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Lipid Synthetic Transcription Factor SREBP-1a Activates p21$^{WAF1/CIP1}$, a Universal Cyclin-Dependent Kinase Inhibitor

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Sterol regulatory element-binding proteins (SREBPs) are membrane-bound transcription factors that regulate lipid synthetic genes. In contrast to SREBP-2, which regulates cellular cholesterol level in normal cells, SREBP-1a is highly expressed in actively growing cells and activates entire programs of genes involved in lipid synthesis such as cholesterol, fatty acids, triglycerides, and phospholipids. Previously, the physiological relevance of this potent activity of SREBP-1a has been thought to regulate the supply of membrane lipids in response to cell growth. Here we show that nuclear SREBP-1a and SREBP-2 bind directly to a novel SREBP binding site in the promoter of the p21$^{WAF1/CIP1}$ gene, the major cyclin-dependent kinase inhibitor, and strongly activate its promoter activity. Only the SREBP-1a isoform consistently causes induction of p21 at both the mRNA and protein levels. Colony formation assays and polyploidy of livers from transgenic mice suggest that activation of p21 by SREBP-1a could inhibit cell growth. Activation of endogenous SREBPs in lipid deprivation conditions was associated with induction of p21 mRNA and protein. Expression of p21 was reduced in SREBP-1 null mice. These data suggest a physiological role of SREBP-1a in p21 regulation. Identification of p21 as a new SREBP target might implicate a new paradigm in the link between lipid synthesis and cell growth.

Sterol regulatory element-binding protein (SREBP) family members have been established as transcription factors regulating transcription of genes involved in cholesterol and fatty acid synthesis (4, 5). 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, and insulin-induced gene 1 (INSIG-1) (18). 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 (SREs) in the promoters of target genes and activate their transcription (4).

Three isoforms of SREBP, 1a, 1c, and 2, are known. Whereas SREBP-2 plays a crucial role in regulation of cholesterol synthesis in any type of cell, SREBP-1c controls gene expression of enzymes involved in synthesis of fatty acids and triglycerides in lipogenic organs (9, 21). Meanwhile, SREBP-1a is highly expressed in cells which are actively growing (24) and has a strong transcriptional activity for a wide range of synthetic genes for cholesterol, fatty acids, and phospholipids. All mammalian cells require these lipids for duplication of membranes in cell division. Depending upon cellular nutritional states and extracellular availability of lipids, nuclear SREBP-1a is induced in growing cells. Therefore, the functional relevance of this potent lipid synthetic regulator to cell proliferation has been believed to be a biological adaptation to meet demands for cellular lipids. However, it has never been intensively explored whether this regulatory system for synthesis of cellular lipids could inversely control cell growth.

p21$^{WAF1/CIP1}$ is the major cyclin-dependent kinase inhibitor, which halts the cell cycle at G_1 (6, 8, 14, 27; reviewed in reference 10). In this study, we found that p21 is an SREBP target, and especially SREBP-1a activates and stabilizes p21 expression, leading to cell growth arrest.

MATERIALS AND METHODS

Construction of the p21 luciferase gene reporter plasmids. The p21 promoter reporter plasmid WWP-Luc (6) was a kind gift from Bert Vogelstein. The promoter cDNAs were subcloned into pGL3-Basic (Promega) and designated p21–2338–Luc and p21–2258–Luc. Other luciferase constructs were generated by PCR.

Transfections and luciferase assay. HepG2 cells and Saos-2 cells were cultured in Dulbecco's modified Eagle's medium containing 25 mM glucose and 50 mM glucose, respectively. All medium contained 100 units/ml penicillin and 100 μg/ml streptomycin sulfate supplemented with 10% fetal calf serum. Cells were plated on 12-well dishes at 5.0×10^4 cells/well. Each luciferase reporter plasmid (0.5 μg) and *Renilla* luciferase control plasmid pRL-SV40 (Promegi; 0.05 μg) were cotransfected into the cells using FuGENE6 transfection reagent (Roche) according to the manufacturer's protocol. The total amount of DNA in each

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transfection was adjusted to 1 μg/well with vector DNA. Luciferase activity in transfectants was normalized to the amount of *Renilla* luciferase activity as measured by a kit (dual-luciferase reporter assay system; Promega).

In vitro translation of SREBPs. The pcDNA3.1(+)-SREBP-1a, -SREBP-1c, and -SREBP-2 vectors were translated in the TNT T7 Quick Coupled transcription/translation reticulocyte lysate system (Promega). Each coupled transcription-translation reaction contained 1 μg of plasmid DNA in a final volume of 50 μl and incubated at 30°C for 1.5 h. For radiolabeling, the vectors were translated in a methionine-free amino acid mixture supplemented with [^{35}S]methionine according to the manufacturer's protocol.

Gel mobility shift assay. The DNA probes were prepared by annealing both strands of the low-density lipoprotein receptor SRE site, GAAAATCAC CCCACTGCAAA; LDLR-SRE, the SRE site in the p21 promoter, GCGGT GGGCCGAGCG; p21-SRE and this mutation version, GCGGTACAAAAT GCG; p21-SRE Mut, GCGGTACGCCGTGCG; p21-SRE Mut1, GCGGTACA CCGAGCG; p21-SRE Mut2, GCGGTGGGCAATGCG; p21-SRE Mut3, GC GTGGGCCAAACG; and p21-SRE Mut4, which were labeled with [α-^{32}P]dCTP (3,000 to 10,000 cpm) using the Megaprime DNA labeling system (Amersham Pharmacia Biotech), followed by purification on mini Quick Spin oligo columns (Roche). The labeled DNA was incubated with 5 μl of in vitro transcription-translation mixture with SREBPs in 40 mM HEPES-KOH (pH 7.8), 50 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 5 mM dithiothreitol, 25 μg/ml poly(dI-dC), 0.1% Triton X-100, and 1 mg/ml skim milk for 1 h at room temperature. The DNA-protein complexes were resolved on a 3.75% polyacrylamide gel at 90 V for 1 h at room temperature. Gels were dried and exposed to BAS 2000 with BAStation software (Fuji Photo Film).

Animals. SREBP transgenic mice overexpressing human SREBP-1a, SREBP-1c, and SREBP-2 under the control of the rat phosphoenolpyruvate carboxykinase promoter were established as described (22). p21 knockout mice were obtained from Jackson Laboratory. SREBP-1a transgenic and p21 knockout mice were generated by intercrossing these mice. The animals were fasted 12 h prior to sacrifice.

Preparation of recombinant adenovirus. cDNAs encoding the active amino-terminal fragment of human SREBP-1a (amino acids 1 to 460), SREBP-1c (amino acids 1 to 436), SREBP-2 (amino acids 1 to 460), and dominant-negative SREBP-1, which lacked the amino-terminal transactivation domain (89 amino acids), were integrated into the adenovirus vector (11). SREBP adenoviral vectors were propagated in 293 cells and purified by cesium chloride density centrifugation.

