEPCK promoter has been extensively studied and shown to be induced by several hormonal stimuli (glucocorticoid, glucagons, and thyroid hormone) and transcriptional factors (cAMP-response element-binding protein, C/EBPα, HNF-3, HNF-4α, and PPARα) (1). Among these, insulin is a dominant negative regulator of PEPCK promoter through several pathways (1). Hepatocyte nuclear factor-4α (HNF-4α) is a highly conserved member of the nuclear receptor superfamily and was initially identified as a transcriptional factor required for liver-specific gene expression (2), although it is also expressed in kidney, intestine, and pancreas (3). Recently, HNF-4α and a coactivator, peroxisome proliferator-activated receptor-γ coactivator-1 (PGC-1), have been shown to activate expression of PEPCK (4) and Glc-6-Pase (5) by binding to HNF-4α-binding cis-elements in their promoters. Crucial roles of HNF-4α and PGC-1 in nutritional regulation of these genes, and thus gluconeogenesis, were confirmed by analysis of liver-specific HNF-4α knockout mice and by adenovirus-mediated overexpression of PGC-1 in mouse livers (6, 7).

The sterol regulatory element-binding protein (SREBP) family has been established as a group of transcription factors regulating transcription of genes involved in cholesterol and fatty acid synthesis. SREBP proteins are initially bound to the rough endoplasmic reticulum membrane and form a complex with SREBP cleavage-activating protein (SCAP), a sterol-sensing molecule. Upon sterol deprivation, SREBP is cleaved to liberate the amino-terminal portion, containing a basic helix-loop-helix leucine zipper domain, and enters the nucleus where it can bind to specific sterol-response elements (SRE) in the promoters of target genes (8–10). Three isoforms of SREBP-1a, -1c, and -2 are known. Although SREBP-2 plays a crucial role in regulation of cholesterol synthesis, SREBP-1c controls gene expression of lipogenic enzymes (11–14). Insulin and glucose have been well known to stimulate lipogenesis, presumably through induction of SREBP-1c expression of which is highly nutritionally regulated in the liver and adipose tissue. Recently, we reported (15, 16) that cross-talk between PPARs and

[1] The abbreviations used are: PEPCK, phosphoenolpyruvate carboxykinase; Glc-6-Pase, glucose-6-phosphatase; HNF-4α, hepatocyte nuclear factor-4α; EMSA, electrophoretic mobility shift assay; PPAR, peroxisome proliferator-activated receptor; LXR, liver X receptor; aa, amino acids; GST, glutathione *S*-transferase; DTT, dithiothreitol; LBD, ligand binding domain; SREBP, sterol regulatory element-binding protein; PBS, phosphate-buffered saline; GRU, glucocorticoid-response unit; SRE, sterol-response elements; RE, response element; TA, transactivation domain.

SREBP-1 Inhibition of HNF-4α/PGC-1 Pathway

LXRs could be involved in reciprocal nutritional regulation of fatty acid metabolism. Considering the physiologically related roles of gluconeogenesis and lipogenesis, their transcriptional regulators HNF-4α and SREBP-1c could reciprocally regulate their target genes. In fact, fasting/feeding response to and insulin/glucose effect on these factors are completely opposite. Despite this knowledge, a mutual interaction between these two pathways has never been explored. Here we investigate the effects of SREBPs on the HNF-4α/PGC-1 pathway, and we examine the potential role of SREBPs in the regulation of gluconeogenesis.

EXPERIMENTAL PROCEDURES

Animals—SREBP transgenic mice used in this study were described previously (17, 18). Mice were housed in colony cages and maintained on a 12-h light/12-h dark cycle. Before time of killing, transgenic animals and littermate controls were placed on the low carbohydrate/high protein diet for 1 week to induce expression of the transgene. Mice were killed after a 12-h fast.

Plasmid Construction—Plasmid constructs were produced by using general molecular biology procedures. A series of PEPECK-LUC vectors were generated by inserting the rat PEPCK promoter region (from −1021 to +278, from −480 to +278, or from −335 to +278) amplified by PCR into the pGL3-basic vector (Promega). The HNF-4α RE LUC vector containing four copies of HNF-4α-response elements was generated by inserting the double-stranded oligonucleotides of HNF-4α-response element upstream of the native PEPCK TATA box linked to a luciferase gene (pUC8). The expression vectors for human SREBP-1a, -1c, and -2 were constructed as described previously (19). Transactivation domain-deleted (ΔTA-SREBP-1) SREBP-1 expression plasmid was constructed by inserting PCR-amplified SREBP-1 fragment (90–460 aa) into pcDNA3.1. The expression vector for mouse PGC-1 was generated by inserting PCR-amplified full-length fragment into pcDNA3.1 (Invitrogen). The expression vector for mouse HNF-4α was generated by inserting PCR-amplified full-length fragment into pCMV7. The expression vector for Gal4-HNF-4α fusion protein was generated by inserting PCR-amplified HNF-4α fragment (115–455 aa) downstream of Gal4 in the pM vector (Clontech). The expression vector for FLAG-tagged HNF-4α was generated by inserting full-length HNF-4α cDNA fragment downstream of the FLAG tag based on pcDNA3.1 (Invitrogen). The expression vector for c-Myc-tagged SREBP-1a was generated by inserting the fragment of the nuclear form of SREBP-1a into pcDNA3.1 Myc/His (Invitrogen). The expression vectors for GST-SREBP-1a, GST-SREBP-1c, or GST-SREBP-1a (1–100 aa) were generated by inserting the PCR-amplified fragment into pGEX-4T (Amersham Biosciences). The expression vectors for the HNF-4α partial fragment were generated by inserting the PCR-amplified fragment into pcDNA3.1 (Invitrogen). The expression vectors for CBP and p300 (pCMV) were a kind gift from Dr. T. Nakajima. All PCR-based constructions were verified by DNA sequencing.

Northern Blotting—Total RNA from mouse livers was prepared with Trizol reagent (Invitrogen). Equal aliquots of total RNA from mice were subjected to Northern blot analysis. cDNA probes for mouse PEPCK, Glc-6-Pase, and acidic ribosomal phosphoprotein PO (36B4) were prepared from reverse transcriptase-PCR of mouse liver total RNA with the following primers: 5′-CACACCATTGCAATTATGCCT-3′ and 5′-GCGTTGAATGCTTTCTCAAAGT-3′ for PEPCK; 5′-GCTTGGACTCAC-TGCACG-3′ and 5′-GACTCTGCATGCCTTACAAAGA-3′ for Glc-6-Pase; and 5′-ATGATTATCCAAAATGCTTCATTG-3′ and 5′-AACAGC-ATATCCCGAATCTCA-3′ for 36B4. Full-length cDNA was used as cDNA probes for PGC-1 and HNF-4α. Blot hybridization was performed with the cDNA probes for mouse PEPCK, Glc-6-Pase, HNF-4α, PGC-1 and 36B4 labeled with [α-^{32}P]dCTP (6000 Ci/mmol) using the Megaprime DNA Labeling System (Amersham Biosciences). Labeled probes were incubated with RNA transferred membranes for 2 h at 65 °C. After washing with washing buffer (0.1× SSC, 0.1% SDS) two times, hybridized membranes were visualized by BAS-2500 (Fuji).

Transient Transfection and Luciferase Assay—HepG2 cells were grown at 37 °C in an atmosphere of 5% CO_2 in minimum Eagle's medium (Sigma) containing non-essential amino acids for minimum Eagle's medium, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin sulfate and supplemented with 10% fetal bovine serum. Before transfection, HepG2 cells were seeded in 24-well plates at a density of 2.5×10^4 cells/well or in 12-well plates at a density of 5.0×10^4 cells/well. After adhesion, cells were transfected with Fu-GENE 6 (Roche Applied Science) according to the manufacturer's protocol. The total amount of DNA was adjusted to 0.5 μg/well in 24-well

plates or 1.0 μg/well in 12-well plates with empty vector DNA. Forskolin (10–30 μM) (a protein kinase A activator) was added to medium after 4 h of transfection. Me_2SO was used as a vehicle-only control. After 24 or 48 h of transfection, cells were washed with phosphate-buffered saline (PBS) and harvested. Luciferase assays were carried out according to the manufacturer's protocol with Dual Luciferase assay kit (Promega), and luciferase activity was quantified by using Wallac 1420 multilabel counter (PerkinElmer Life Sciences). As the internal standard, SV40 *Renilla* luciferase control vector was also cotransfected to normalize for transfection efficiency. All experiments were performed in triplicate (mean ± S.D.)

