SREBP-1-independent regulation of lipogenic gene expression in adipocytes

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Abstract Sterol regulatory element-binding protein (SREBP)-1c is now well established as a key transcription factor for the regulation of lipogenic enzyme genes such as FAS in hepatocytes. Meanwhile, the mechanisms of lipogenic gene regulation in adipocytes remain unclear. Here, we demonstrate that those in adipocytes are independent of SREBP-1c. In adipocytes, unlike in hepatocytes, the stimulation of SREBP-1c expression by liver X receptor agonist does not accompany lipogenic gene upregulation, although nuclear SREBP-1c protein is concomitantly increased, indicating that the activation process of SREBP-1c by the cleavage system is intact in adipocytes. Supportively, transcriptional activity of the mature form of SREBP-1c for the FAS promoter was negligible when measured by reporter analysis. As an underlying mechanism, accessibility of SREBP-1c to the functional elements was involved, because chromatin immunoprecipitation assays revealed that SREBP-1c does not bind to the functional SRE/E-box site on the FAS promoter in adipocytes. Moreover, genetic disruption of SREBP-1 did not cause any changes in lipogenic gene expression in adipose tissue. In summary, in adipocytes, unlike in hepatocytes, increments in nuclear SREBP-1c are not accompanied by transactivation of lipogenic genes; thus, SREBP-1c is not committed to the regulation of lipogenesis.—Sekiya, M., N. Yahagi, T. Matsuzaka, Y. Takeuchi, Y. Nakagawa, H. Takahashi, H. Okazaki, Y. Iizuka, K. Ohashi, T. Gotoda, S. Ishibashi, R. Nagai, T. Yamazaki, T. Kadowaki, N. Yamada, J-i. Osuga, and H. Shimano. **SREBP-1-independent regulation of lipogenic gene expression in adipocytes.** ***J. Lipid Res.*** **2007.** 48: **1581–1591.**

Supplementary key words sterol regulatory element-binding protein • lipogenesis • fatty acid synthase

The fatty acid biosynthetic pathway, composed of some 25 enzymes, has been elucidated in detail (1). Among these enzymes, FAS, the main synthetic enzyme that catalyzes the condensation of malonyl-CoA to produce the 16 carbon saturated fatty acid palmitate, and acetyl-coenzyme A carboxylase (ACC), which synthesizes malonyl-CoA from acetyl-CoA, are of particular importance. The regulation of these lipogenic enzymes has two remarkable features. First, their overall enzymatic activities largely depend on the amount of expressed protein that is primarily controlled at the transcriptional level, although regulation through phosphorylation is also important for some enzymes, such as ACC. Second, their rates of transcription are coordinately regulated (2). Therefore, it has been presumed that these genes share a regulatory sequence in their promoters that interacts with common *trans*-acting factors. In the liver, the most likely factor conducting this coordinated transcriptional regulation has been revealed to be sterol regulatory element-binding protein (SREBP)-1 (3, 4).

SREBPs are transcription factors that belong to the basic helix-loop-helix leucine zipper family and are considered to be profoundly involved in the transcriptional regulation of cholesterogenic and lipogenic enzymes (5, 6). Unlike other members of the basic helix-loop-helix leucine zipper family, SREBPs are synthesized as precursors bound to the endoplasmic reticulum and nuclear envelope. Upon activation, SREBPs are released from the membrane into the nucleus as mature protein by a sequential two-step cleavage process. To date, three SREBP isoforms, SREBP-1a, -1c, and -2, have been identified and characterized. SREBP-1a and -1c are transcribed from the same gene, each by a distinct promoter, and the predominant SREBP-1 isoform in liver and adipose tissue is 1c rather than 1a (7).

The role of SREBP-1c for the regulation of lipogenesis in the liver has been well established by several lines of

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Abbreviations: ACC, acetyl-coenzyme A carboxylase; ALLN, *N*-acetyl-leucyl-leucyl-norleucinal; ChIP, chromatin immunoprecipitation; LXR, liver X receptor; RXR, retinoid X receptor; SREBP, sterol regulatory element-binding protein.

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evidence, especially from transgenic and knockout mouse models (4, 8). In these models, mature hepatic SREBP-1c protein levels determine mRNA expression levels for a battery of lipogenic genes in the liver. Moreover, mature hepatic SREBP-1c is physiologically regulated by nutrient availability (i.e., it is downregulated when animals are starved and upregulated when they are refed), thereby adjusting lipogenic gene expression levels to the nutritional conditions (9). Thus, regarding its role in the liver, SREBP-1c is now well established as a key transcription factor for the regulation of lipogenic gene expression and by extension triglyceride storage in liver (10, 11).

Despite the extensive knowledge gained in recent years regarding the role of SREBP-1 in lipogenesis in liver, its physiological role in adipocytes remains obscure. Although the mRNA expression of SREBP-1c in adipocytes is also drastically altered by dietary conditions, we have reported that targeted disruption of the SREBP-1 gene scarcely affected the dynamic changes of lipogenic gene expression in adipose tissue (4). Conversely, the impact of SREBP-1c overexpression in adipocytes was also evaluated in transgenic mice; however, it disrupted the differentiation processes of adipocytes, resulting in lipodystrophy. Hence, the effect of SREBP-1c on lipogenic gene regulation in adipocytes was not able to be evaluated (12), although ectopic overexpression of SREBP-1a drives fatty acid synthesis in the adipose tissue of transgenic mice (13).

These situations prompted us to investigate the effects of SREBP-1c activation in adipocytes by stimulating the SREBP-1c promoter. We and others have found that liver X receptors (LXRs), nuclear receptor-type transcription factors, activate the transcription of the SREBP-1c gene through binding to LXR elements in the promoter, together with retinoid X receptors (RXRs) (14, 15).

Here, we show that the stimulation of the SREBP-1c gene with LXR agonist has negligible effects on the expression of lipogenic enzymes in adipocytes, despite a concomitant increase in nuclear active SREBP-1c, indicating that the transcriptional activity of SREBP-1c against lipogenic genes is almost nil in adipocytes in contrast to hepatocytes. In fact, luciferase reporter gene assays demonstrated that the recombinant nuclear active form of SREBP-1c had far lower activity for the FAS promoter in 3T3-L1 adipocytes than in HepG2 hepatoma cells. Consistent with these observations, chromatin immunoprecipitation (ChIP) assays revealed that SREBP-1 is not recruited to the functional *cis* element on the FAS promoter in LXR-activated adipocytes. Therefore, lipogenic genes are controlled almost independently of SREBP-1c in adipocytes, and the triglyceride biosynthetic pathway is differently regulated between liver and adipose tissue.

MATERIALS AND METHODS

Materials

The synthetic LXR agonist T0901317 was purchased from Cayman Chemical (Ann Arbor, MI). The RXR agonist LG100268 was synthesized as described elsewhere (16). Standard laboratory chow (MF; composed of 60% carbohydrate, 13% fat, and 27% protein on a caloric basis) was obtained from Oriental Yeast (Tokyo, Japan). Other materials were purchased from Sigma unless indicated otherwise.