Northern blot analysis. Total RNA was prepared from mouse livers and culture cells using TRIzol reagent (Life Technologys, Inc.); 24 h after transfection, the mRNA of HEK293 cells was extracted by Oligotex-dT30 <Super> kit (Roche). For Northern gel analysis, equal aliquots of total RNA from three mice and culture cells were pooled (total 10 μg or 300 ng of mRNA), denatured with formaldehyde and formamide, subjected to electrophoresis in a 1% agarose gel, and transferred to Hybond N membranes (Amersham Pharmacia Biotech) for hybridization. cDNA probes were labeled with [α-^{32}P]dCTP (3,000 to 10,000 cpm) using Megaprime DNA labeling system (Amersham Pharmacia Biotech). The filters were hybridized with the radiolabeled probe in Rapid-hyb buffer (Amersham Pharmacia Biotech) at 65°C and washed in 0.1× (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at 65°C. Blots were exposed to Kodak BIOMAX MS1 (EASTMAN KODAK COMPANY) and BAS 2000 with BAStation software (Fuji Photo Film).

Western analysis. Cells were cultured for 24 or 48 h after transfection or treated with 0.1 mM isopropylthiogalactopyranoside (IPTG) and harvested in NP-40 lysis buffer [50 mM Tris (pH 7.5), 250 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 1 mM DTT and protease inhibitor (Roche)]. Protein concentrations determined by the BCA protein assay kit (PIERCE). The samples were fractionated on 8, 12, or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by transfer to Immobilon Transfer membranes (MILLIPORE). Blots were subsequently incubated in TBS-T (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) overnight at 4°C with 1:2,500 anti-SREBP1 anti-p21^{WAF1} (Santa Cruz). Following incubation with horseradish peroxidase-conjugated secondary antibodies, blots were detected by the ECL or ECL Advance Western blotting detection kit (Amersham).

Colony formation assay. Saos-2 cells which achieved ~70% confluence were transfected with each plasmids (pcDNA3.1[+]-empty, -SREBPs, and -p21^{waf1}). The cells growth media were supplemented with G418 (500 μg/ml) 48 h later. Approximately 3 weeks later, when microscopic colonies become detectable, the cells were washed with phosphate-buffered saline, fixed in 10% acetic acid/10% methanol for 15 min, and stained with 0.4% crystal violet, 20% ethanol for 15 min (17) (31).

Flow cytometry analysis. For flow cytometry, livers were suspended in phosphate-buffered saline and through a cell strainer. The hepatocytes were diluted in 0.1% Triton X-100 and stained with 25 μg/ml propidium iodide and 1 mg/ml RNase. Total cellular DNA content was determined on flow cytometry and analyzed by FACScalibur (Becton Dickinson).

RESULTS

p21 is a novel target gene for SREBP-1a and SREBP-2. DNA microarray analysis revealed that the bulk of lipid synthetic genes are highly upregulated in livers overexpressing nuclear SREBP-1a from transgenic mice, confirming that SREBP-1a is a potent transactivator of lipid synthetic genes. Intriguingly, it was also found that expression of p21, a universal cyclin-dependent kinase inhibitor, was highly (7.5-fold) increased compared to wild-type mouse livers. To see whether this induction was due to direct transactivation by SREBP, promoter analysis on the human p21 gene was performed.

The human p21 promoter (2.4 kb) has been shown to fully contain known elements for its gene regulation, including p53 binding sites (Fig. 1) (6, 7, 13). In Saos-2 cells, deficient in p53, the basal p21 promoter activity, as estimated by luciferase reporter assays, was very low and coexpression of p53 caused a marked induction of p21 promoter activity, confirming that p53 plays a dominant role in a basal p21 expression (Fig. 1a). Expression of nuclear SREBP-1a induced p21 promoter activity even more strongly than p53. When the p53 binding sites were deleted, the p53 activation of the p21 promoter was eliminated, whereas the absence of p53 binding sites did not influence activation of p21 by SREBP-1a. These data suggest that SREBP activation of the p21 promoter is completely independent of p53. In HepG2 cells, which endogenously express p53, the p21 promoter activity was robust, but still highly activated by SREBP-1a (Fig. 1b).

To identify the promoter region responsible for SREBP activation, sequential deletion constructs were tested for SREBP-1a activation. Through these assays in HepG2 cells (Fig. 1b) and Saos-2 cells (data not shown), an SREBP activation site was identified far downstream of p53 sites between −98 bp and −90 bp. This region of the p21 promoter is not related to any other known *cis* elements for reported p21 activators (7). As depicted in Fig. 1c, the DNA sequence of this region, TGGGCCGAG, was novel as an SREBP binding site with slight similarity to that of Diazepam binding protein/acyl CoA binding protein, another SREBP target (25). The direct and specific binding of SREBP to this region was also shown by gel mobility retardation assays (Fig. 1d). Several mutations in this region caused loss of SREBP binding and confirmed the specificity of this sequence as p21-SRE (data not shown). The three isoforms of the SREBP family: SREBP-1a, SREBP-1c, and SREBP-2 have different target specificities and activities depending upon target gene promoters (1). Upon comparison of p21 activation among the isoforms in HepG2 cells (Fig. 2a) and Saos-2 cells (data not shown), SREBP-2 had similar or even stronger activity than SREBP-1a whereas SREBP-1c was the weakest. Consistently, in vitro-translated SREBP proteins all bound to this p21-SRE (Fig. 2b).

Transcriptional regulation of p21 is mediated through transactivation domain of SREBPs. A mutation of tyrosine for arginine in the basic region of the basic helix-loop-helx portion of SREBP (YR mutation) is known to abolish the binding