GST Pull-down Assay—GST and GST fusion proteins were expressed in *Escherichia coli* (BL21, DE3) and purified using standard techniques using pGEX-4T according to the manufacturer's protocol. Bacteria culture in ampicillin were grown at 37 °C to A_{600} of 0.6 before induction with 100 μM isopropyl-1-thio-β-D-galactopyranoside at 25 °C for 6 h. Cells were pelleted and resuspended in buffer A (25 mM Hepes (pH 7.9), 50 mM KCl, 6% (v/v) glycerol, 5 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, 0.05% Triton X-100, protease inhibitor mixture (Roche Applied Science), (*N*-acetyl-leucyl-leucyl-norleucinal 15 μg/ml). The cells were lysed on ice by sonication and centrifuged at $14,000 \times g$ for 10 min at 4 °C. The supernatant was mixed for 10 min at 4 °C for 1 h on a rotator with glutathione-Sepharose beads that were preswollen in buffer A. After absorption, beads were wash three times with buffer A. GST or GST fusion proteins were eluted with elution buffer (10 mM glutathione, 50 mM Tris-HCl (pH 8.0)) at room temperature for 10 min. The concentration and sizes of GST and GST fusion proteins were estimated by SDS-PAGE and immunoblotting, using a known quantity of molecular weight standards. After dialysis, the purified samples were stored at −80 °C until pull-down assays were performed.

L-[^{35}S]Methionine-labeled proteins were prepared by using TNT T7 quick-coupled transcription/translation system (Promega). After GST or GST fusion proteins were incubated with glutathione-Sepharose beads (Amersham Biosciences) for 1 h at 4 °C and washed extensively, a 50% slurry of GST or GST fusion protein-coupled beads were incubated with radiolabeled HNF-4α or PGC-1 in binding buffer (2 mM Hepes-KOH (pH 7.8), 10 mM KCl, 0.2 mM EDTA, 1 mM $MgCl_2$, 10% glycerol, 1 mM DTT) for 2 h at 4 °C or room temperature. After beads were washed extensively three times with PBS containing 0.1% Triton X-100 or 0.1% Nonidet P-40, GST or GST fusion proteins were eluted with elution buffer (10 mM glutathione, 50 mM Tris-HCl (pH 8.0)) at room temperature for 10 min, and labeled proteins were analyzed by SDS-PAGE, and gels were dried and visualized with BAS-2500.

Coimmunoprecipitation—293T cells were grown at 37 °C in an atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium (Sigma) containing 100 units/ml penicillin, 100 μg/ml streptomycin sulfate and supplemented with 10% fetal bovine serum. 293T cells were transfected with the expression vector for FLAG-HNF-4α and c-myc-SREBP-1a by using FuGENE 6 (Roche Applied Science). Before 5 h of harvesting cells, *N*-acetyl-leucyl-leucyl-norleucinal (15 μg/ml) was added to the medium as a protease inhibitor. After 48 h of transfection, cells were harvested on ice, and nuclear proteins were prepared as described previously (19). Nuclear extracts (800 μg) were incubated for 1 h at 4 °C with anti-FLAG antibody (mouse monoclonal antibody, Promega) and incubated overnight at 4 °C with protein G-agarose matrix. After incubation, the beads were washed three times with PBS containing 0.1% Nonidet P-40 and analyzed on 6% gel by SDS-PAGE. Western blot analysis was performed by using anti-c-Myc antibody (mouse monoclonal antibody, Roche Applied Science) and anti-HNF-4α antibody (goat polyclonal antibody sc-6556, Santa Cruz Biotechnology).

Electrophoresis Mobility Shift Assay—*In vitro* translated SREBP-1a and HNF-4α proteins were produced using the TNT T7 quick-coupled transcription/translation system (Promega). Double-stranded oligonucleotides were prepared by combining equal amounts of the complementary single-stranded DNA in TE buffer followed by heating to 95 °C for 5 min and then gradually cooling to 37 °C. The annealed oligonucleotides were labeled by using the Klenow element of *E. coli* DNA polymerase in the presence of [α-^{32}P]dCTP. The binding reactions (final volume of 15 μl) were carried out in binding buffer (2 mM Hepes-KOH (pH 7.8), 10 mM KCl, 0.2 mM EDTA, 1 mM $MgCl_2$, 10% glycerol, 1 mM DTT) with labeled probe and 0.4 μg of poly(dI-dC) and TNT reaction lysate. Reaction mixtures were incubated for 1 h at 4 °C and then analyzed on 4% polyacrylamide gel in 0.5× TBE at 90 V for 1 h at room temperature. Supershift reactions were performed by adding antibody to the reaction mixture. Antibodies used were sc-6556 (Santa Cruz Biotechnology) for HNF-4α and sc-8984 for SREBP-1 (Santa Cruz Biotechnology). After drying, gels were analyzed by BAS-2500 (Fuji). The probe sequence for EMSA is as follows: PEPCK GRU probe (5′-GAAT-
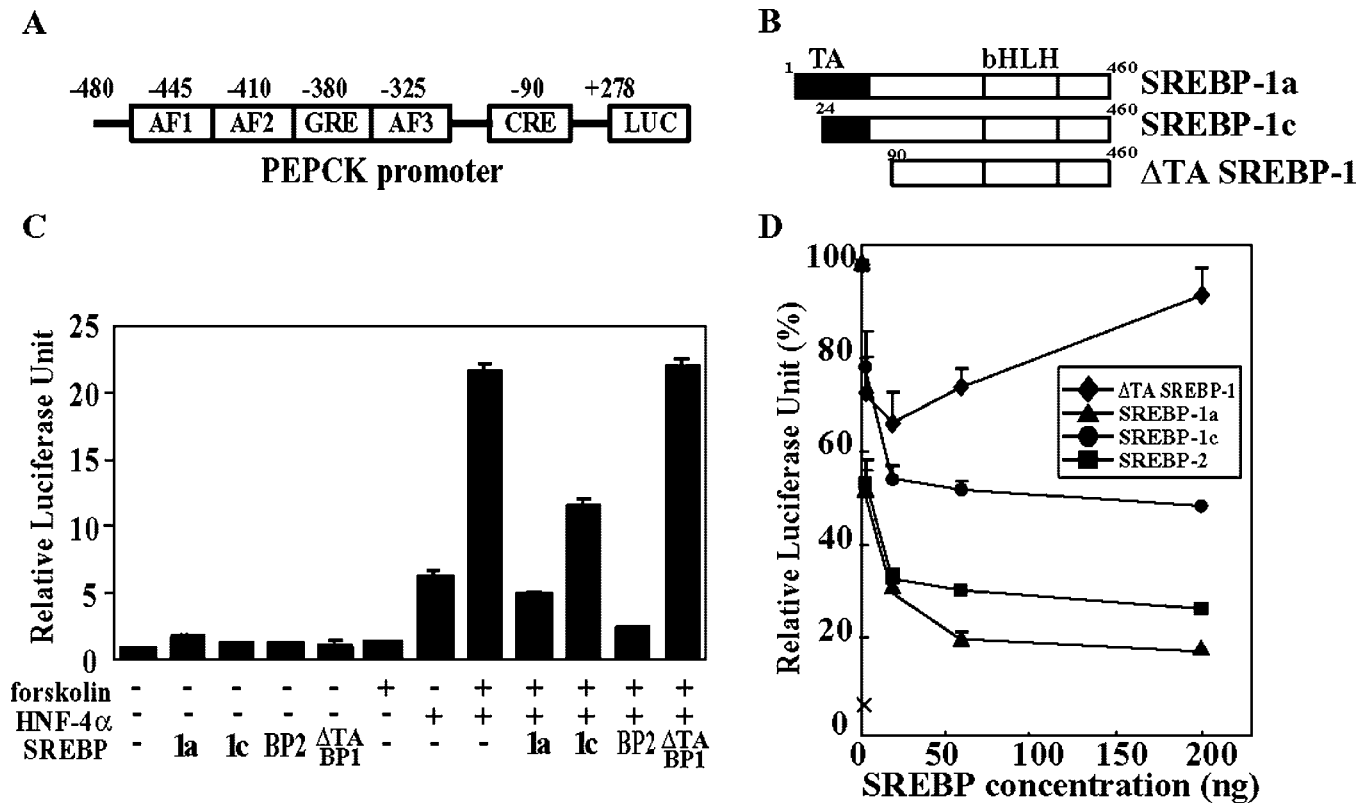
FIG. 1. **PEPCK promoter activity is induced by HNF-4α and forskolin and is repressed by SREBP-1a, -1c, and -2.** *A*, schematic representation of glucocorticoid-response unit in PEPCK promoter. *B*, schematic representation of the isoforms and the transactivation domain-deleted mutant form of nuclear SREBP-1 (*ΔTA-SREBP-1*). *C*, regulation of PEPCK promoter by HNF-4α and SREBPs. HepG2 cells in a 12-well plate were cotransfected with PEPCK-LUC (−480 to +278) vector (400 ng/well), expression vectors for HNF-4α (300 ng/well), SREBP, or ΔTA-mutant SREBP-1 (300 ng/well). After 4 h of transfection, forskolin (30 μM) or vehicle (Me₂SO) was added to medium. Cells were harvested after 24 h of transfection. The luciferase activity was measured and normalized by *Renilla* luciferase activity. *D*, dose-dependent suppression of HNF-4α-induced PEPCK promoter by SREBPs. HepG2 cells in a 24-well plate were cotransfected with PEPCK-LUC (−480 to +278) vector (100 ng/well) and expression vector for HNF4α (200 ng/well), together with expression vector for SREBP or mutant SREBP-1 (0, 5, 20, 60, and 200 ng/well). Cells were harvested after 24 h of transfection. The luciferase activity was measured and normalized by *Renilla* luciferase activity. HNF-4α-induced PEPCK promoter activity without SREBPs was set at 100%. The basal promoter activity with empty vector is indicated by ×. All experiments were performed in triplicate.