Animals

Eight week old male Wister rats and 8 week old male C57BL/6J mice were purchased from CLEA (Tokyo, Japan). All animals were maintained in a temperature-controlled environment with a 12 h light/dark cycle and were given free access to standard chow and water. The dosage of T0901317 (suspended with 0.9% carboxymethylcellulose, 9.95% polyethylene glycol 400, and 0.05% Tween 80, administered orally) was 10 mg/kg for rats and 50 mg/kg for mice. Animals were euthanized at 12 h after administration. The protocol of dietary manipulation was as follows: for the fasting group, mice were starved for 24 h and rats were starved for 48 h; for the refeeding group, they were refed at 12 h or 24 h after a 24 h (for mice) or 48 h (for rats) starvation, respectively, unless stated otherwise. All groups of animals in one experiment were euthanized at the same time. SREBP-1-null mice (16 weeks of age, female) on the C57BL/6J background have been reported previously (4).

RNA isolation and Northern blotting

Total RNA from liver, epididymal fat pads, and cultured cells was isolated with Trizol Reagent (Invitrogen), and a 10 μg RNA sample equally pooled among each group was run on a 1% agarose gel containing formaldehyde and transferred to a nylon membrane. cDNA probes were cloned as described previously (4, 17). The probes were labeled with [α-^{32}P]dCTP using the Megaprime DNA Labeling System (Amersham Biosciences). The membranes were hybridized with the radiolabeled probe in Rapid-hyb buffer (Amersham Biosciences) at 65°C and washed in 0.1× SSC buffer with 0.1% SDS at 65°C. Blots were exposed to both Kodak XAR-5 film and the imaging plate for the BAS2000 BIO Imaging Analyzer (Fuji Photo Film, Tokyo, Japan). The quantification results obtained from the BAS2000 system were normalized to the signal generated from 36B4 (acidic ribosomal phosphoprotein P0) mRNA.

Quantitative real-time PCR analysis

Two micrograms of total RNA was reverse-transcribed using the ThermoScript RT-PCR system (Invitrogen). Quantitative real-time PCR was performed using SYBR Green dye (Applied Biosystems, Foster City, CA) in an ABI Prism 7900 PCR instrument (Applied Biosystems). The relative abundance of each transcript was calculated by a standard curve of cycle thresholds for serial dilutions of a cDNA sample and normalized to 36B4. Primer sequences are available upon request.

Nuclear protein extraction from liver and fat

Nuclear extract protein from mouse or rat liver was prepared as described previously (18). Briefly, excised livers (0.5 g) were homogenized in a Polytron in 5 ml of buffer A, which consisted of 10 mM HEPES at pH 7.9, 25 mM KCl, 1 mM EDTA, 2 M sucrose, 10% glycerol, 0.15 mM spermine, and 2 mM spermidine, supplemented with protease inhibitors [6 μg/ml *N*-acetyl-leucyl-leucyl-norleucinal (ALLN; Calbiochem), 2.5 μg/ml pepstatin A, 2 μg/ml leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, and 2.5 μg/ml aprotinin]. Pooled homogenate was then subjected to one stroke of a Teflon pestle in a Potter-Elvejiem homogenizer, followed by filtration through two layers of cheesecloth, and layered over 10 ml of buffer A. After centrifugation at 24,000 rpm on a Beckman SW28 rotor for 1 h at 4°C, the resulting nuclear pellet

was resuspended in a buffer containing 10 mM HEPES at pH 7.9, 100 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol, supplemented with protease inhibitors, after which 0.1 volume of 5 M NaCl was added. Each mixture was agitated gently for 30 min at 4°C and then centrifuged at 89,000 rpm on a Himac S120AT2 rotor (Hitachi, Tokyo, Japan) for 30 min at 4°C. The supernatant was used as nuclear extract.

Nuclear protein from white adipose tissue was prepared as described previously (19). In brief, fresh epididymal fat pads (~3 g) were rinsed in ice-cold PBS, minced, and homogenized with 10 strokes of a Teflon homogenizer in 15 ml of NDS buffer at 4°C (10 mM Tris-HCl at pH 7.5, 10 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 14 mM mercaptoethanol, 0.5 mM EGTA, 2 mM EDTA, 0.5% Nonidet P-40, and 1 mM dithiothreitol) supplemented with protease inhibitors (6 μg/ml ALLN, 2 μg/ml leupeptin, 2.5 μg/ml aprotinin, 2.5 μg/ml pepstatin A, and 0.1 mM phenylmethylsulfonyl fluoride). The Nonidet P-40 concentration was increased to 1%, and nuclei were pelleted at 700 *g* for 10 min, washed once with 25 ml of NDS buffer (1% Nonidet P-40), filtered through 70 μm mesh, pelleted at 500 *g* for 10 min, resuspended in 1 volume of 1% citric acid, lysed by the addition of 2.5 volumes of 0.1 M Tris-HCl, 2.5% SDS, and 0.1 M dithiothreitol, sonicated briefly, and heated to 90°C for 5 min. Aliquots of nuclear protein (20 μg) were subjected to SDS-PAGE.

Primary culture of rat preadipocytes

Fibroblastic preadipocytes were isolated from epididymal fat pads of Wister rats by collagenase digestion as described previously (20). In brief, the epididymal fat pads from male Wister rat were removed and minced in KRBH buffer (130 mM NaCl, 5.2 mM KCl, 1.3 mM KH_2PO_4, 2.7 mM $CaCl_2$, 1.3 mM $MgSO_4$, 24.8 mM $NaHCO_3$, and 10 mM HEPES at pH 7.4) supplemented with 3% (w/v) BSA, 2 mM glucose, and 200 nM adenosine. After digestion by collagenase (type II; 1.5 mg/ml) at 37°C for 1 h in a shaking water bath, the digest was filtered through sterile 250 μm nylon mesh. The adipocytes were allowed to float to the top of the tube and the infranatant was collected, passed through a 25 μm stainless steel filter, and centrifuged at 250 *g* for 10 min. The pellet was resuspended in MEM-α medium (Invitrogen) with 10% FBS supplemented with penicillin and streptomycin (100 U/ml and 100 μg/ml, respectively; Invitrogen). Red blood cells were lysed by hypotonic shock (21). The cells were plated on 60 mm culture dishes at a density of 6×10^5 cells/well. Medium was changed every 2 days. After the cells reached confluence, differentiation was induced in MEM-α medium with 10% FBS by the addition of 0.5 mM isobutylmethylxanthine (Wako), 0.25 μM dexamethasone, 5 μg/ml bovine insulin, and 1 μM pioglitazone (provided by Takeda Pharmaceutical). The differentiation of cells was morphologically confirmed. Differentiated adipocytes were treated with either vehicle (ethanol) or 10 μM T0901317 for 12 h in MEM-α medium containing the indicated amounts of glucose and insulin.