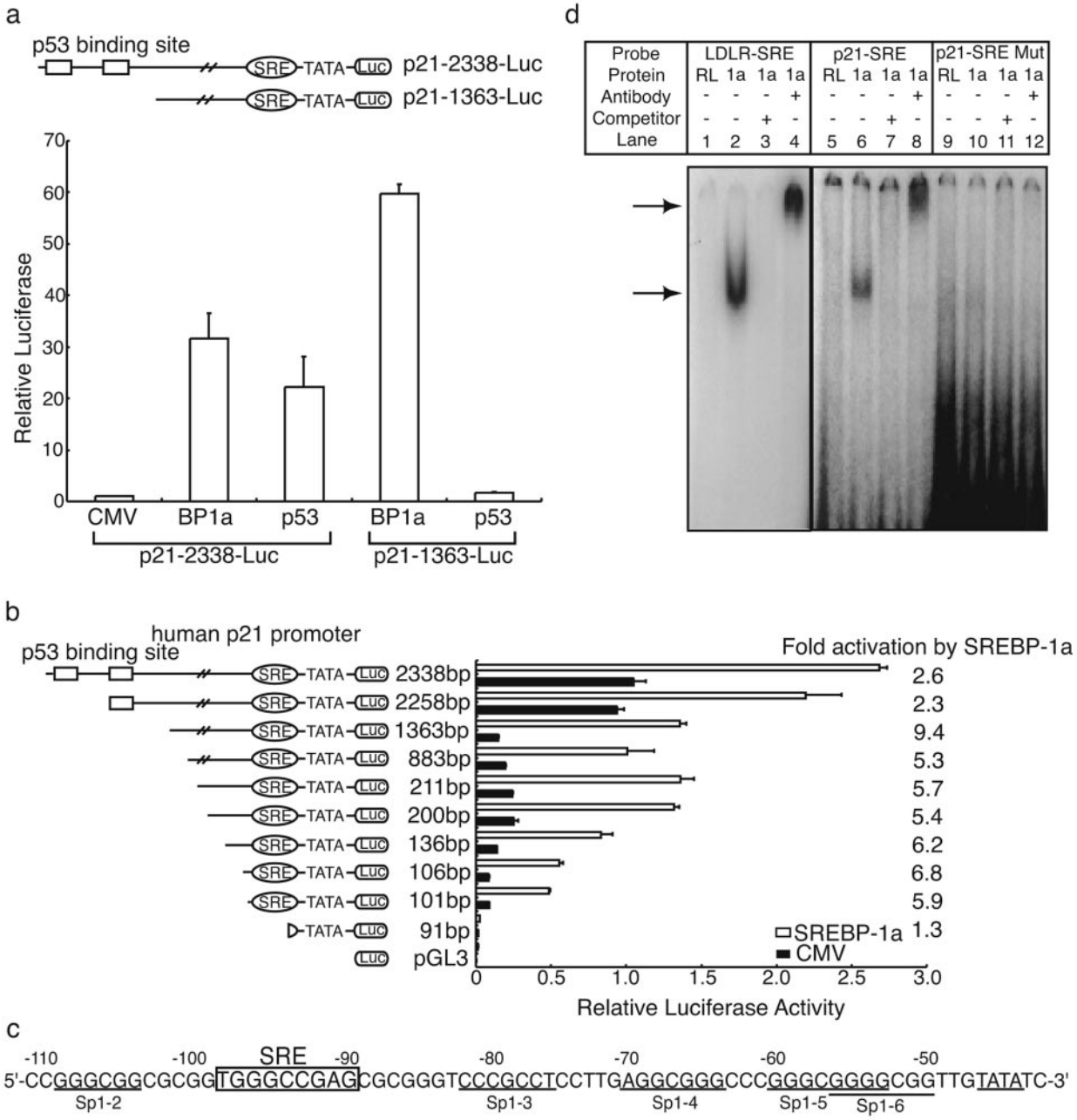

FIG. 1. Activation of p21 gene promoter by SREBP-1a. a. Activation of p21 promoter-luciferase by SREBP-1a and p53. Saos-2 cells, p53-deficient osteosarcoma cell line, were transfected with the human p21 promoter firefly luciferase plasmid (p21–2338–Luc or p21–1363–Luc), expression vector for nuclear SREBP-1a or p53, and a *Renilla* luciferase reference plasmid, pRL-SV40. The luciferase activity was measured and normalized to the *Renilla* luciferase activity after 24 h incubation. p21–2338–Luc contains and p21–1363–Luc lacks two p53-binding sites. b. Identification of an SREBP binding site (SRE) in p21 gene promoter. A series of deletion luciferase constructs for the human p21-promoter are indicated in the left panel. HepG2 cells, human hepatocellular carcinoma cell line, were transfected with the indicated reporter plasmid, a cytomegalovirus expression plasmid for SREBP-1a or empty plasmid, and a reference plasmid, pRL-SV40. Relative luciferase activity was measured after 24 h incubation. Fold activation represents ratio of values for SREBP-1a versus a cytomegalovirus empty vector. c. The SREBP binding site (p21-SRE) and its neighboring region in human p21 gene promoter. d. SREBP-1a directly binds to p21-SRE. Gel mobility shift assays was performed to show the direct binding of SREBP-1a to the p21 promoter. Double-stranded DNA fragments corresponding to the LDLR-SRE (1 to 4), the original binding site for SREBP, p21-SRE (5 to 8), and p21-SRE-Mut (9 to 12) where the GTGGGCCGAG was replaced by GTACAAAATG, labeled with [α-^{32}P]dCTP and incubated in the reaction mixture with (lanes 2 to 4, 6 to 8, and 10 to 12) or without (lane 1, 5, and 9) in vitro synthesized nuclear SREBP-1a protein. Specificity of SREBP-1a binding to the p21 SRE probe was confirmed by a supershift after the addition of SREBP-1 antibody (lane 8). In competition assays (Competitor), a 50-fold molar excess of an unlabeled SRE complex DNA (lane 7) was added to the incubation. RL, reticulocyte lysate; 1a, SREBP-1a.

