Sterol-independent, sterol response element-dependent, regulation of low density lipoprotein receptor gene expression

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Abstract **Stimulation with phorbol 12-myristate 13-acetate (PMA) and the calcium ionophore ionomycin increased native low density lipoprotein (LDL) receptor gene expression in the human leukemic T cell line Jurkat when cells were cultured in the absence of sterols and also increased nuclear accumulation of sterol regulatory element binding protein (SREBP)-1. PMA and ionomycin likewise increased LDL receptor mRNA levels when cells were cultured in the presence of suppressive concentrations of sterols, when neither SREBP-1 nor SREBP-2 was detectable in the nucleus. These findings indicated that mitogen-induced up-regulation of the LDL receptor gene could be independent of sterol-regulated transcription factors. The involvement of sterol regulatory element (SRE)-1 was analyzed by transfection of LDL receptor promoter constructs. Promoter fragments of either the 5′ 1472 or 142 base pairs induced reporter gene expression after mitogenic stimulation when cells were cultured in the absence or presence of sterols. Mutation of the SRE-1 sequence in either construct abolished sterol-mediated regulation of transcription. However, mutation of the SRE-1 sequence in the 1472 base pair promoter fragment did not alter mitogenic induction of transcription, whereas mutation of SRE-1 in the 142 base pair promoter fragment completely prevented up-regulation of transcription. Taken together, these results demonstrate that the LDL receptor promoter contains at least one 5′ SRE-independent as well as an SRE-dependent response element. Furthermore, the data suggest that the SRE-dependent response may not involve the action of either SREBP-1 or -2. Thus, mitogen-induced transcription of the LDL receptor promoter is regulated by diverse sterol-independent mechanisms.—** Makar, R. S. J., P. E. Lipsky, and J. A. Cuthbert. **Sterol-independent, sterol response element-dependent, regulation of low density lipoprotein receptor gene expression. *J. Lipid. Res.*** 39: **1647–1654.**

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Studies of the regulation of low density lipoprotein (LDL) receptor gene expression have generally centered on the mechanism of sterol-mediated feedback repression of gene transcription. A model system has evolved that attempts to account for all alterations in LDL receptor gene expression as a function of cellular sterol balance (1–3 and references therein). However, in several different cell lines, a variety of non-sterol signaling molecules, including growth factors, cytokines, and calcium ionophores, can also induce LDL receptor gene expression (4–10). For example, mitogenic activation of resting human T lymphocytes increases LDL receptor mRNA levels and LDL receptor activity (6, 11). Similarly, stimulation of the human leukemic T cell line Jurkat with the mitogenic combination of the phorbol ester, phorbol 12-myristate 13-acetate (PMA), and the calcium ionophore, ionomycin, activates LDL receptor gene expression (10). Of importance, the induction of LDL receptor gene expression in Jurkat cells by PMA in combination with ionomycin is not prevented by exogenous sterols, suggesting that LDL receptor gene transcription in T cells, and perhaps in many other cell types, can be regulated by non-sterol signals independent of ambient sterol concentrations.

Studies with reporter constructs transfected into Jurkat cells demonstrated that a 6.5 kb fragment from the 5′ promoter region of the LDL receptor gene was sufficient to mediate gene transcription in response to non-sterol stimuli (10). As this promoter fragment contains the sterol regulatory element (SRE)-1, which is required for cholesterol-mediated feedback repression of LDL receptor gene expression, a role for the SRE-1 sequence in non-sterol regulation of LDL receptor gene expression could not be excluded. In order to identify the regions of the 6.5 kb promoter fragment required for induction of LDL receptor gene expression by mitogenic stimulation and to define the precise role of SRE-1, experiments with different

Abbreviations: BCS, bovine calf serum; CAT, chloramphenicol acetyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LDL, low density lipoprotein; LPP, lipoprotein-poor plasma; PMA, phorbol 12-myristate 13-acetate; SRE, sterol regulatory element; SREBP, sterol regulatory element binding protein.