TCCCTTCTCATGACCTTTGGCCGTGGGAGTGACACCTCACAGCTG-TGGTGTTTTGACAAC-3′), AF1 site probe (5′-GAATTCCTTCTCATG-ACCTTTGGCCGTGGGAGTGA-3′), and LDL-R SRE probe (5′-G-AAAATCACCCCACTGCAAA-3′).

RESULTS

SREBP Inhibits the Transcriptional Activity of HNF-4α Induced by Forskolin—In the process of seeking genes down-regulated by SREBPs, we identified the PEPCK gene by DNA microarray analysis of liver RNAs from SREBP-1a transgenic and wild type mice. The hepatic expression level of PEPCK was 5.3-fold decreased by SREBP-1a overexpression. This result was consistent with the previous report that adenovirus-mediated overexpression of SREBP-1c in primary hepatocytes represses mRNA of PEPCK gene induced by cAMP and dexamethasone (20). The glucocorticoid-response unit (GRU) has been designated as a functional region to regulate PEPCK gene transcription by cooperation between glucocorticoid receptor and other transcriptional factors in the liver (Fig. 1*A*). It encompasses the glucocorticoid-response element, AF1, AF2, and AF3 sites. AF1 is the binding site of HNF-4α and is responsible for HNF-4α activation of the PEPCK promoter (4). To investigate the role of SREBP as a regulator of PEPCK promoter activity, we tested whether SREBP could affect the PEPCK promoter in HepG2 cells by using a luciferase reporter gene linked to the rat PEPCK promoter region containing GRU. As shown in Fig. 1*C*, HNF-4α expression caused induction of PEPCK promoter activity. The HNF-4α transactivation of the PEPCK promoter was markedly enhanced by addition of fors-

kolin, a protein kinase A activator (Fig. 1*C*), presumably due to protein kinase A phosphorylation of cAMP-response element-binding protein, another PEPCK transactivator through the cAMP-response element site (Fig. 1*A*). Addition of SREBPs resulted in a marked inhibition of the PEPCK promoter activity induced by HNF-4α and forskolin (Fig. 1*C*). Among the isoforms of SREBPs, SREBP-1a and -2 had the stronger inhibitory effect than SREBP-1c. The difference is likely due to the shorter amino-terminal transactivation domain of SREBP-1c as compared with that of SREBP-1a (Fig. 1*B*). When the amino-terminal transactivation domain (TA) of SREBP-1 was deleted (ΔTA-SREBP-1, Fig. 1*B*), the SREBP-1 inhibitory effect on the PEPCK promoter was completely abolished, although this mutant still possessed binding activity to the sterol regulatory element (SRE). These results suggest that the amino-terminal transactivation domain of SREBP is essential for the suppression of HNF-4α/forskolin-induced PEPCK promoter activity. As shown Fig. 1*D*, the PEPCK promoter activity induced solely by HNF-4α was also repressed by SREBPs in a dose-dependent manner (Fig. 1*C*). These data indicated that inhibitory action of SREBPs on PEPCK expression is transcriptional repression mediated through interference with HNF-4α activation.

Suppression of PEPCK Promoter by SREBPs Was Mediated through GRU—To identify the cis-acting element responsible for the inhibitory effect of SREBP on HNF-4α, a series of deleted PEPCK promoter LUC plasmids was constructed for transfection studies in HepG2 cells (Fig. 2). In the presence of GRU, luciferase activity was increased greater than 6-fold by
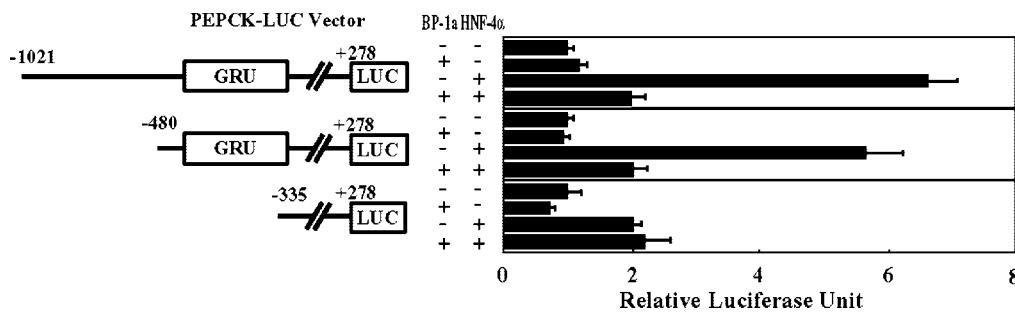
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FIG. 2. **GRU is responsible for the inhibitory effect of SREBP-1a on PEPCK promoter activity.** HepG2 cells in a 24-well plate were cotransfected with sequentially deleted PEPCK-LUC vectors as indicated in the *left panel* (100 ng/well), expression vectors for HNF-4α (200 ng/well) and SREBP-1a (200 ng/well). Cells were harvested after 24 h of transfection. The luciferase activity was measured and normalized by *Renilla* luciferase activity. All experiments were completed in triplicate.

HNF-4α, and the absence of the GRU (from −335 to +278) considerably decreased the HNF-4α induction, which is consistent with the previous report that the effective cis-acting element for HNF-4α is the AF1 site in GRU. However, there still remained a slight HNF-4α induction (2-fold) in this construct, which is presumably due to another DR-1 site (AF3). The HNF-4α induction, as observed in GRU-containing luciferase constructs, was equally suppressed by SREBP-1a. Deletion of GRU completely abolished this SREBP suppression. These results demonstrate that GRU is essential for the inhibitory effect of SREBP on HNF-4α activation of the PEPCK promoter.

SRE-like Sequences in GRU Are Not Responsible for the Inhibitory Effect of SREBP on HNF-4α—SREBP could function as a negative regulator of transcription by direct binding to the SRE as reported for the promoter of microsomal triglyceride transfer protein (21). As depicted in Fig. 3A, the SRE-like sequences were identified in the GRU of the PEPCK promoter region. However, EMSAs demonstrated that SREBP-1a did not bind to the GRU of the PEPCK promoter, whereas HNF-4α specifically bound to this region (Fig. 3B), excluding the possibility that SREBPs might suppress PEPCK promoter through their direct binding.