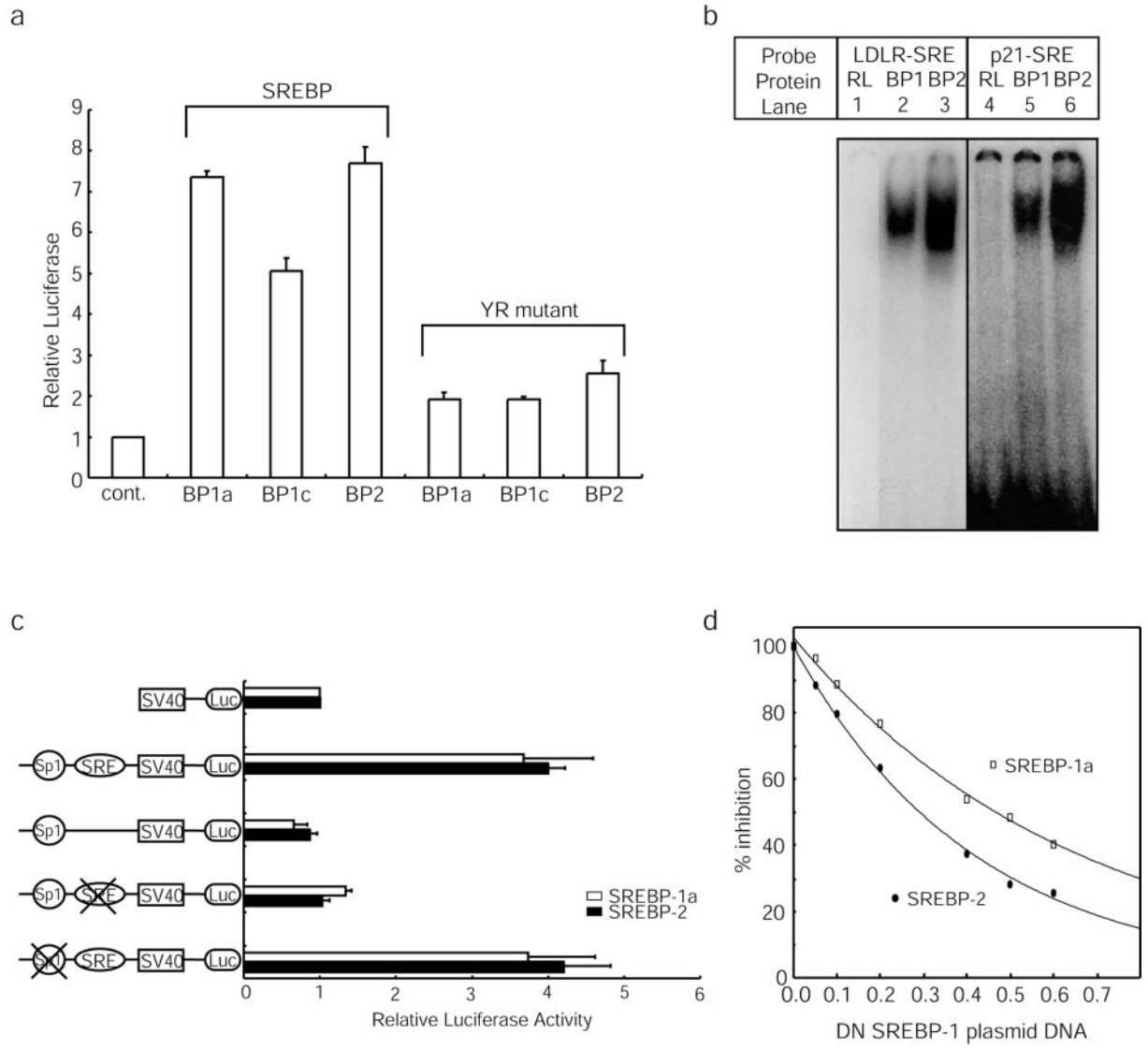

FIG. 2. Binding characteristics of SREBP isoforms to the p21 gene promoter. a. Effects of SREBP isoforms and YR mutants on the p21 promoter. Luciferase assays for the p21–2338–Luc were compared among nuclear SREBP isoforms, and their respective YR mutants (SREBP-1aM −1 cM and −2 M), in which the tyrosine residue in the basic region of each SREBP isoform was replaced by arginine. These mutants were capable to bind to E-boxes as an innate feature of basic helix-loop-helx protein, but not to the SRE sites, SREBP-specific binding sites (12). cont, cytomegalovirus empty vector; BP1a, SREBP-1a; BP1c, SREBP-1c; BP2, SREBP-2. b. Gel mobility shift assay of nuclear SREBP-1 and -2. A double-stranded DNA fragment corresponding to p21-SRE was labeled with [α-^{32}P]dCTP and incubated in the reaction mixture with (lanes 2, 3, 5, and 6) or without (lanes 1 and 4) in vitro synthesized nuclear SREBP-1a (1 to 3) or -2 (4 to 6) protein. Since SREBP-1a and -1c share the common basic helix-loop-helix domain, these data indicate that all the SREBP isoforms bind to the p21-SRE with similar affinities. c. The p21-SRE has an enhancer activity in the activation of p21 gene promoter by SREBPs. An SV40 promoter luciferase reporter for estimation of a region containing the upstream Sp1 site and p21-SRE as an enhancer was constructed as indicated. Deletion and mutation analysis were performed with reporter genes in the indicated positions of the promoter. HepG2 cells were transfected with each reporter plasmid, an expression plasmid for nuclear SREBP-1a or SREBP-2, reference plasmid pRL-SV40. Luciferase activity was measured and normalized to pRL-SV40 activity after 24 h incubation. Fold induction of luciferase activity by SREBPs is shown. d. Deletion of transactivation domain of SREBP-1 suppresses activation of p21 promoter by SREBP-1a and SREBP-2 in dominant negative fashion. SREBP-1 dominant negative form (DN-SREBP-1) lacks the amino-terminal transactivation domain (89 amino acids) and has the ability to bind to SRE site but inhibits transcriptional activities by native SREBPs for known SREBP target promoters (23). HepG2 cells were transfected with the p21–2338–Luc, SREBP expression vector (SREBP-1a or -2), and the indicated DNA amount of the dominant-negative SREBP-1 expression vector. Luciferase activity was measured after 24 h incubation.

activity of SREBP to SRE, but retains binding to the E-box as a general property of basic helix-loop-helix proteins (12). The YR mutation of SREBPs markedly reduced activation of the p21 promoter activity by SREBPs, suggesting that SREBPs bind to the p21-SRE in an SREBP-specific manner. This p21-SRE possessed an enhancer activity when it was put next to the simian virus promoter for the test of SREBP activation (Fig. 2c).

Previous reports demonstrated that coactivators such as Sp1 and NF-Y are essential for SREBP activation in the promoters