Primary culture of rat hepatocytes

Hepatocytes were isolated from nonfasted 4 week old Wister rats by the collagenase perfusion method as described previously (22). Cells were resuspended in DMEM containing 25 mM glucose supplemented with 5% FBS and seeded on collagen-coated 100 mm dishes at a final density of 4×10^4 cells/cm^2. After 4 h, attached cells were cultured with Medium 199 containing Earle's salts (Invitrogen) and 5% FBS. After incubation for 20 h, cells were treated with either vehicle (ethanol) or 10 μM T0901317 for 12 h in similar medium containing the indicated amounts of glucose and insulin.

Preparation of nuclear extracts from cultured cells

Nuclear proteins from cultured cells were extracted as described previously (23). In brief, 2 h before collection, ALLN (6 μg/ml) was added to the medium. After collection, cells were resuspended in buffer A (10 mM HEPES at pH 7.6, 1 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, and 1 mM EGTA) and passed through a 26 gauge needle 20 times and then briefly centrifuged. The pellet, containing the nuclei, was resuspended in buffer B (20 mM HEPES at pH 7.6, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and 0.5 mM dithiothreitol) and rotated at 4°C for 15 min and then centrifuged at 15,000 *g* for 20 min. The supernatant was collected as nuclear extract. Whole cell lysates were harvested with a buffer (20 mM Tris-HCl at pH 7.4) containing detergents (1% Triton X-100, 0.1% SDS, and 1% sodium deoxycholate) and protease inhibitors according to a standard protocol.

Immunoblotting of SREBP proteins

Membrane fractions from livers and epididymal fat pads were prepared as described previously (18). Aliquots of nuclear extract (20 μg) and membrane fraction (50 μg) proteins were subjected to SDS-PAGE. Immunoblot analysis was performed using the ECL Western Blotting Detection System (Amersham Biosciences) and exposed to Kodak XAR-5 film. The primary antibodies (rabbit polyclonal; No. 931 for mouse SREBP-1a and -1c, No. 772 for SREBP-1c, which does not bind to SREBP-1a, and No. 528 for SREBP-2) were used as described previously (11, 17, 24). The precursor and mature SREBP-1 bands are ~125 and 65 kDa, respectively.

Transfection and luciferase assays

An expression plasmid for the human nuclear form of SREBP-1c constructed in pcDNA3.1(+) (Invitrogen) was described previously (25). Luciferase reporter plasmids for the SRE promoter (SRE-Luc) and the fatty acid synthase gene promoter (FAS-Luc) were prepared as described previously (8, 24). Human hepatoma HepG2 cells were cultured in DMEM containing 25 mM glucose, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate supplemented with 10% FBS. On day 0, cells were plated on a 12-well plate at 4.5×10^4 cells/well. On day 2, the indicated amounts of mature SREBP-1c expression plasmids, mock plasmids [empty pcDNA3.1(+)] to adjust total DNA amount, and luciferase reporter plasmids (FAS-Luc or SRE-Luc; 0.25 μg each) mixed with an SV-β-galactosidase reference plasmid (0.1 μg; p-SV-β-gal; Promega) were cotransfected into HepG2 cells using SuperFect Transfection Reagent (Qiagen) according to the manufacturer's protocol. The luciferase activity in transfectants was measured on a luminometer and normalized to β-galactosidase activity measured by standard kits (Promega).

3T3-L1 adipocytes were transfected by electroporation as described previously (26, 27). In brief, after cells became confluent (day 0), adipose conversion was induced in DMEM containing 25 mM glucose, 10% FBS, 0.5 mM isobutylmethylxanthine, 5 μg/ml bovine insulin, 0.25 μM dexamethasone, 1 μM pioglitazone, 33 μM biotin (Wako), 17 μM pantothenate (Wako), and antibiotics. After 48 h, the induction medium was removed and replaced by DMEM containing 25 mM glucose, 10% FBS, insulin, pioglitazone, biotin, and pantothenate. This medium was changed every 2 days. The adipocytes at day 8 of differentiation grown on 60 mm dishes were detached from dishes with 0.25% trypsin and 0.5 mg/ml collagenase (Wako) in PBS, washed twice, and resuspended in PBS. The indicated amounts of SREBP-1c expression plasmids, mock plasmids to adjust total DNA amount, luciferase reporter plasmids (FAS-Luc or SRE-Luc; 15 μg each), and control plasmids (pSV-β-gal; 10 μg) were cotransfected to

the cells by a pulse current generated from the electroporator (Cell-Porator; Invitrogen) at 160 V with 880 μF capacitance and a low ohm setting. After electroporation, cells were immediately mixed with fresh medium for 10 min before being reseeded onto 24-well collagen-coated plates and assayed at 40 h after transfection.

ChIP assays

ChIP assays were performed as described by Boyd and Farnham (28) with minor modifications. The supernatant of soluble chromatin derived from 1×10^7 cells was used. Briefly, rat primary hepatocytes and adipocytes were treated with 1% formaldehyde for 10 min. The cross-linking reaction was stopped by the addition of glycine to a final concentration of 0.125 M for 5 min. After washing with cold PBS, the cells were suspended in cell lysis buffer containing 10 mM HEPES at pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.5% Nonidet P-40, and protease inhibitors. After incubation for 30 min on ice, the cells were passed through a 26 gauge needle 20 times on ice. The nuclei were collected by microcentrifugation at 5,000 rpm and resuspended in nuclear lysis buffer (50 mM Tris-HCl at pH 8.1, 1% SDS, 10 mM EDTA, and protease inhibitors) on ice for 10 min. Samples were sonicated with a Branson sonifier at power 2 for six 10 s pulses to an average DNA length of <2 kb and then microcentrifuged at 15,000 rpm for 10 min. The supernatant was diluted 10-fold with buffer containing 0.01% SDS, 16.7 mM Tris-HCl at pH 8.1, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, and protease inhibitors and precleared for 1.5 h with Protein G-Sepharose. The supernatant was incubated with normal rabbit IgG or anti-SREBP-1 antibody overnight at 4°C.