SREBP Is a Negative Regulator of HNF-4α via PGC-1—Next, we focused on the HNF-4α-response element (HNF-4α-RE) in GRU as an SREBP suppression site. The enhancer construct containing HNF-4α-RE (AF1 site) was transfected into HepG2 cells for these studies (Fig. 4A). Activation of HNF-4α RE-LUC by HNF-4α was markedly enhanced by PGC-1 coexpression in a dose-dependent and saturable manner (Fig. 4B). This result is consistent with the previous report that PGC-1 is an essential coactivator of HNF-4α (7). As shown in Fig. 4C, both SREBP-1a and SREBP-1c efficiently repressed this PGC-1-mediated HNF-4α activity. These data demonstrate the potential for a direct interaction between HNF-4α and SREBP in this inhibition.

SREBP Physically Interacts with HNF-4α in Vitro and in Vivo—To test this possibility, we used GST pull-down assays with GST-SREBP-1 fusion proteins and *in vitro* translated HNF-4α as the target. As shown Fig. 5A, both GST-SREBP-1a and GST-SREBP-1c, but not GST, were able to pull-down HNF-4α. These results indicate that SREBP-1 could bind HNF-4α *in vitro*. The amino-terminal region of SREBP-1a GST-SREBP-1a (1–100 aa) was also able to pull down HNF-4α. These data support the concept that the amino-terminal region of SREBP-1 is responsible for its inhibitory effect on HNF-4α. Additionally, under the same conditions, the interaction between SREBP-1a and PGC-1 could not be detected, as shown in Fig. 5A. Lack of SREBP-1/PGC-1 interaction precludes the possibility of competition between HNF-4α and SREBP-1 in PGC-1 recruitment as a plausible mechanism for SREBP-1 inhibition of HNF-4α activity. Next, to determine the binding

region in HNF-4α to SREBP, we constructed expression plasmids for different domains of HNF-4α. As shown in Fig. 5B, SREBP-1a-GST protein pulled down both the ligand binding domain (LBD) (115–359 aa) and AF2 of HNF-4α (342–455 aa) with slightly less efficiency, whereas the amino-terminal AF1 and DNA binding domains of HNF-4α (1–130 aa) did not bind to SREBP-1a. The LBD/AF2 part (115–455 aa) showed a comparable signal to the whole molecule of HNF-4α. These data indicated that the binding of these two molecules is mediated through interaction between the TA domain of SREBP and LBD/AF-2 domains of HNF-4α. Finally, the direct interaction between SREBP-1a and HNF-4α was confirmed *in vivo*; as shown in Fig. 5C, FLAG-HNF-4α was able to coimmunoprecipitate myc-SREBP-1a in 293T cells.

SREBP Competes with PGC-1 Recruitment for Activation of HNF-4α—Based upon the direct interaction between SREBP-1 and HNF-4α, two hypotheses for the mechanism of the SREBP inhibitory effect on the HNF-4α-induced PEPCK promoter could be considered: 1) SREBP-HNF-4α interaction impairs the affinity of HNF-4α to bind to HNF4α-RE, or 2) it interferes with the recruitment of PGC-1 by HNF-4α. To examine the first hypothesis, we performed EMSA by using AF1 (HNF-4α-RE) in the PEPCK promoter as a probe. As shown in Fig. 6, *A* and *B*, the binding of HNF-4α to HNF-4α-RE was not influenced by addition of either SREBP-1a or PGC-1 at the concentrations tested here. Next, to determine whether SREBP-1 has an inhibitory effect on the interaction between HNF-4α and PGC-1, a Gal4-luciferase assay system for evaluation of the recruitment of PGC-1 by HNF-4α was established. HNF-4α LBD and AF2 domain (115–455 aa) were fused to the carboxyl terminus of Gal4 (Fig. 7A). This assessment of coactivator recruitment clearly highlighted PGC-1 and not CBP or p300 as an important coactivator for HNF-4α (Fig. 7B). Fig. 7C shows that PGC-1 dose-dependently increased HNF-4α signaling and that both SREBP-1a and SREBP-1c competitively repressed the PGC-1-dependent luciferase activities. In contrast, the amino-terminal TA-deleted SREBP mutant had no inhibitory effect on this activity. These results indicate that SREBP-1 effectively inhibits the interaction between HNF-4α and PGC-1 through the amino-terminal TA region of SREBP-1. In this inhibition, SREBP-1a was more effective than SREBP-1c.

SREBP-1 Competes with PGC-1 in Activation of HNF-4α—As observed above, SREBP-1a repressed the HNF-4α activity more efficiently than SREBP-1c. When dose-dependent inhibition curves for the PGC-1 recruitment were compared between SREBP-1a and -1c in HepG2 cells, the difference was cancelled at high doses of SREBP-1, suggesting that SREBP-1a has a higher affinity in the inhibition than SREBP-1c (Fig. 8A). To investigate further the kinetics between SREBP-1 and PGC-1 on competition of HNF-4α activation, the dose-dependent inhibition by SREBP-1a was also compared with different
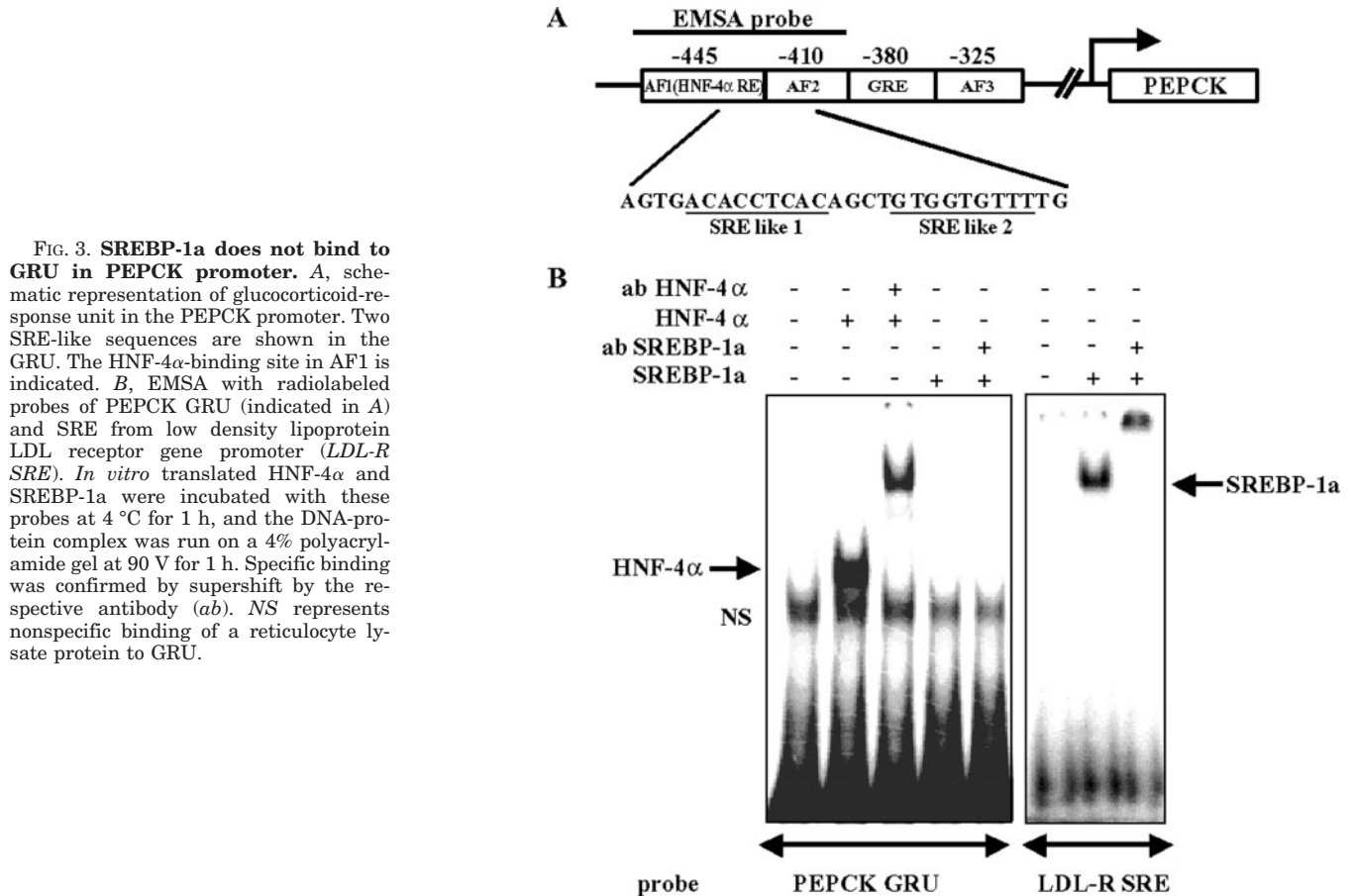
FIG. 3. **SREBP-1a does not bind to GRU in PEPCK promoter.** *A*, schematic representation of glucocorticoid-response unit in the PEPCK promoter. Two SRE-like sequences are shown in the GRU. The HNF-4α-binding site in AF1 is indicated. *B*, EMSA with radiolabeled probes of PEPCK GRU (indicated in *A*) and SRE from low density lipoprotein LDL receptor gene promoter (*LDL-R SRE*). *In vitro* translated HNF-4α and SREBP-1a were incubated with these probes at 4 °C for 1 h, and the DNA-protein complex was run on a 4% polyacrylamide gel at 90 V for 1 h. Specific binding was confirmed by supershift by the respective antibody (*ab*). *NS* represents nonspecific binding of a reticulocyte lysate protein to GRU.
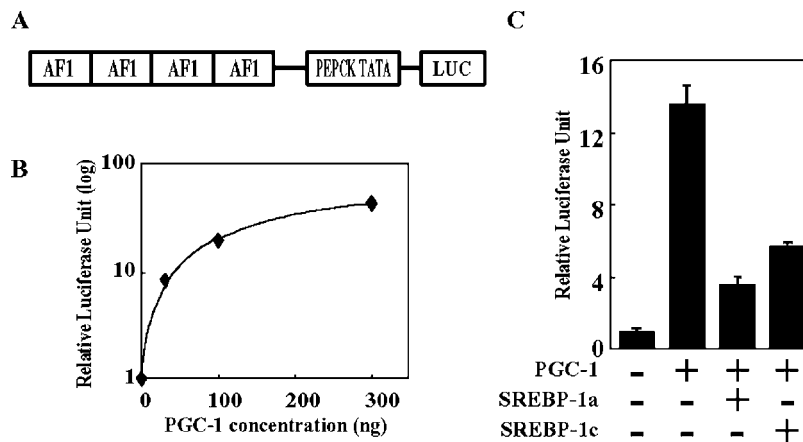