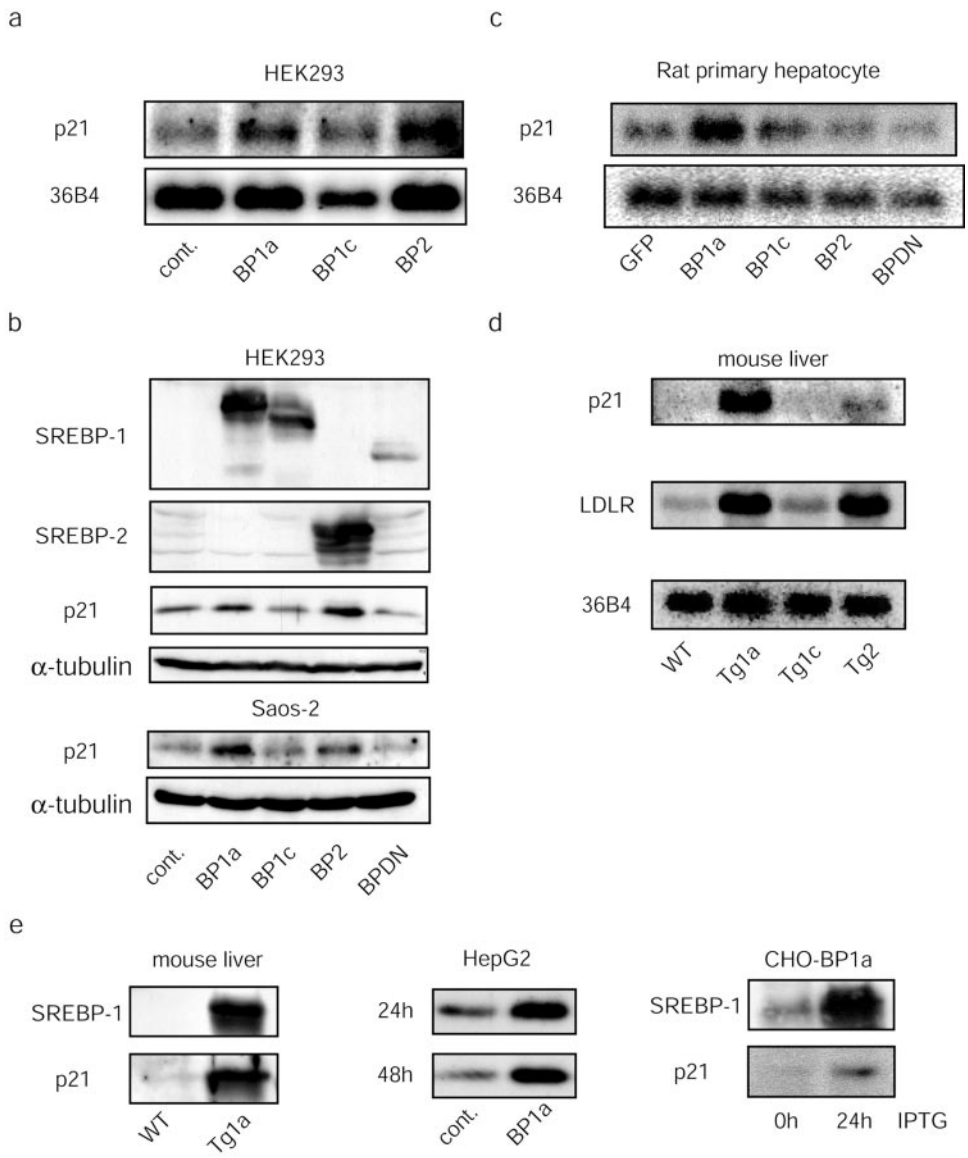

FIG. 3. SREBP-1a induces p21 at mRNA and protein levels. a. p21 mRNA induction by overexpression of SREBPs in HEK293 cells. HEK293 cells, human kidney cell line were transfected with the indicated cytomegalovirus promoter expression plasmids. Total RNA was isolated from cells 24 h after transfection for Northern blot analysis (10 μg per lane). cont, cytomegalovirus empty vector; BP1a, SREBP-1a; BP1cm, SREBP-1c; BP2, SREBP-2. b. p21 protein induction by overexpression of SREBPs in HEK293 and Saos-2 cells. The cells were transfected with indicated cytomegalovirus promoter expression plasmid. Total cellular proteins from cells, 24 h after transfection of indicated expression plasmids. The protein was subjected to immunoblot analysis for SREBP-1 and SREBP-2 and p21 (42.5 μg per lane). c. p21 mRNA induction by overexpression of SREBPs in hepatocytes. Rat primary hepatocytes were infected with control adenovirus expressing GFP or an adenoviral vector expressing SREBPs at an MOI of 100. Total RNA was isolated from cells 48 h after infection for Northern blot analysis (10 μg per lane). GFP, green fluorescent protein for controls; DNBP, dominant-negative form of SREBP-1 described in the legend to Fig. 2d. d. p21 gene expression in livers from SREBP transgenic mice. Mice overexpressing a nuclear active form of human SREBP-1a, SREBP-1c, and SREBP-2 in the liver were fed a high-protein/low-carbohydrate diet for a week and fasted overnight to induce the transgene under the phosphoenolpyruvate carboxykinase (PEPCK) promoter. Total liver RNAs were subjected to Northern blot analysis to estimate gene expression with the indicated cDNA probes. WT, wild-type; BP1a, SREBP-1a transgenic mice; BP1c, SREBP-1c transgenic mice; BP2, SREBP-2 transgenic mice. e. Cellular p21 protein level in SREBP-1a overexpression. In the left panel, nuclear extract proteins from livers of wild-type or SREBP-1a transgenic mice were subjected to immunoblot analysis with antibody against SREBP-1 and p21. In the center panel, total cellular proteins from HepG2, 24 or 48 h after transfection of SREBP-1a expression plasmid, were subjected to immunoblot analysis with antibody against p21 (40 μg per lane). In the right panel, CHO stable cell lines that inducibly express nuclear SREBPs under IPTG-regulated promoter (18) were incubated in 0.1 mM IPTG-containing medium. Each cell was harvested in NP-40 buffer at the indicated time. Proteins were subjected to immunoblot analysis for SREBP-1 and p21 (100 μg per lane).

of most of SREBP target genes (3) (26) (2). Although there are several Sp1 sites in the p21 promoter which were reported to be involved in p21 gene regulation (Fig. 1c), Sp1 was dispensable in SREBP activation of p21 promoter, as shown in deletion and mutation analyses (Fig. 2c). A dominant negative mutant of SREBP-1 in which the amino-terminal transactivation domain was deleted had no activity for the p21 promoter, and further inhibited activation of the p21-SRE construct by cotransfected native SREBP-1 or SREBP-2 in a dose-dependent manner (Fig. 2d). These data imply that the transactivation domain of SREBP-1 is essential for its activation of the p21 promoter, as reported in other regular SREBP target genes, presumably through recruitment of coactivators such as CBP/p330 (3).

Only SREBP-1a strongly and consistently induces p21 protein. Expression of p21 following SREBP activation of the p21 promoter was investigated. In 293 cells, both endogenous p21 mRNA and protein were detected by Northern and immunoblot analyses, respectively (Fig. 3a and b). After the transient expression, SREBP-1a and SREBP-2 but not SREBP-1c elevated p21 mRNA levels with resultant increases in cellular p21 levels. Induction of p21 protein by SREBP-1a and SREBP-2 expression was more prominent in p53-deficient Saos-2 cells (Fig. 3b). Conversely, overexpression of dominant-negative SREBP reduced endogenous p21 protein in these cell lines, confirming the specific SREBP activation of p21. These data suggest that overexpression of SREBP-1a and SREBP-2 can activate p21 expression in a manner consistent with the results from the luciferase assays. In contrast, when rat primary hepatocytes were infected with adenoviral SREBP expression vectors, only SREBP-1a caused appreciable activation of p21 expression (Fig. 3c).