Samples were subsequently washed four times using wash buffer A (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, and 20 mM Tris-HCl at pH 8.1), wash buffer B (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, and 20 mM Tris-HCl at pH 8.1), wash buffer C (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl at pH 8.1), and wash buffer D (1 mM EDTA and 10 mM Tris-HCl at pH 8.0) and were eluted by 30 min of incubation with 100 μl of elution buffer (1% SDS, 50 mM $NaHCO_3$, and 10 mM DTT). Then, NaCl was added at a final concentration of 0.3 M and RNA was removed by the addition of RNase A, and the samples were incubated at 65°C for 4 h to reverse the formaldehyde-induced cross-linking. Protein was digested by proteinase K in 2× PK buffer (20 mM Tris-HCl at pH 7.5, 10 mM EDTA, and 1% SDS) at 45°C for 2 h. The resulting chromatin DNA was further purified on silica beads (Wizard DNA Clean-Up System; Promega) and used as a template for PCR. Primers used to amplify FAS promoter regions were as follows: for the SRE/E-box site at −65 (−109 to +63 was amplified), 5′-GACGCTCATTGGCCTGG-3′ and 5′-CTCTGGAGGCAGACGACAAG-3′; for the SRE-1 site at −150 (−94 to −299 was amplified), 5′-AGGACAGAGATGAGGGCGTC-3′

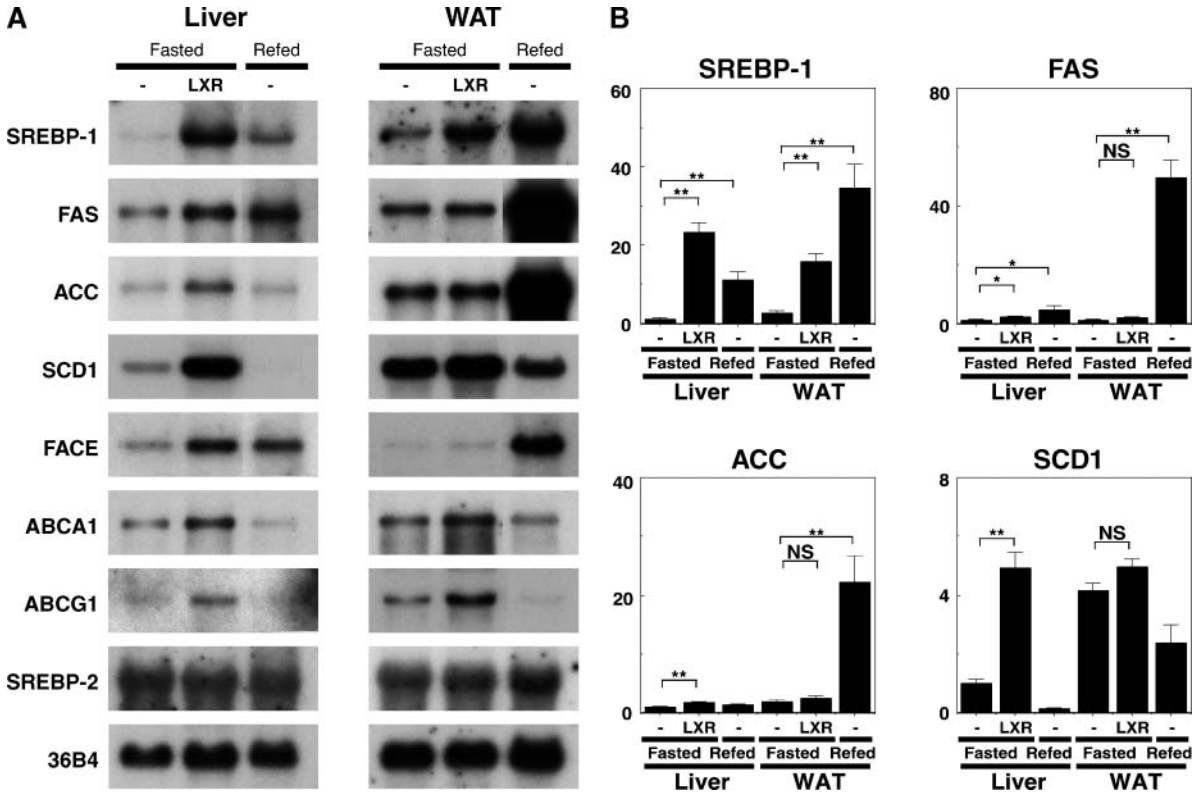

Fig. 1. Sterol regulatory element-binding protein (SREBP)-1c activation by liver X receptor (LXR) agonists does not increase lipogenic gene expression in mouse adipose tissue. Northern blot (A) and real-time PCR (B) analyses of liver and adipose tissue of mice treated with LXR agonist are shown. C57BL/6J mice (8 weeks old, male, four animals for each group) were treated with the LXR agonist T0901317 (50 mg/kg) as indicated and euthanized at 12 h after treatment in a 24 h fasted state. Refed control mice were refed for 12 h after 24 h of starvation. A: Total RNA (10 μg) pooled equally was subjected to Northern blotting. A cDNA probe for 36B4 (acidic ribosomal phosphoprotein P0) was used to confirm equal loading. B: Real-time PCR analysis of RNA isolated from liver and adipose tissue. mRNA levels of target genes were normalized to 36B4. Values are means ± SEM presented as fold changes relative to liver controls. ACC, acetyl-coenzyme A carboxylase; FACE, fatty acyl-coenzyme A elongase; SCD1, stearoyl-coenzyme A desaturase 1; WAT, white adipose tissue. * $P < 0.05$, ** $P < 0.01$ versus controls.

and 5′-CCAGGCCAATGAGCGTC-3′; for the nonspecific site (−1,076 to −904), 5′-AAGCCACTGCCCATAAGGTT-3′ and 5′-TTAAAGGGAGGGAGGGTGAG-3′. For the SRE/E-box and nonspecific sites, PCR was performed using AmpliTaq Gold (Applied Biosystems) under the following reaction conditions: after 9 min at 95°C, 35 cycles of 1 min at 95°C, 30 s at 62°C, and 1 min at 72°C. For the SRE-1 site, PCR was performed using Advantage-GC Genomic Polymerase Mix (Clontech) under the following reaction conditions: after 1 min at 95°C, 35 cycles of 30 s at 94°C, 30 s at 62°C, and 2.5 min at 68°C. After amplification, PCR products were electrophoresed on a 3% NuSieve agarose gel and visualized by ethidium bromide staining. These experiments were performed at least three times.

RESULTS

SREBP-1c activation by LXR agonist does not induce lipogenic gene expression in adipose tissue

In our first series of experiments, we examined the effects of SREBP-1c overexpression on lipogenic gene expression in liver and adipose tissue. As expected, administration of synthetic LXR agonist T0901317 to animals (mice in **Fig. 1** and rats in **Fig. 2**) upregulated SREBP-1 expression and thereby the downstream lipogenic enzyme genes, such as FAS, ACC, and stearoyl-coenzyme A desaturase 1, in their livers. In contrast, quite unexpectedly, lipogenic genes in their adipose tissues were not influenced by the treatment, although SREBP-1 was markedly upregulated in the same way as in liver. These results from Northern blotting were confirmed by quantitative real-time PCR analyses (Fig. 1B). It is notable that SREBP-1 mRNA levels quantified by real-time PCR are equivalent in both tissues, as shown in Fig. 1B, denying the possibility that a lack of lipogenic gene induction in adipose tissue is a quantitative effect. The effect of LXR activation was further verified by the marked increase of ABCA1 and ABCG1, representatives of LXR-activated genes (29, 30), which were strongly induced in both tissues. These results were not altered by combined administration of both LXR agonist and the RXR agonist LG100268 (30 mg/kg), which produced additive effects on the SREBP-1 pathway in the liver but did not influence lipogenic gene expression in adipose tissue (data not shown).