FIG. 4. **SREBP-1 suppresses the activity of HNF-4α induced by PGC-1.** *A*, schematic representation of HNF-4α-responsive element enhancer plasmid (HNF-4α RE-LUC vector). Four tandem copies of AF1 containing HNF-4α-RE (see Fig. 3*A*) were fused to the native TATA box of the PEPCK promoter. *B*, HepG2 cells in a 24-well plate were cotransfected with HNF-4α RE-LUC vector (200 ng/well), expression vector for PGC-1 (0, 30, 100, and 300 ng/well). Cells were harvested after 24 h of transfection. The luciferase activity was measured and normalized by *Renilla* luciferase activity. *C*, HepG2 cells in a 24-well plate were transfected with HNF-4α RE-LUC vector (200 ng/well), expression vectors for PGC-1 (100 ng/well), and SREBP-1a and -1c (100 ng/well). Cells were harvested after 24 h of transfection. The luciferase activity was measured and normalized by *Renilla* luciferase activity. All experiments were performed in triplicate.

amounts of PGC-1 in HepG2 cells. As shown Fig. 8*B*, PGC-1 competitively restored HNF-4α activation repressed by SREBP-1a in a dose-dependent manner. Thus, it could be concluded that SREBP-1 can compete with PGC-1 in its HNF-4α activation through SREBP-1 and HNF-4α interaction.

Overexpression of SREBP-1 Repressed Expression of Gluconeogenic Enzyme Genes in the Liver—The series of *in vitro* and cell culture experiments shown above strongly suggest that SREBP negatively regulates the PEPCK promoter. To investigate the influence of SREBP-1 on PEPCK expression *in vivo*, livers of transgenic mice overexpressing SREBP-1a

and -1c were used. Northern blot analysis demonstrated that the high hepatic PEPCK mRNA level as observed in wild type mice at fasting was reduced by overexpression of SREBP-1a and -1c (Fig. 9). Moreover, the mRNA level of Glc-6-Pase, another HNF-4α target gene involved in hepatic glucose output, was also decreased in these transgenic mice. Expression of HNF-4α and *Pgc-1* genes was not affected by SREBP-1 overproduction. These results support the hypothesis that SREBP-1 negatively regulates gluconeogenic genes *in vivo*, presumably through interference with PGC-1 recruitment to HNF-4α.
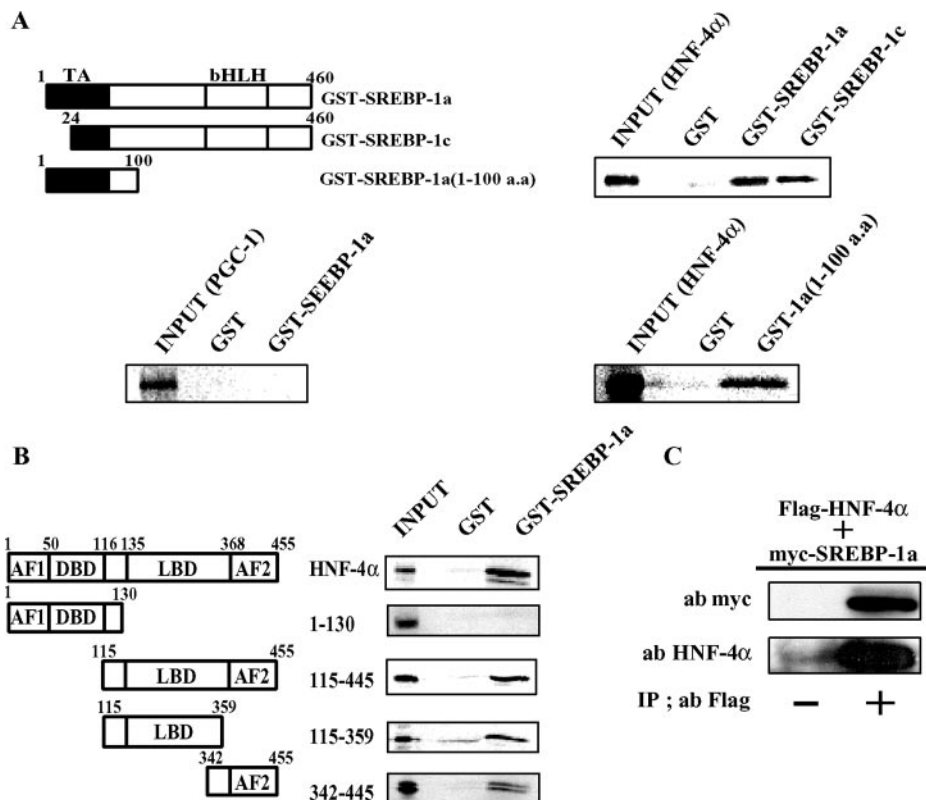
FIG. 5. **SREBP physically interacts with HNF4α *in vivo* and *in vitro*.** *A*, *E. coli* expressed GST-SREBP-1a, -1c, -1a(1–100 aa), or GST were incubated at 4 °C for 2 h with *in vitro* translated ^{35}S-radiolabeled HNF-4α. *E. coli* expressed GST-SREBP-1a was incubated in binding buffer at 4 °C for 2 h with *in vitro* translated ^{35}S-radiolabeled PGC-1. After washing three times with PBS containing 0.1% Triton X-100, the protein-bound beads were eluted and analyzed on 10% gel by SDS-PAGE and visualized by autoradiography. *B*, *in vitro* translated ^{35}S-radiolabeled HNF-4α domains were incubated in binding buffer containing BSA (0.1 μg/μl) with GST-SREBP-1a at room temperature for 2 h. After washing three times with PBS containing 0.1% Nonidet P-40, the protein-bound beads were eluted and analyzed on 12% gel by SDS-PAGE and visualized by autoradiography. *C*, 293T cells were cotransfected with expression vectors for Myc-tagged SREBP-1a and FLAG-HNF-4α. After 48 h of transfection, nuclear extracts were prepared and subjected to coimmunoprecipitation (*IP*) with anti-FLAG antibody. After incubation at 4 °C overnight and washing three times with PBS containing 0.1% Nonidet P-40, the samples were analyzed on 6% gel by SDS-PAGE and Western blot with anti-c-Myc antibody (*ab*) and anti-HNF-4α antibody.
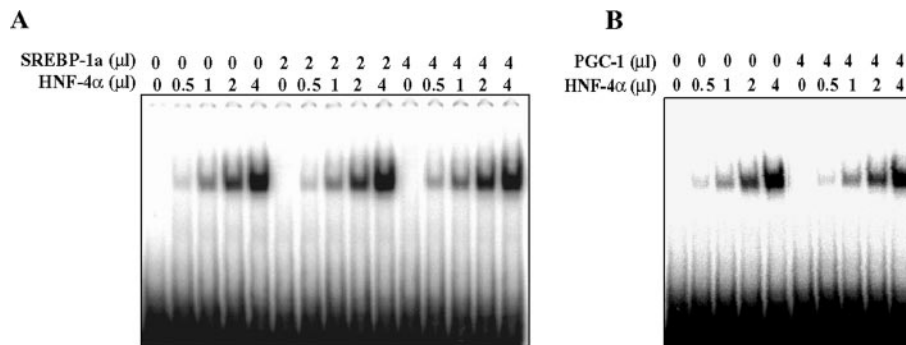