As a model for sustained SREBP activation, hepatic expression of p21 was compared in livers overexpressing nuclear SREBPs from transgenic mice (22). SREBP-1a markedly increased hepatic p21 mRNA levels, consistent with DNA microarray results (Fig. 3d). The p21 protein was also markedly induced in SREBP-1a-expressing livers (Fig. 3e). Elevation of p21 protein by SREBP-1a was also observed in HepG2 cells after the transient transfection and stably inducible CHO cells (19). In contrast, constitutive activation of SREBP-2 only modestly elevated p21 expression in transgenic livers, although mRNA for LDLR, an authentic SREBP target gene, was fully activated in this transgenic line, whereas SREBP-1c was essentially inactive for p21 (Fig. 3d). These data suggest that although both SREBP-1a and SREBP-2 have similar transcription activities for the p21 promoter, there is differential posttranscriptional regulation of p21 depending upon SREBP isoform and cell type.

Nuclear SREBP-1a inhibits cell growth. To see the consequences of SREBP activation of p21 expression in cell growth, colony formation assays were performed in stably transfected Saos-2 cells. As shown in Fig. 4a, permanent transfection of a control vector containing the neomycin resistance gene generated a substantial number of surviving colonies in the presence of G418. Cotransfection of the p21 expression plasmid significantly decreased the number of resistant colonies, showing its inhibitory effect on cell growth. SREBP-1a and SREBP-2 exhibited an inhibitory effect slightly less than that of p21, whereas SREBP-1c and dominant-negative SREBP-1 had essentially no effect. These data suggest that p21 activation by SREBP-1a and SREBP-2 could cause cell growth inhibition.

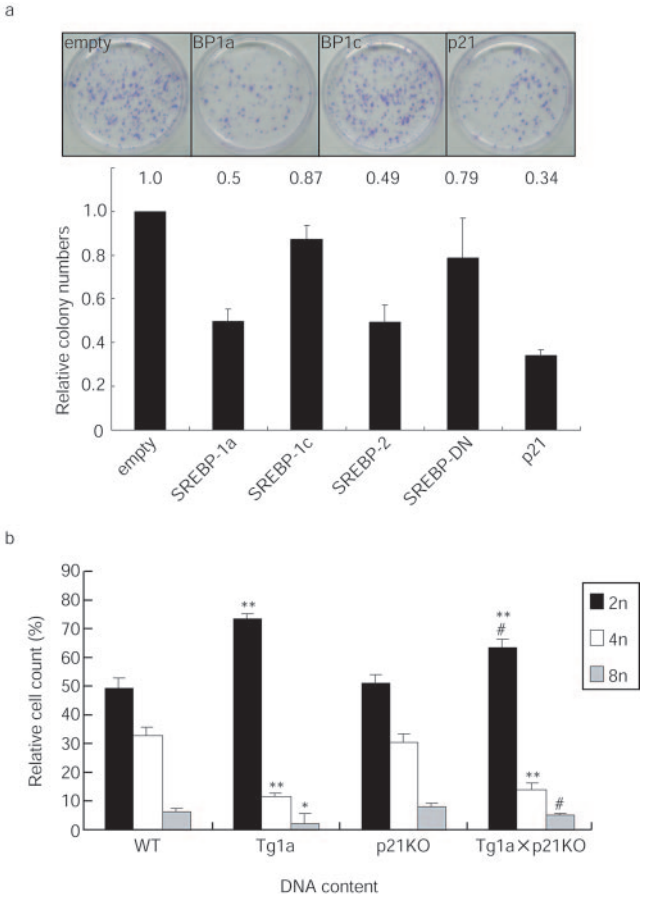

FIG. 4. Induction of p21 by SREBP-1a leads to cell growth arrest as evidenced by colony formation assays with Saos-2 cells (a) and hepatocyte polyploidy of SREBP-1a transgenic and p21 null mice (b). a. Saos-2 cells were transfected with 10 μg of the indicated expression plasmids containing neomycin resistance gene; 24 h after transfection, the cells were cultured in medium containing 500 μg/ml of G418 for 3 weeks. The resultant survival cell colonies were fixed and stained with violet blue as representatively indicated in the upper panel. The values represent relative colony numbers normalized to an empty vector control. b. The hepatocytes were prepared for livers from the indicated mouse lines. The DNA content was determined by flow cytometry after DNA staining with propidium iodide. Hepatocyte ploidy is shown as 2n, 4n, and 8n. WT, C57BL/6J; Tg1a, SREBP-1a transgenic mice; p21KO, p21 knockout mice; Tg1a×p21KO, SREBP-1a transgenic and p21 knockout double mutant mice. **, $P < 0.01$, and *, $P < 0.05$ versus wild-type; #, $P < 0.05$ versus SREBP-1a transgenic mice (Student's *t* test).

The effects of p21 on cell cycle were also estimated in the livers of SREBP-1a transgenic mice. As shown in Fig. 4b, flow cytometry demonstrated that a considerable proportion of normal hepatocytes exhibit polyploidy (4n and 8n). The absence of p21 did not changes these patterns of polyploidy in normal hepatocytes. Compared to control livers, SREBP-1a expression caused a significant increase in 2n cells, indicating the G_0/G_1 stage, with concomitant decreases in 4n and 8n cells, supporting increased activity of p21 for G_1 arrest induced by SREBP-1a. p21 contributed roughly 50% to these changes of polyploidy by SREBP-1a, as estimated in transgenic SREBP-