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LDL receptor promoter–reporter gene constructs were carried out. Transfection analysis of both wild-type LDL receptor promoter fragments and promoter fragments containing a mutated SRE-1 sequence revealed that the LDL receptor promoter contains two *cis*-acting mitogen-inducible elements. One element, within the 142 base pair promoter fragment, is dependent on an intact SRE-1 sequence for mitogenic induction. This SRE-dependent response, however, does not require nuclear localization of SREBPs, suggesting the existence of alternative mechanisms of mitogen-induced SRE-dependent responses. The second element, located upstream of the 5′ 142 base pairs, functions independently of SRE-1. These findings indicate that mitogenic stimuli and ambient sterol concentrations induce LDL receptor gene transcription through distinct, as well as interactive, mechanisms.

MATERIALS AND METHODS

Cell preparation and culture

Jurkat cells were maintained in RPMI-1640 medium (Gibco BRL Life Technologies, Gaithersburg,MD) containing gentamicin (10 μg/ml), penicillin G (200 units/ml), and 10% v/v iron-supplemented bovine calf serum (Hyclone Laboratories, Logan, UT). For experiments examining sterol regulation, cells were cultured in lipoprotein-depleted medium (supplementation of RPMI-1640 medium with either lipoprotein-depleted serum or lipoprotein-poor plasma (12)) with or without 0.5 μg/ml 25-hydroxycholesterol (Steraloids Inc., Wilton, NH) and 10 μg/ml cholesterol (Eastman Kodak Co., Rochester, NY) dissolved in ethanol for 48 hours. The mature (nuclear) forms of SREBPs were markedly decreased 2 h after addition of this combination of suppressive sterols (1). In some experiments, the cells were initially incubated in RPMI-1640 medium supplemented with 10% v/v human serum (200 μg cholesterol/ml) before being cultured in lipoprotein-depleted medium. Cells were harvested after varying lengths of incubation and assayed for CAT activity. For experiments examining mitogen responses, cells maintained in medium containing 10% v/v bovine calf serum were stimulated for 2 h with 10 ng/ml PMA (Calbiochem, San Diego, CA) and 2.5 μM ionomycin (Calbiochem).

Vector construction

The vectors 1472-CAT and 142-CAT were constructed by inserting *Hind*III fragments from the LDL receptor promoter isolated from the plasmids pLDLR-CAT 1563 and pLDLR-CAT 234 (13) into the *Hind*III site of pML4 (10). The vectors contain 1472 and 142 base pairs, respectively, from the LDL receptor promoter and each contains 36 base pairs from exon 1. The orientation of each promoter fragment was confirmed by restriction mapping or DNA sequencing.

Mutations in SRE-1 (**Fig. 1** and reference 14) were generated using the technique of Kunkel, Roberts, and Zakour (15). Briefly, the LDL receptor promoter fragment from 1472-CAT was subcloned into the *Hind*III site of M13mp19 and the orientation of the insert was determined by DNA sequencing. The M13mp19 was then transformed into *Escherichia coli* dut$^-$ ung$^-$ strain CJ236 (Muta-Gene M13 *In Vitro* Mutagenesis kit, Bio-Rad Laboratories, Hercules, CA) and single-strand M13mp19 DNA was isolated. A mutant oligonucleotide incorporating 9 mismatches in the central 10 bases of SRE-1, that resulted in the formation of a new *Cla*I site, was synthesized (Applied Biosystems Inc., division of Perkin-Elmer Cetus, Emoryville, CA), treated with T4 polynucleotide kinase (Pharmacia Biotech Inc., Piscataway, NJ), annealed and used for an in vitro DNA replication reaction catalyzed by T7 DNA polymerase and T4 DNA ligase. The *E. coli* strain TG-1 was