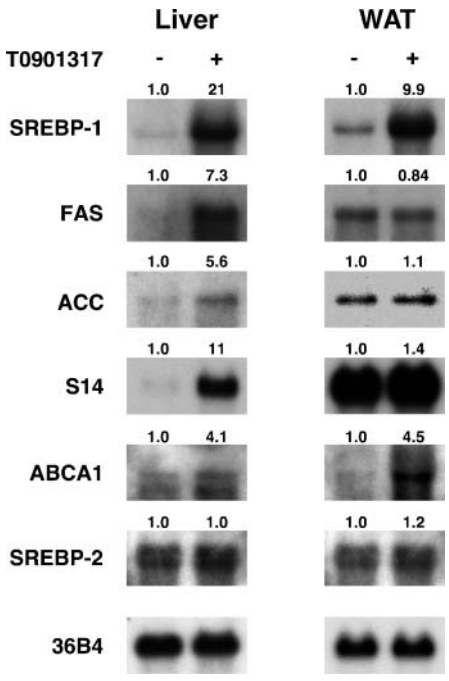

Fig. 2. SREBP-1c activation by LXR agonist does not induce lipogenic gene expression in rat adipose tissue. Northern blot analysis of liver and adipose tissue of rats treated with LXR agonist is shown. Wister rats (8 weeks old, male, three animals for each group) were administered the LXR agonist T0901317 (10 mg/kg) or vehicle alone and euthanized at 12 h after treatment in a fasted state. Total RNA (10 μg) pooled equally was subjected to Northern blotting. A cDNA probe for 36B4 was used to confirm equal loading. The quantification results were obtained with the BAS2000 system and normalized to the signal generated from 36B4 mRNA, and the fold changes against control are displayed above each blot. S14, Spot 14; WAT, white adipose tissue.

Posttranslational activation of SREBP-1 is intact, and mature protein is generated in adipose tissue

SREBP-1 is synthesized as a precursor bound to the endoplasmic reticulum and nuclear envelope and is released from the membrane into the nucleus as a mature protein by a cleavage process (6). Therefore, it is possible that defective posttranslational processing of SREBP-1 in adipocytes might result in this blunted response of lipogenic genes to SREBP-1 overexpression. To test this possibility, we separated nuclei from liver and adipose tissue and quantified the amount of mature SREBP-1 by immunoblot analysis. As shown in **Fig. 3**, nuclear contents of SREBP-1 were increased similarly by LXR agonist in both liver and adipose tissue, indicating that posttranslational processing of SREBP-1 is intact in adipose tissue (Fig. 3A, mice; Fig. 3B, rats). It was further confirmed by SREBP-1c-specific antibody that isoform 1c was increased in the nuclei of adipose tissue. These findings demonstrate that the defective response of lipogenic genes to SREBP-1 overexpression in adipose tissue is not attributable to an impairment of the SREBP-1c cleavage process.

To deny the possibility that the expression levels of the nuclear form of SREBP-1 protein in adipose tissue stimulated by LXR agonist are too low to increase lipogenic gene expression, the effect of T0901317 was compared with that of refeeding. As shown in Fig. 3C, the mature form of SREBP-1 in the nuclei of LXR-stimulated adipocytes is more abundant than that observed in adipocytes from refed animals. However, this abundant SREBP-1 protein of the active form did not induce FAS expression at all (Fig. 3D), demonstrating that the amount of SREBP-1 protein is not the cause of the inability of SREBP-1 to upregulate FAS expression in adipocytes.

SREBP-1 and lipogenic gene expression in primary cultured cells

LXR widely modifies lipid and glucose metabolism, affecting serum levels of insulin and fatty acids (31), which are known to influence the SREBP-1 pathway (32). To