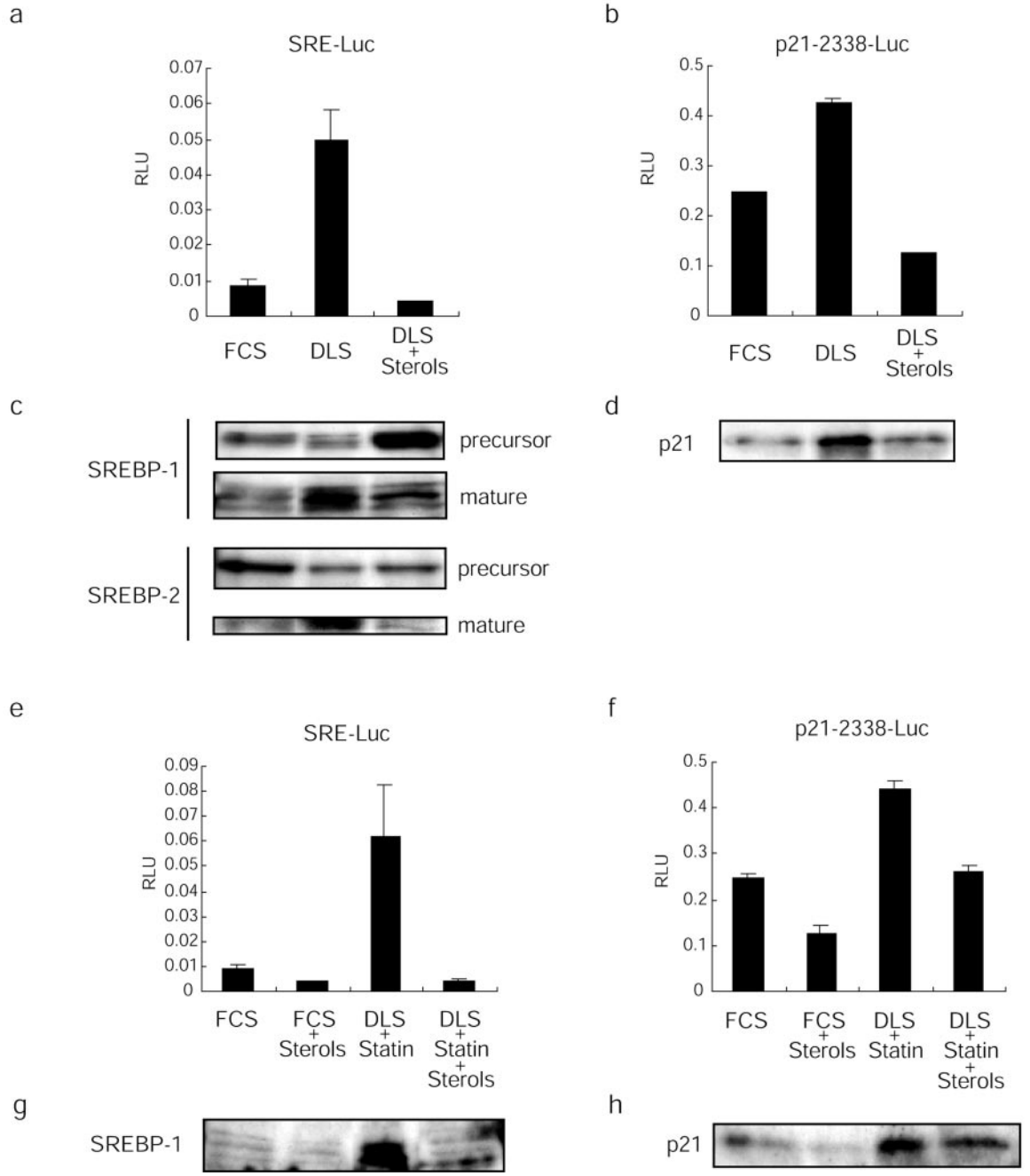

FIG. 5. Activation of p21 promoter and induction of p21 protein by endogenous SREBPs in Saos-2 cells. Saos-2 cells were transfected with each reporter plasmid, SRE-Luc (a and c) or p21–2338–Luc (b and d), a reference plasmid pRL-CMV, and incubated at the indicated conditions to activate endogenous SREBPs by delipidated serum (a to d) and further pravastatin, an HMGCoA reductase inhibitor (e to h). Luciferase activity was measured and normalized to pRL-CMV activity after 24 h incubation (a, b, e, and f). Total cellular (40 μg) (c and d) and nuclear (10 μg) (g and h) proteins were subjected to immunoblot analysis for SREBP-1 and SREBP-2 (c and g), and p21 (d and h). FCS, 5% fetal calf serum; DLS, 5% delipidated serum; statin, 50 μM pravastatin; sterols, 10 μg/ml cholesterol and 1 μg/ml 25-hydroxychoresterol.

1a/p21-knockout double mutant mice. These data provide supporting evidence that SREBP-1a overexpression could activate p21 and could affect cell proliferation in vivo as well as in cultured cells.

Endogenous SREBP-1a regulates p21 expression. To test the physiological relevance to SREBP regulation of p21, the effects of endogenous SREBPs on p21 expression were estimated. Saos-2 cells were cultured in delipidated serum and further treated with pravastatin, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor. These treatments are known to increase endogenous nuclear SREBP-1a and SREBP-2 through activation of the SCAP/INSIG system. As shown by luciferase assays in Fig. 5a, the SRE-Luc reporter as an indicator of endogenous SREBPs was strongly activated by delipi-