FIG. 6. **SREBP-1a and PGC-1 do not affect DNA binding activity of HNF-4α.** EMSA with radiolabeled HNF-4α-binding site (AF1, HNF-4α-RE) probe in PEPCK promoter (*A* and *B*) was performed. Indicated amounts of *in vitro* translated HNF-4α protein and SREBP-1a protein (*A*) or PGC-1 protein (*B*) were pre-incubated with this radiolabeled probe at 4 °C for 1 h, and the DNA-protein complex was run on a 4% polyacrylamide gel at 90 V for 1 h.

DISCUSSION

This study clearly demonstrates that SREBP inhibits HNF-4α signaling in the induction of PEPCK promoter activity. A series of reporter assays, EMSA, coimmunoprecipitation, and coactivator recruitment assays, indicate that the SREBP inhibition is mediated through its direct interaction with HNF-4α, leading to impaired recruitment of PGC-1. The direct interaction is through the TA of SREBP and the LBD/AF2 site of HNF-4α. It has been shown that the TA of SREBPs determines transcriptional activity of the SREBP family; SREBP-1c with the shortest TA has a lower transactivation potential for many SREBP target genes as compared with SREBP-1a and -2 with

long TA (17, 18). It is plausible that SREBP-1c was less effective in inhibition of HNF-4α signaling due to its weaker affinity for HNF-4. The kinetics in dose-dependent inhibition curves for SREBP-1a and -1c and lack of suppressive activity in TA-deleted SREBP mutant all support this hypothesis.

Our data indicate that the protein interaction between HNF-4α and SREBP impairs PGC-1 recruitment for HNF-4α activation and does not inhibit HNF-4α binding to its cis-element. Considering that PGC-1 has been reported to bind to the AF2 domain of HNF-4α (7), identification of LBD/AF2 and not LBD of HNF-4α as an SREBP-binding site can explain the mechanism of inhibition. The mode of inhibition is likely to be
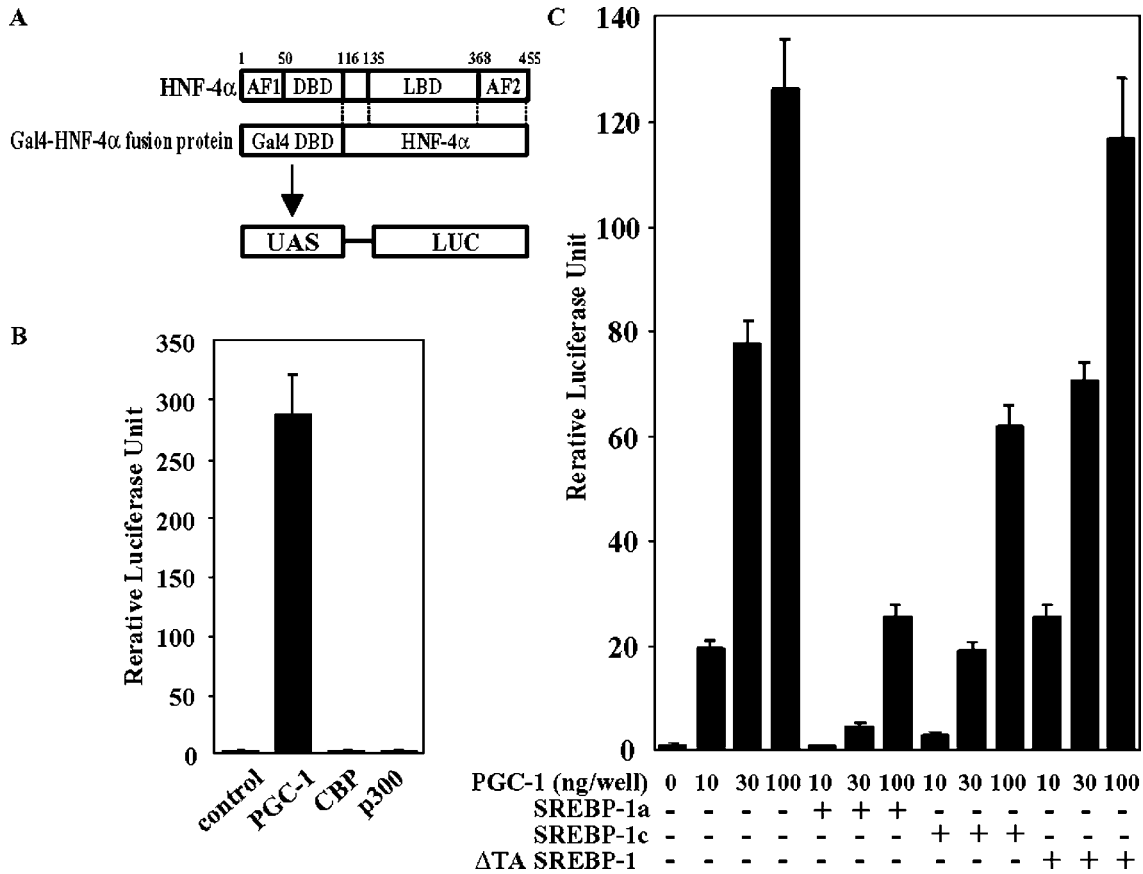
FIG. 7. **PGC-1 recruitment and coactivation of HNF-4α was inhibited by SREBPs.** *A*, schematic representation of Gal4-HNF-4α fusion protein expression vector and UAS-LUC vector containing eight copies of UAS, Gal4-binding site. Carboxyl-terminal region (115–455 aa) of HNF-4α protein was fused to Gal4-DNA binding domain for estimation of PGC-1 recruitment and coactivation of HNF-4α. *B*, coactivation of HNF-4α by PGC-1. HepG2 cells in 12-well plates were cotransfected with UAS-LUC vector (300 ng/well), expression vectors for Gal4-HNF-4α fusion protein (300 ng/well), and PGC-1, CBP, or p300 (400 ng/well). Cells were harvested after 24 h of transfection. Luciferase activity was measured and normalized by *Renilla* luciferase activity. *C*, SREBP suppression of PGC-1 coactivation. HepG2 cells in 24-well plates were cotransfected with UAS-LUC vector (150 ng/well), expression vectors for Gal4-HNF4-α fusion protein (150 ng/well), PGC-1 (0, 10, 30, and 100 ng/well), and SREBP-1a, SREBP-1c, or ΔTA-SREBP-1 (30 ng/well). Cells were harvested after 48 h of transfection. Luciferase activity was measured and normalized by *Renilla* luciferase activity. All experiments were performed in triplicate.
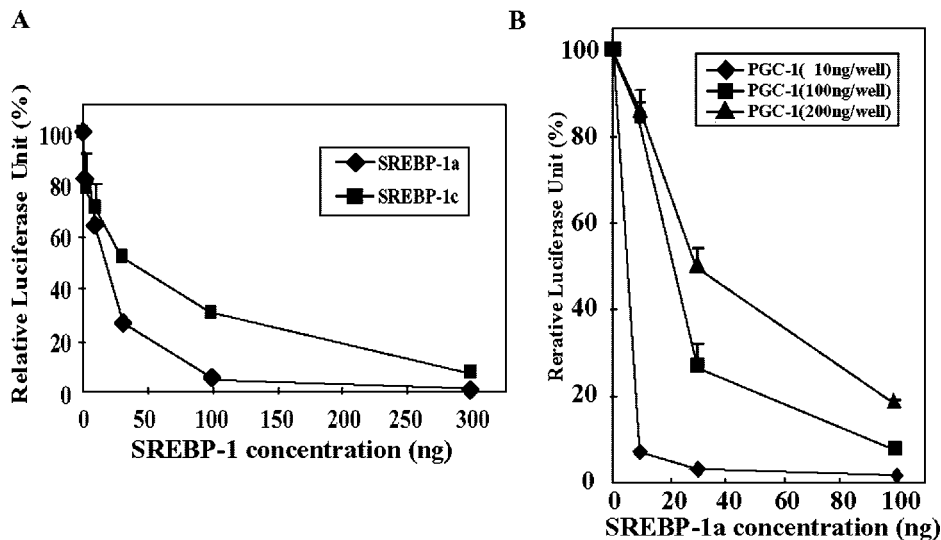