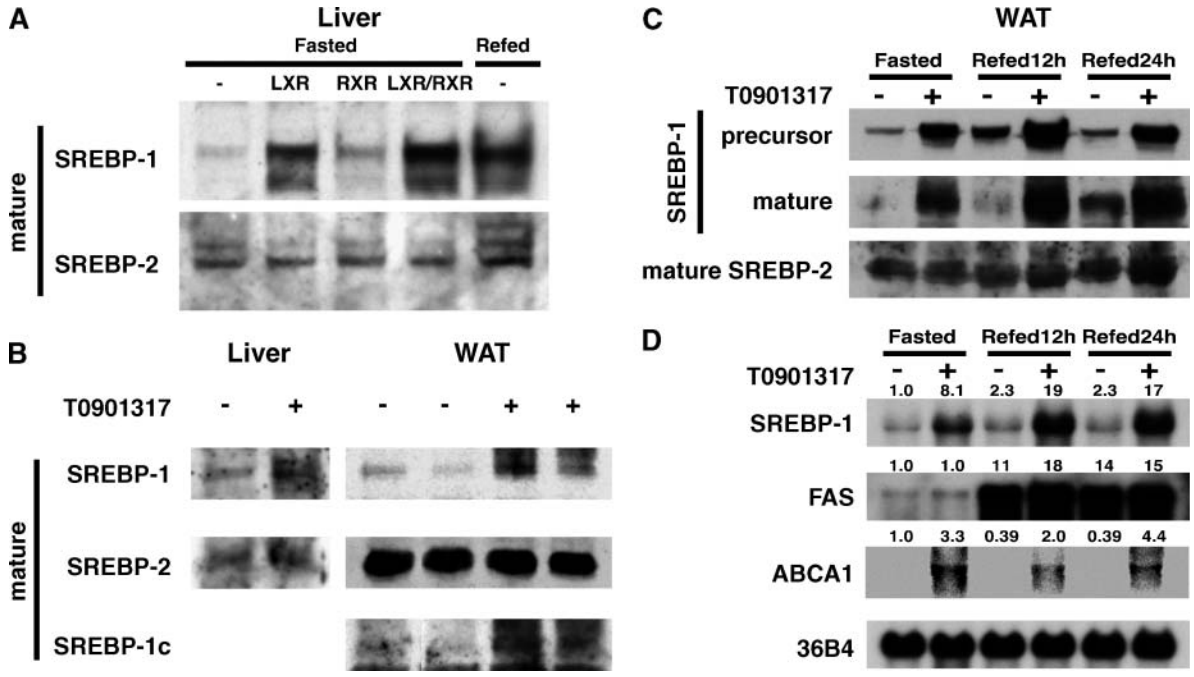

Fig. 3. Posttranslational activation of SREBP-1 is intact and mature protein is generated in adipose tissue. Immunoblot analyses of SREBPs in nuclear proteins from liver (A, B) and adipose tissue (B, C) are shown. A: Mature forms of SREBP-1 and -2 in the liver. C57BL/6J mice (8 weeks old, male, four mice for each group) were administered the LXR agonist T0901317 (50 mg/kg) and/or the retinoid X receptor (RXR) agonist LG100268 (30 mg/kg) as indicated and euthanized at 12 h after treatment in a 24 h fasted state. Refed control mice were refed for 12 h after 24 h of starvation. B: Mature forms of SREBP-1 and -2 in liver and adipose tissue were quantified from Wister rats (8 weeks old, male) administered T0901317 (10 mg/kg) or vehicle alone and euthanized at 12 h after treatment in a fasted state. Nuclei were isolated from liver (pooled within groups) and adipose tissue (individually), and aliquots of nuclear proteins (20 μg) were subjected to immunoblot analysis. SREBP-1c-specific antibody was also used. C, D: Effect of administering LXR agonist compared with that of refeeding rats. C: Precursor and mature forms of SREBP-1 in adipose tissue were visualized by immunoblots. D: Northern blotting of SREBP-1 and FAS. Wister rats (8 weeks old, male, three animals for each group) were administered T0901317 (10 mg/kg) or vehicle alone at 12 h before euthanization. WAT, white adipose tissue.

eliminate these indirect effects through systemic factors and reinforce the in vivo results, we also examined the effects of SREBP-1c activation in primary cultured hepatocytes and adipocytes in vitro, whose isoform patterns of SREBP-1 are 1c-predominant as assessed by quantitative real-time PCR assays (data not shown). In these primary models, it was demonstrated that the overexpression of SREBP-1 mRNA and thereby precursor proteins induced by LXR agonist led to the increase of mature SREBP-1 proteins in both hepatocytes and adipocytes (**Fig. 4**). However, the resulting activation of SREBP-1 produced totally different effects on lipogenic gene expression between hepatocytes and adipocytes; consistent with the in vivo results described above, FAS mRNA expression was upregulated by SREBP-1c in hepatocytes but was not induced in adipocytes.

Transcriptional activity of SREBP-1c for the FAS promoter is negligible in adipocytes

As described above, the contribution of SREBP-1 to lipogenic gene regulation was demonstrated to be different between hepatocytes and adipocytes. Because SREBP-1c activation processes are revealed to be equally intact in both cell types, it is presumed that nuclear SREBP-1 would have different transcriptional activity in hepatocytes and adipocytes. To test this hypothesis, we performed luciferase reporter gene assays and compared transcriptional activities of nuclear SREBP-1c between HepG2 hepatoma cells and 3T3-L1 adipocytes. We estimated the transcriptional activity of nuclear SREBP-1c against the FAS promoter by transfecting cells with FAS-Luc reporter plasmids along with mature SREBP-1c expression plasmids. Transfection efficiency was evaluated using an optimum SRE-Luc as a control. As shown in **Fig. 5**, in HepG2 cells, FAS-Luc and SRE-Luc were vigorously activated by SREBP-1c. In contrast, activation of FAS-Luc by the active form of SREBP-1c was faint in 3T3-L1 adipocytes, whereas SRE-Luc was highly activated. These data demonstrate that the transcriptional activity of SREBP-1c against the FAS promoter is far lower in adipocytes compared with hepatocytes.

SREBP-1 does not bind to the functional *cis* element on the FAS promoter in adipocytes

To further investigate the molecular mechanism against the inability of SREBP-1 to activate transcription from the FAS gene in adipocytes, we evaluated the direct binding of SREBP-1 to the FAS promoter using ChIP assays. In the FAS promoter, two potential binding sites for SREBP-1 have been identified: one is −65 SRE/E-box, which contains two tandem copies of SREs and the E-box; the other

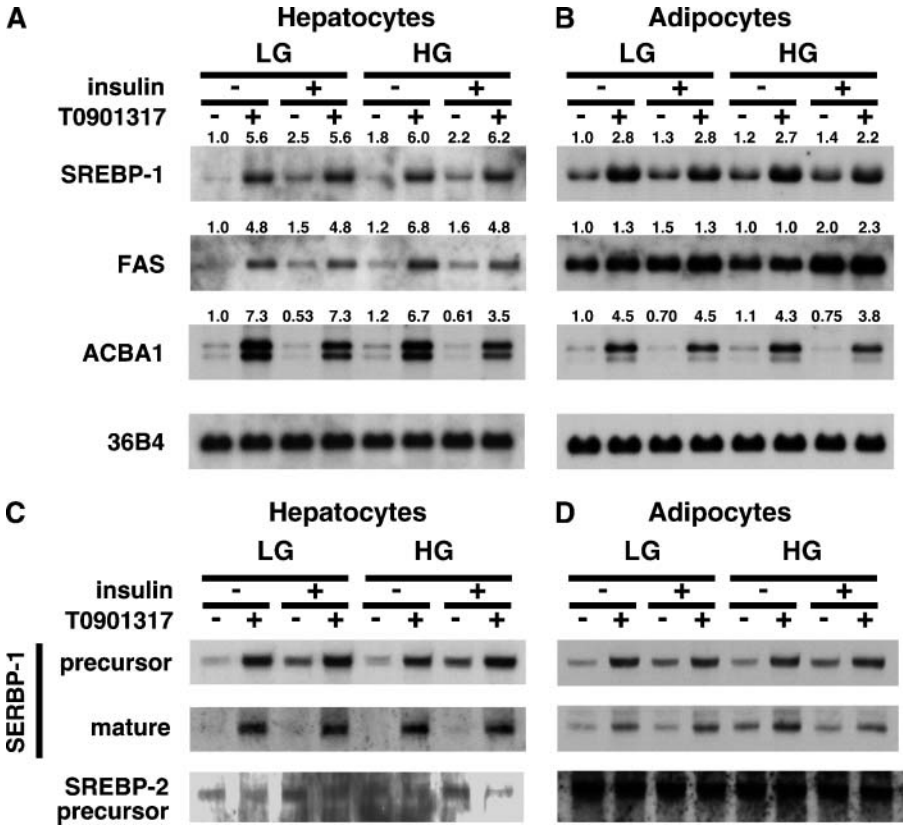

Fig. 4. Northern and Western blot analyses of SREBP-1 in primary hepatocytes and adipocytes. A, B: mRNA quantification of SREBP-1 and lipogenic genes in primary hepatocytes (A) and adipocytes (B). Hepatocytes isolated from rat livers (A) and adipocytes differentiated from rat preadipocytes (B) were incubated for 12 h with or without T0901317 (10 μM) and insulin (100 nM). Incubation medium contained either 5 mM glucose (LG; low glucose) or 25 mM glucose (HG; high glucose). Total RNA (10 μg) was subjected to Northern blotting. A cDNA probe for 36B4 was used to confirm equal loading. The quantification results were obtained with the BAS2000 system and normalized to the signal generated from 36B4 mRNA, and fold changes against control are displayed above each blot. C, D: Precursor and mature forms of SREBP-1 proteins from primary hepatocytes (C) and adipocytes (D) are shown. Cells were stimulated as described above, whole cell lysates (50 μg of protein) were subjected to immunoblot analysis, and the bands corresponding to precursor and mature SREBP-1 proteins are presented as indicated. These experiments were repeated twice, and reproducibility was confirmed.

is −150 SRE-1, which is not functional because of the lack of cofactor binding sites nearby, although the sequence itself is a complete SRE (5′-TCACCCCAC-3′) (33, 34). In our ChIP assays, SREBP-1 activation augmented its binding to −65 SRE/E-box in rat primary hepatocytes, as expected (**Fig. 6**). In sharp contrast to this, SREBP-1 activation did not result in its recruitment to −65 SRE/E-box in adipocytes, demonstrating that SREBP-1 does not bind to the functional *cis* element on the FAS promoter in adipocytes. Meanwhile, SREBP-1 activation led to its binding to −150 SRE-1 in both hepatocytes and adipocytes. These results were also confirmed using mouse primary cells (data not shown).