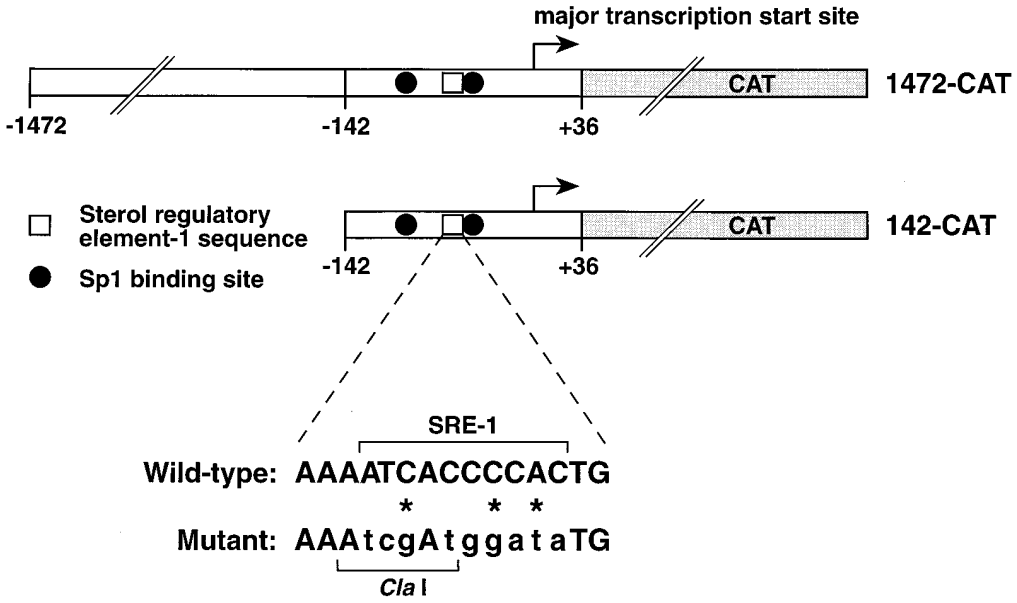

Fig. 1. Structure of human LDL receptor promoter–CAT constructs. Two fragments of the human LDL receptor promoter were subcloned into the plasmid pML4. The fragments share the same 3′ sequences but extend different lengths 5′. The arrow indicates the position of the major transcription start site. The locations of the two Sp1 binding sites (also called repeats 1 and 3) and the SRE-1 sequence (also called repeat 2) are indicated. Mutations introduced into the wild-type SRE-1 are indicated by lower case letters. Point mutations of each of the bases noted by an asterisk in the wild-type SRE-1 abolish sterol regulation in Chinese hamster ovary cells (14).

transformed with the resulting double-stranded DNA and the replicative form of the mutated M13mp19 was isolated and analyzed by restriction mapping. The entire promoter fragment containing the appropriate mutation was subcloned into pML4 (mutant SRE-1 1472-CAT) and sequenced (Applied Biosystems 373A DNA sequencer) to verify that only the SRE-1 was mutated. An *Eag I* fragment of mutant SRE-1 1472-CAT containing the SRE-1 mutation was isolated and used to generate mutant SRE-1 142-CAT by replacing the same fragment in wild-type SRE-1 142-CAT. The orientation of this promoter fragment was verified by restriction analysis.

Preparation of transfectants

Jurkat T cells were transfected by electroporation, as previously described (16). Briefly, cells were washed, resuspended in RPMI-1640, and mixed with 20 μg of linearized plasmid DNA in a Gene Pulser electroporation cuvette (Bio-Rad). Electroporations were conducted with a Gene Pulser apparatus (Bio-Rad) at 0.25 kV and 960 μF. Twenty-four hours after electroporation, G418 (Gibco Life Technologies, Grand Island, NY) was added to the transfected cells (final concentration 2 mg/ml for the first week, 1 mg/ml thereafter) and cultures were maintained in G418 until resistant cells grew out, usually after 3–4 weeks.