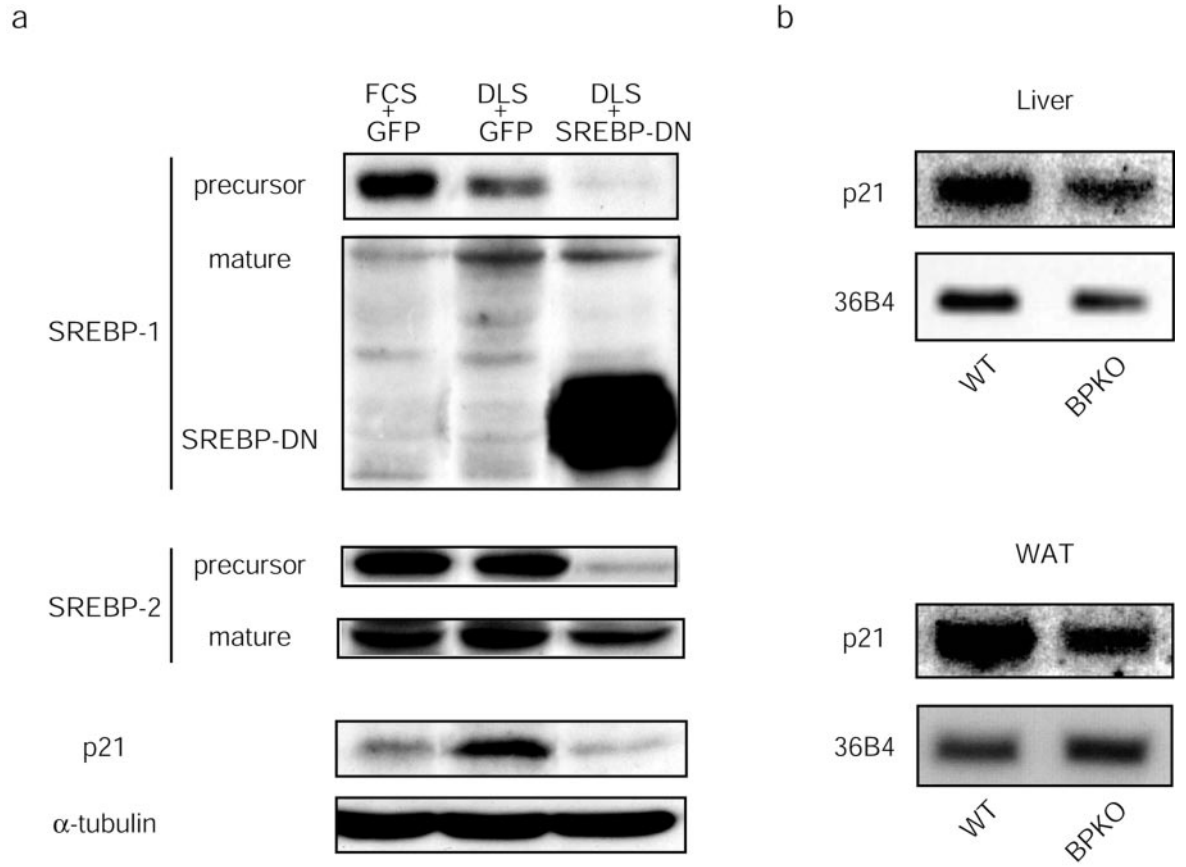

FIG. 6. Disruption of endogenous SREBPs suppressed p21 expression. a. Saos-2 cells infected with control adenovirus expressing GFP or an adenoviral vector expressing dominant-negative SREBP-1 at a multiplicity of infection of 30. Total cellular proteins were isolated from cells incubated at the indicated conditions 24 h after infection. Total proteins (35 μg) were subjected to immunoblot analysis for SREBP-1, SREBP-2, and p21. b. Northern blot analysis was performed to measure p21 mRNA in livers and white adipose tissue (WAT) from wild-type (WT) and SREBP1 knockout (BPKO) mice. The animals were refed a high-sucrose/fat-free diet for 12 h after 24 h of starvation.

dated serum. Addition of sterols (25OH cholesterol and cholesterol) completely cancelled this activation. With a similar pattern, the p21 promoter was also activated and repressed by sterol deprivation and supplementation, respectively (Fig. 5b).

In the same set of experiments using immunoblot analysis, it was shown that p21 protein was consistently regulated in accordance with changes in endogenous nuclear SREBP-1a and SREBP-2 proteins depending upon cellular sterol states (Fig. 5c and d). Treatment with pravastatin, another manipulation for sterol deprivation, and sterols provided similar results (Fig. 5e to h). The regulation of p21 promoter activity by endogenous SREBPs by delipidated serum, statin, and sterols was also observed in 293 cells (data not shown). Endogenous SREBPs were responsible for the induction of p21 by incubation in delipidated serum because adenoviral overexpression of the dominant-negative form of SREBP-1 cancelled the induction of p21 observed in adenovirus-green fluorescent protein-infcted Saos-2 cells incubated in delipidated serum (Fig. 6a). These data demonstrate that endogenous SREBPs can regulate p21 promoter in a physiological range of sterol regulation. Finally, p21 expression was significantly decreased in livers and adipose tissue of SREBP-1 null mice compared to wild-type mice, providing more support that SREBP-1 can participate in endogenous p21 expression (Fig. 6b and c).

DISCUSSION

The current studies first demonstrate that the cyclin-dependent kinase inhibitor p21 is an SREBP target gene. SREBPs activate the p21 promoter as strongly as p53. The p21 mRNA promoter contains a new SREBP binding site (p21-SRE). Promoter analysis with mutated SREBPs and DNA probes supports that p21 activation is mediated through transcriptional activity of SREBPs by binding to this site. Although both SREBP-1a and SREBP-2 highly activate the p21 promoter, there is a discrepancy in the resultant elevation of p21 mRNA and protein levels depending upon the experimental conditions and cell types. Only SREBP-1a strongly and consistently activated p21 protein in liver. To sustain high p21 expression, it is likely to require some posttranscriptional regulation where SREBP-1a but not other isoforms could stabilize p21 mRNA and protein levels.

The importance of posttranscriptional regulation of p21 has

been recognized, especially with regard to the involvement of mitogen-activated protein kinases in stabilization of p21 (15). Supportively, we observed that induction of SREBP-1a and not SREBP-2 activates ERK1/2 (p42/44) in the inducibly expressing CHO cell line, which might contribute to high p21 protein level in the CHO-SREBP-1a line (data not shown). Neither p38 nor JNK was changed in these cells. Further studies are needed to clarify the precise mechanism by which nuclear SREBPs determine the p21 protein level.

The physiological relevance to p21 activation of SREBP-1a is yet to be fully clarified. Reduced p21 expression in SREBP-1-deficient mice suggested that SREBP-1 could physiologically contribute to p21 regulation. The data from cultured cells in conditions of lipid deprivation demonstrated that SREBP activation of p21 can occur in a physiological range of sterol regulation. For cellular cholesterol homeostasis, nuclear SREBP-2 is a key player that is strictly regulated by the sterol-regulated cleavage system composed of SCAP/INSIG (30) depending upon the cellular cholesterol balance. However, actively growing cells could still be in danger of a deficiency of lipids, where SREBP-1a should be activated to support SREBP-2 and in some severe conditions could transiently sustain cell growth at G_1 for lipid synthesis through p21 activation. This hypothesis is applicable to cells and tissues highly expressing SREBP-1a: cultured cells, embryonic tissues, adult intestine, and lymphoid tissues.

SREBP-1a is partly under control of the sterol-sensing SCAP/INSIG system, but seems to be activated independently, which suggests the presence of an unidentified regulatory function(s) of SREBP-1a other than sterol regulation. In contrast, SREBP-1c is the major SREBP-1 isoform in differentiated tissues and not in cultured cells, is nutritionally regulated, and controls synthesis of fatty acids and triglycerides (9, 20). p21 was not induced by this nutritional regulator.

p21 gene is also known as a p53 target, induced by DNA damage such as radiation and UV (6). One potential mechanism to be considered is that accumulation of lipids in cells caused by SREBP-1a could be a cellular stress to induce p53 and thus p21. We recently reported that p53 and p21 are activated in nutritionally dysregulated tissues such as in enlarged adipose tissues and fatty livers of leptin-deficient ob/ob mice (28, 29). In fatty livers from mutant mice with different genotypes of leptin and SREBP-1 genes, there was a strong correlation between hepatic triglyceride and p21 mRNA levels. This hepatosteatosis-associated mechanism, presumably related to some stress signal, could contribute to the marked induction of p21 in SREBP-1a transgenic fatty livers in addition to the direct and p53-independent induction by SREBP-1a.

p21 is also induced by contact inhibition and differentiation, and numerous factors such as STAT family, C/EBP, retinoic acid receptor, vitamin D receptor, MyoD, Sp1, Sp3, AP2, and calcitonin have been identified by extensive promoter analysis (reviewed in reference 7). In this aspect, activation of p21 by SREBP-1a is unique because SREBP-1a is linked to growth, which is opposed to the generally accepted functions of p21. In our preliminary data (M. Nakakuki and H. Shimano, unpublished observation), nuclear SREBP-1a causes G_1 cell cycle arrest and inhibits cell proliferation through diverse mechanisms which are currently under investigation. Activation of p21 as a new SREBP target at least partially explains this unexpected antiproliferative activity of SREBP-1a. Our current data imply that lipid synthesis is not simply under regulation of growth, but is actively involved in the regulation of cell growth. Further investigations on the link between lipid synthesis and cell growth, especially in the light of the cell cycle, should be pursued.

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