Genetic disruption of SREBP-1 decreases lipogenic gene expression as well as the responses to LXR activation in the liver, but not in adipose tissue

To further validate that SREBP-1 is not involved in the regulation of lipogenic genes in adipocytes, we evaluated FAS expression in SREBP-1 knockout mice with or without LXR stimulation. As shown in **Fig. 7**, genetic deletion of SREBP-1 impaired the transactivation of FAS in response to LXR activation in liver, whereas in adipose tissue, LXR activation did not alter the expression levels of these lipogenic genes in either SREBP-1 knockout or wild-type mice. These results demonstrate that SREBP-1 does not substantially contribute to the regulation of FAS expression in adipose tissue, whereas in the liver, SREBP-1 actually regulates the expression level of FAS.

DISCUSSION

This study has clearly demonstrated that lipogenic gene regulation is primarily independent of SREBP-1c in adipocytes. Hence, the involvement of SREBP-1c in fatty acid synthesis differs fundamentally between liver and adipose tissue, the two major lipogenic organs.

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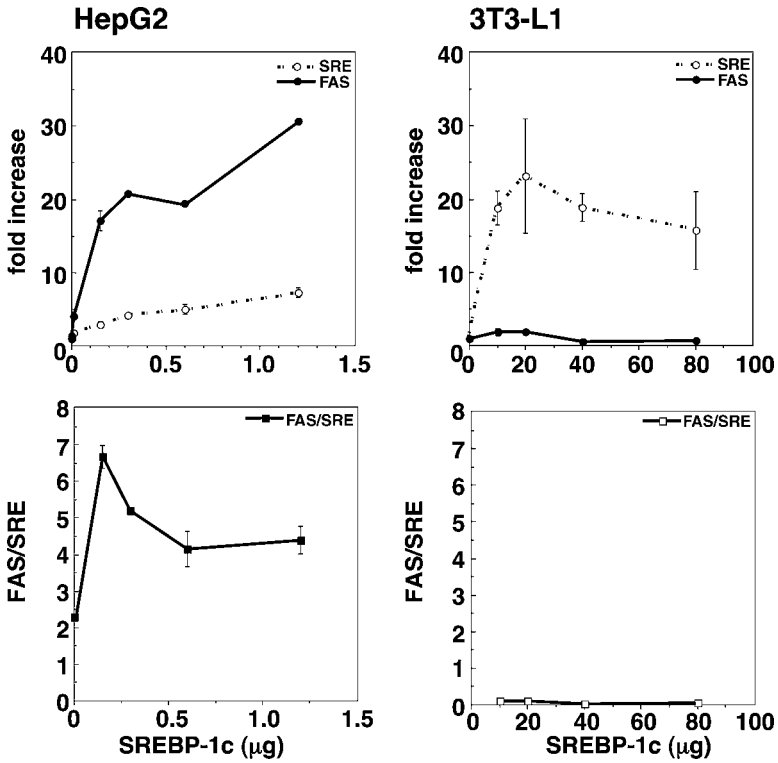

Fig. 5. Transcriptional activity of SREBP-1c against the FAS promoter is negligible in adipocytes. Transcriptional activities of the mature form of SREBP-1c in HepG2 hepatoma cells (left panels) and 3T3-L1 adipocytes (right panels) were measured by luciferase reporter gene assay. Both types of cells were transfected with the indicated amounts of mature SREBP-1c expression plasmids and mock plasmids [pcDNA3.1(+)] to adjust total DNA amount in combination with FAS-Luc or SRE-Luc and pSV-β-gal (note that transfection methods and scales are different between HepG2 and 3T3-L1 and that the amounts of transfected DNA cannot be compared between them). Luciferase activities were measured and normalized to β-galactosidase activities. In upper panels, values are expressed as fold changes against control (i.e., no SREBP-1c). Error bars represent SEM. These experiments were repeated twice, and reproducibility was confirmed.

Recently, a microarray analysis of mouse liver and adipose tissue stimulated by LXR agonist was reported by others (35). In that study, SREBP-1 mRNA expression was induced in both liver and adipose tissue by LXR agonist, whereas lipogenic genes such as fatty acid synthase, malic enzyme, and Spot 14 were upregulated only in liver and not in adipose tissue, supporting our data. We further proceeded to explore the underlying mechanism for this discrepancy and have revealed that the active form of SREBP-1 is definitely generated but does not transactivate lipogenic genes in adipocytes, as a result of defective binding to functional SRE in promoter regions in vivo.

In our first series of experiments, we demonstrated that in both adipocytes and hepatocytes the nuclear content of mature SREBP-1 protein parallels the amount of the precursor form of SREBP-1, whether its expression is increased by LXR activation or by refeeding. This observation indicates that the proteolytic activation of SREBP-1 precursor protein is intact in adipocytes and that SREBP-1 is basically activated when its mRNA expression is upregulated. This feature of SREBP-1 activation provides a sharp contrast to that of SREBP-2, which is tightly regulated through the cleavage processes as a pivotal part of the negative feedback system to maintain cholesterol homeostasis (6). In fact, SREBP-1 and -2 are reported to be regulated distinctly under some specific conditions (18, 36). It is intriguing that there is so much difference in the regulation of the proteolytic activation of SREBP-1 and -2, although they are both subject to the same cleavage machineries, consisting of SREBP cleavage-activating protein and site-1/2 proteases (37).

Nevertheless, in contrast to hepatocytes, increases of the active form of SREBP-1c protein failed to activate lipogenic genes in adipocytes. This ineffectiveness of SREBP-1c in adipocytes was validated by four lines of evidence: *1*) overexpression of active SREBP-1c had little effect on mRNA levels of lipogenic genes; *2*) transcriptional activity of SREBP-1c against the FAS promoter measured by reporter analysis was faint; *3*) nuclear SREBP-1c failed to bind to the functional *cis* element of the FAS promoter in adipocytes; and *4*) genetic disruption of SREBP-1 hardly affected mRNA levels of lipogenic genes. These results are further supported by the recent report claiming that mRNA levels of SREBP-1c do not coincide with the changes in adipose lipogenic gene expression (38, 39).