FIG. 8. **SREBP-1a and -1c competes with PGC-1 in coactivation of HNF-4α.** *A*, dose-dependent inhibition of SREBP-1a and -1c with PGC-1 recruitment to HNF-4α. HepG2 cells in 24-well plates were cotransfected with UAS-LUC vector (50 ng/well), expression vectors for Gal4-HNF-4α fusion protein (50 ng/well), PGC-1(100 ng/well), and SREBP-1a or SREBP-1c (0, 3, 10, 30, 100, and 300 ng/well). Cells were harvested after 48 h of transfection. PGC-1-induced relative luciferase activity was set at 100%. *B*, competition between SREBP-1a and PGC-1 in PGC-1 recruitment to HNF-4α. HepG2 cells in 24-well plates were cotransfected with UAS-LUC vector (50 ng/well), expression vectors for Gal4-HNF-4α fusion protein (50 ng/well), PGC-1 (10, 100, and 200 ng/well), and SREBP-1a (0, 10, 30, and 100 ng/well). Cells were harvested after 48 h of transfection. PGC-1-induced relative luciferase activity is set at 100%. Luciferase activity was measured and normalized by *Renilla* luciferase activity. All of the experiments were performed in triplicate.
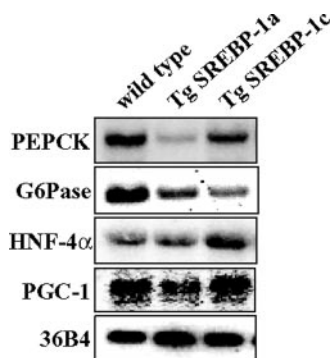
FIG. 9. **Expression of gluconeogenic genes was repressed in livers of SREBP-1 transgenic mouse.** Total RNA (10 μg/lane) extracted from wild type or transgenic mouse liver was subjected to electrophoresis and Northern blot hybridization with the indicated radiolabeled probe.

FIG. 10. **Role of SREBP in the transcriptional regulation of gluconeogenic genes.** Molecular mechanism in which SREBP inhibits the HNF-4α-dependent PEPCK promoter activity. SREBP competition with PGC-1 recruitment to HNF-4α could be either due to physical hindrance or impaired accessibility of HNF-4 ligands.

a competition between SREBP-1a and PGC-1, based upon the dose-dependent kinetic study of SREBP-1 and PGC-1. Meanwhile, it is also possible that binding of SREBP to the LBD of HNF-4α could affect the access of endogenous ligands to HNF-4α, resulting in impairment of PGC-1 recruitment. Physiological HNF-4α ligand and the molecular mechanism for HNF-4α activation by the ligands are currently an enigma, although some studies including analysis of the three-dimensional structure of HNF-4α LBD indicate that the endogenous HNF-4α ligand could be palmitate (22). Polyunsaturated fatty acids have been reported to regulate the transcription of Glc-6-Pase through the repression of HNF-4α activity in hepatoma cell (5). Therefore, it is possible that SREBP binding to HNF-4α LBD might affect HNF-4α signaling through accessibility of these potential ligands or antagonists.

In the livers of SREBP-1a and -1c transgenic mice, mRNA levels of gluconeogenic enzymes, PEPCK and Glc-6-Pase, were decreased. Gene expression of Glc-6-Pase as well as PEPCK seem to be regulated by the HNF-4α/PGC-1 pathway based upon recent studies (6, 7) with adenovirus-mediated overexpression of PGC-1 and liver-specific HNF-4α knockout mice. Taken together with our findings on SREBP inhibition of the HNF-4α/PGC-1 pathway, SREBPs could be involved in regulation of gluconeogenesis through inhibition of gluconeogenic genes. From a physiological standpoint, SREBPs, in particular SREBP-1c, could play a role in regulation of gluconeogenesis, because it is highly induced in a fed condition for lipogenesis after the ingestion of excessive carbohydrates as shown in Fig. 10 (23). SREBP-1c inhibition of gluconeogenesis could minimize hepatic glucose output, saving hepatic glucose for lipogenesis. Conversely, in a fasted condition, SREBP-1c expression was completely suppressed (24, 25) allowing gluconeogenesis to be sustained for the maintenance of glucose homeostasis. Recently, synthesis of LXR ligand improved glucose tolerance in a model of diet-induced obesity and insulin resistance. In mice treated with LXR ligand, mRNAs of PEPCK and Glc-6-Pase were decreased as compared with that of control mice. Since we and other groups (16, 26) demonstrated previously that the transcription of SREBP-1c is strongly induced through LXR *in vitro* and *in vivo*, it could be speculated that suppression of gluconeogenic genes by the LXR ligand could be mediated through a concomitant increase in SREBP-1c.

Recently we reported that cross-talk between PPARs and LXRs could be involved in reciprocal nutritional regulation of fatty acid metabolism (15, 16). Our current finding that the protein interaction between HNF-4α and SREBP-1c affects HNF-4α signaling suggests that transcription factors involved in glucose and lipid metabolism are highly related in mutual regulatio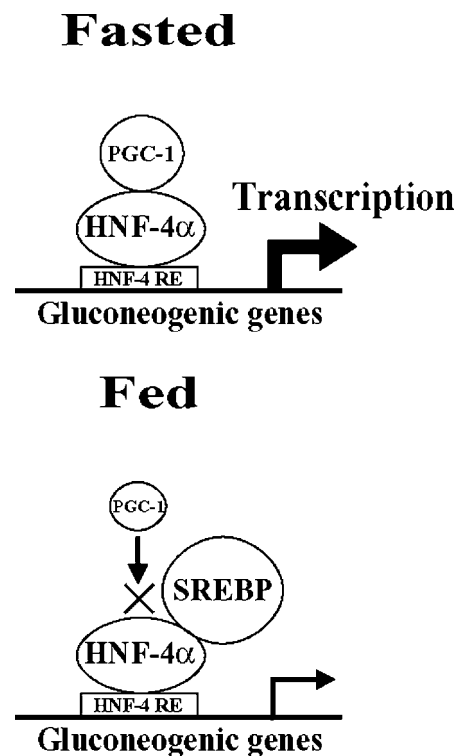n, and provides another example for cross-talk in a network of nutritional transcription factors. Although the role of PGC-1 in HNF-4α regulation of gluconeogenic genes has been established, the mechanism for HNF-4α signaling in other HNF-4α target genes is not fully understood. Modes of SREBP effects on other HNF-4α signaling could be different from SREBP inhibition of HNF-4α signaling in gluconeogenesis. In fact, SREBP overexpression did not affect hepatic expression of apolipoprotein genes that have been shown to be HNF-4α targets in HNF-4α knockout mice (27). Conversely and very intriguingly, in the light of SREBP signaling, HNF-4α/SREBP interaction enhances SREBP transactivation of sterol regulatory genes in some conditions (33). The physiological consequences of HNF-4α/SREBP interaction could depend upon target genes, coactivators, and cell types.