Regarding why mature SREBP-1c poorly transactivates lipogenic genes in adipocytes, there are two possibilities: *1*) SREBP-1c cannot reach its binding sites on the promoters; and *2*) SREBP-1c properly binds to its cognate sites, but the absence of required coactivator(s) or the presence of corepressor(s) specific to adipocytes prevents the genes from being transcribed. Our results from ChIP assays favor the former hypothesis. SREBP-1 was able to access both functional −65 SRE/E-box and nonfunctional −150 SRE-1 site in hepatocytes. In contrast, SREBP-1 could not bind to −65 SRE/E-box in adipocytes, although binding to −150 SRE-1 site was intact and similar to that observed in hepatocytes.

Another question our studies bring up is what transcription factors then regulate lipogenic gene expression in adipocytes. Unfortunately, we have few clues on this issue, except that this factor should be functionally activated by refeeding. Upstream stimulatory factors-1/2 (40–42), carbohydrate response element binding protein (43), and CCAAT/enhancer binding protein α (44) are potential candidates, but an as yet unknown

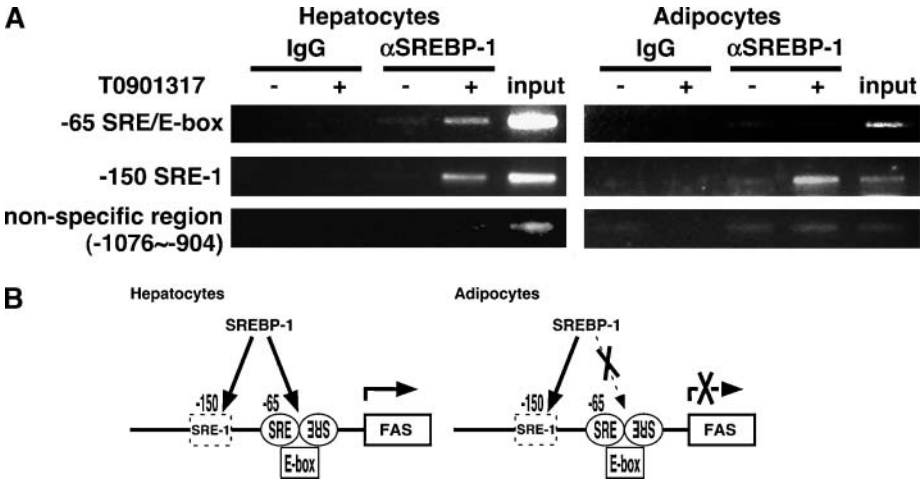

Fig. 6. SREBP-1 does not bind to the functional *cis* element on the FAS promoter in adipocytes. A: Chromatin immunoprecipitation (ChIP) assays using antibody to SREBP-1 and amplifying DNA fragments from the FAS promoter. Primary hepatocytes and adipocytes differentiated from preadipocytes were incubated for 12 h with or without T0901317 (10 μM). After cross-linking, the nuclei were isolated and sonicated, and chromatin protein-DNA complexes were immunoprecipitated with control rabbit IgG or anti-SREBP-1 antibodies. The resultant DNA was analyzed by PCR with primers amplifying −150 SRE-1, −65 SRE/E-box, and a nonspecific region (from −1,076 to −904) on the FAS promoter. The results shown here are representative of three independent experiments. B: Schematic representations of the FAS promoter. The FAS promoter contains two SREBP-1 recognition sites: −150 SRE-1 and −65 SRE/E-box. ChIP assays revealed tissue-dependent recruitment of SREBP-1; SREBP-1 can bind to both functional SRE/E-box and nonfunctional SRE-1 in hepatocytes, whereas the binding to SRE/E-box is prevented in adipocytes.

factor might be the answer. Further studies in adipocytes are imperative.

It has been reported by others that fatty acid synthase gene expression is increased by SREBP-1c through adenovirus-mediated gene transduction to 3T3-L1 adipocytes (45). In our experiments, FAS-Luc reporter activity was not increased by SREBP-1c transfection (Fig. 5). The cause of this discrepancy is not clear, but it is possible that the conditions of our transfection by electroporation method resulted in a more physiological level of SREBP-1c increase compared with adenovirus-mediated overexpression.

The importance of the LXR pathway in the regulation of lipogenesis and adipogenesis in adipocytes has been controversial; some reports have concluded that LXR stimulation leads to lipogenic gene activation (46, 47), whereas others have presented opposing data (35, 48). The current data support no impact of the LXR ligand on the gene expression of lipogenic enzymes, but the discrepancy

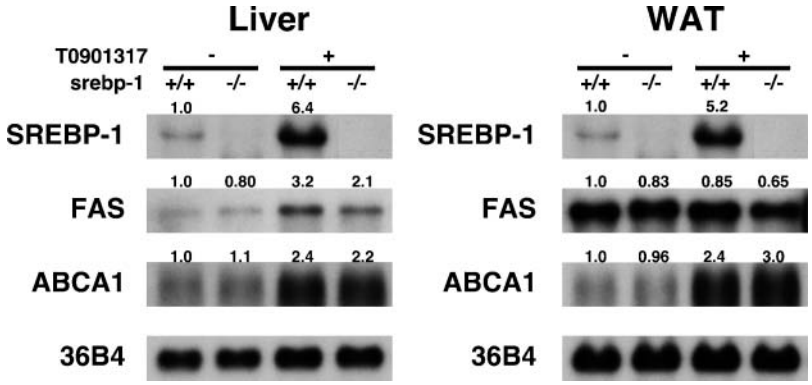

Fig. 7. Effects of the genetic disruption of SREBP-1. SREBP-1 disruption decreased lipogenic gene expression as well as the responses to LXR activation in the liver (left panel), whereas it did not do so in adipose tissue (right panel). SREBP-1 knockout mice in the C57BL/6J background (16 weeks old, female, three mice for each group) and their control littermates were treated with T0901317 (50 mg/kg) and analyzed by Northern blot. Mice were euthanized at 12 h after treatment in a 24 h fasted state. WAT, white adipose tissue.

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might originate from differences in experimental conditions, such as variable glucose availability, that might indirectly influence lipogenesis, considering that the LXR pathway is reported to upregulate glucose transport (49). Our main conclusion here is that adipocyte lipogenesis is independent of SREBP-1c even if upregulated by LXR activation, consistent with our previous and current data from SREBP-1-null mice.

In summary, we have found that lipogenic genes in adipocytes are primarily regulated by factor(s) other than SREBP-1. Although proteolytic activation of SREBP-1 is intact and the resulting active form of SREBP-1 is upregulated whenever the SREBP-1 gene is stimulated, it does not function as a transactivator for lipogenic genes in adipocytes.

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