Insulin is the most potent negative regulator of PEPCK gene expression. SREBP-1c has been thought to be induced as consequences of insulin/glucose effects in the liver (28, 29). Thus, it is tempting to speculate that insulin suppression of PEPCK expression could be at least partly mediated through SREBP inhibition of PEPCK expression. However, this concept awaits clarification of the precise molecular mechanism for nutritional induction of SREBP-1c. The HNF-4α and SREBP-1c interaction in hepatic glucose output should be estimated in relevance to the pathophysiology of hepatic insulin resistance and diabetes. Considering a role of HNF-4α as a causative gene for maturity onset diabetes of young (MODY1) (30–32), it would also be interesting to investigate roles of SREBP and HNF-4α in pancreatic β cells in terms of insulin expression, secretion, and diabetes.

The current studies propose a novel role of SREBP in the transcriptional regulation of gluconeogenesis and suggest that the SREBP-1 functions not only as a lipid regulator but also as a regulator of glucose metabolism via the transcriptional regulation of gluconeogenesis.

REFERENCES

1. Hanson, R. W., and Reshef, L. (1997) *Annu. Rev. Biochem.* **66,** 581–611
2. Sladek, F. M., Zhong, W. M., Lai, E., and Darnell, J. E., Jr. (1990) *Genes Dev.* **4,** 2353–2365
3. Drewes, T., Senkel, S., Holewa, B., and Ryffel, G. U. (1996) *Mol. Cell. Biol.* **16,** 925–931
4. Hall, R. K., Sladek, F. M., and Granner, D. K. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 412–416
5. Rajas, F., Gautier, A., Bady, I., Montano, S., and Mithieux, G. (2002) *J. Biol. Chem.* **277,** 15736–15744
6. Rhee, J., Inoue, Y., Yoon, J. C., Puigserver, P., Fan, M., Gonzalez, F. J., and Spiegelman, B. M. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 4012–4017
7. Yoon, J. C., Puigserver, P., Chen, G., Donovan, J., Wu, Z., Rhee, J., Adelmant, G., Stafford, J., Kahn, C. R., Granner, D. K., Newgard, C. B., and Spiegelman, B. M. (2001) *Nature* **413,** 131–138
8. Brown, M. S., and Goldstein, J. L. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 11041–11048
9. Brown, M. S., and Goldstein, J. L. (1997) *Cell* **89,** 331–340
10. Brown, M. S., Ye, J., Rawson, R. B., and Goldstein, J. L. (2000) *Cell* **100,** 391–398
11. Horton, J. D., Goldstein, J. L., and Brown, M. S. (2002) *J. Clin. Investig.* **109,** 1125–1131
12. Shimano, H. (2000) *Trends Cardiovasc. Med.* **10,** 275–278
13. Shimano, H. (2001) *Prog. Lipid Res.* **40,** 439–452
14. Shimano, H. (2002) *Vitam. Horm.* **65,** 167–194
15. Yoshikawa, T., Ide, T., Shimano, H., Yahagi, N., Amemiya-Kudo, M., Matsuzaka, T., Yatoh, S., Kitamine, T., Okazaki, H., Tamura, Y., Sekiya, M., Takahashi, A., Hasty, A. H., Sato, R., Sone, H., Osuga, J. I., Ishibashi, S., and Yamada, N. (2003) *Mol. Endocrinol.* **7,** 1240–1254
16. Ide, T., Shimano, H., Yoshikawa, T., Yahagi, N., Amemiya-Kudo, M., Matsuzaka, T., Nakakuki, M., Yatoh, S., Iizuka, Y., Tomita, S., Ohashi, K., Takahashi, A., Sone, H., Gotoda, T., Osuga, J. I., Ishibashi, S., and Yamada, N. (2003) *Mol. Endocrinol.* **7,** 1255–1267
17. Shimano, H., Horton, J. D., Hammer, R. E., Shimomura, I., Brown, M. S., and Goldstein, J. L. (1996) *J. Clin. Investig.* **98,** 1575–1584
18. Shimano, H., Horton, J. D., Shimomura, I., Hammer, R. E., Brown, M. S., and Goldstein, J. L. (1997) *J. Clin. Investig.* **99,** 846–854
19. Shimano, H., Yahagi, N., Amemiya-Kudo, M., Hasty, A. H., Osuga, J., Tamura, Y., Shionoiri, F., Iizuka, Y., Ohashi, K., Harada, K., Gotoda, T., Ishibashi, S., and Yamada, N. (1999) *J. Biol. Chem.* **274,** 35832–35839
20. Chakravarty, K., Leahy, P., Becard, D., Hakimi, P., Foretz, M., Ferre, P., Foufelle, F., and Hanson, R. W. (2001) *J. Biol. Chem.* **276,** 34816–34823
21. Sato, R., Miyamoto, W., Inoue, J., Terada, T., Imanaka, T., and Maeda, M. (1999) *J. Biol. Chem.* **274,** 24714–24720
22. Dhe-Paganon, S., Duda, K., Iwamoto, M., Chi, Y. I., and Shoelson, S. E. (2002) *J. Biol. Chem.* **277,** 37973–37976
23. Horton, J. D., Bashmakov, Y., Shimomura, I., and Shimano, H. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 5987–5992
24. Yahagi, N., Shimano, H., Hasty, A. H., Amemiya-Kudo, M., Okazaki, H., Tamura, Y., Iizuka, Y., Shionoiri, F., Ohashi, K., Osuga, J., Harada, K., Gotoda, T., Nagai, R., Ishibashi, S., and Yamada, N. (1999) *J. Biol. Chem.* **274,** 35840–35844
25. Yoshikawa, T., Shimano, H., Yahagi, N., Ide, T., Amemiya-Kudo, M., Matsuzaka, T., Nakakuki, M., Tomita, S., Okazaki, H., Tamura, Y., Iizuka, Y., Ohashi, K., Takahashi, A., Sone, H., Osuga Ji, J., Gotoda, T., Ishibashi, S., and Yamada, N. (2002) *J. Biol. Chem.* **277,** 1705–1711
26. Yoshikawa, T., Shimano, H., Amemiya-Kudo, M., Yahagi, N., Hasty, A. H., Matsuzaka, T., Okazaki, H., Tamura, Y., Iizuka, Y., Ohashi, K., Osuga, J., Harada, K., Gotoda, T., Kimura, S., Ishibashi, S., and Yamada, N. (2001) *Mol. Cell. Biol.* **21,** 2991–3000
27. Hayhurst, G. P., Lee, Y. H., Lambert, G., Ward, J. M., and Gonzalez, F. J. (2001) *Mol. Cell. Biol.* **21,** 1393–1403
28. Hasty, A. H., Shimano, H., Yahagi, N., Amemiya-Kudo, M., Perrey, S., Yoshikawa, T., Osuga, J., Okazaki, H., Tamura, Y., Iizuka, Y., Shionoiri, F., Ohashi, K., Harada, K., Gotoda, T., Nagai, R., Ishibashi, S., and Yamada, N. (2000) *J. Biol. Chem.* **275,** 31069–31077
29. Shimomura, I., Bashmakov, Y., Ikemoto, S., Horton, J. D., Brown, M. S., and Goldstein, J. L. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 13656–13661
30. Yamagata, K., Furuta, H., Oda, N., Kaisaki, P. J., Menzel, S., Cox, N. J., Fajans, S. S., Signorini, S., Stoffel, M., and Bell, G. I. (1996) *Nature* **384,** 458–460
31. Furuta, H., Iwasaki, N., Oda, N., Hinokio, Y., Horikawa, Y., Yamagata, K., Yano, N., Sugahiro, J., Ogata, M., Ohgawara, H., Omori, Y., Iwamoto, Y., and Bell, G. I. (1997) *Diabetes* **46,** 1652–1657
32. Hani, E. H., Suaud, L., Boutin, P., Chevre, J. C., Durand, E., Philippi, A., Demenais, F., Vionnet, N., Furuta, H., Velho, G., Bell, G. I., Laine, B., and Froguel, P. (1998) *J. Clin. Investig.* **101,** 521–526
33. Misawa, K., Horiba, T., Arimura, N., Hirano, Y., Inoue, J., Emoto, N., Shimano, H., Shimizu, M., and Sato, R. (2003) *J. Biol. Chem.* **278,** 36176–36182