Measurement of CAT activity

Transfected cells were washed in Dulbecco's phosphate-buffered saline (Sigma Chemical Co., St. Louis, MO), resuspended in 0.25 M Tris-Cl (pH 7.8), and whole cell lysates were prepared by three cycles of freeze-thawing. After removing cellular debris by centrifugation at 14,000 *g* for 5 min at 4°C, the cleared supernatant was incubated for 10 min at 65°C and centrifuged again at 14,000 *g* for 5 min at 4°C. Protein content of the cell lysates was determined using Bradford reagent (Bio-Rad) and aliquots (0.2–20 μg of protein) were incubated for 2 h at 37°C in a standard CAT assay (17) in a final volume of 0.15 ml containing 0.53 mM acetyl CoA and 0.35–0.7 nCi of [^{14}C] chloramphenicol (~60 mCi/mmol) (DuPont NEN, Boston, MA). After the incubation, the acetylated reaction products were extracted in ethyl acetate, chromatographed on Polygram Sil G silica gel plates (Brinkmann Instruments, Inc., Westbury, NY) using a chloroform–methanol (95:5, v/v) solvent system, and the fraction of radioactive acetylated versus unacetylated reaction products was quantitated using the AMBIS 100 radioanalytical scanner. Preliminary experiments confirmed the linearity of the assay system over the incubation time for the amount of protein used.

Measurement of mRNA by nuclease protection

Total RNA was isolated from Jurkat T cells solubilized in a solution of guanidinium thiocyanate (Fluka Chemical Corp., Ronkonkoma, NY). RNA was extracted from the guanidinium thiocyanate cell lysate using acid phenol-chloroform as detailed by Chomczynski and Sacchi (18). Single-stranded cDNA probes for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), LDL receptor, and CAT were prepared as previously described (10). Single-stranded ^{32}P-labeled probes were synthesized with an oligonucleotide primer in the presence of 0.75 μM [α-^{32}P]dCTP (~3000 Ci/mmol, Amersham Co., Arlington Heights, IL), unlabeled dCTP (0.75 μM for CAT, 1.5 μM for LDL receptor, ≥270 μM for GAPDH probes), 0.1 mM dATP, dTTP, dGTP, and the Klenow fragment of *E. coli* DNA polymerase. The sizes of the probes (including M13 sequences) were: GAPDH = 598 nucleotides, LDL receptor = 337 nucleotides, CAT = 319 nucleotides. Total RNA (10–40 μg) was hybridized with ^{32}P-labeled probes as described. Hybridization was carried out overnight at 37°C (CAT) or 48°C (LDL receptor), after which the samples were digested with 5 units mung bean nuclease/10 μg total RNA (Gibco BRL) and separated by gel electrophoresis as detailed (19). The [^{32}P]cDNA content was analyzed with an AMBIS 100 radioanalytical scanner (10). In the experiments described, results are presented as relative CAT or LDL receptor mRNA levels, normalized using the amount of GAPDH mRNA to correct for procedural losses, and expressed as fold induction compared to unstimulated control cells calculated by the following formula: GAPDH-normalized CAT or LDL receptor mRNA levels in mitogen-stimulated cells/GAPDH-normalized CAT or LDL receptor mRNA levels in control unstimulated cells.

Isolation of nuclear SREBPs

For assays of the levels of nuclear SREBPs, cells were processed as described (20, 21) with minor modifications. Briefly, cells were harvested in phosphate-buffered saline containing protease inhibitors (50 μg/ml calpain inhibitor I, 10 μg/ml leupeptin, 2.2 μg/ml aprotinin, 5 μg/ml pepstatin, and 0.5 mM phenylmethylsulfonyl fluoride) and 0.5 mM dithiothreitol, centrifuged at 3,000 rpm for 5 min and the pellet was resuspended in hypotonic buffer (10 mM HEPES-KOH pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.5 mM dithiothreitol) supplemented with protease inhibitors. After incubation on ice, cells were sheared by multiple passages through a 23-gauge needle followed by a 27-gauge needle, centrifuged at 6,000 rpm for 5 min and the nuclear pellet was resuspended in high salt buffer (0.4 M NaCl, 20 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 25% glycerol) containing all protease inhibitors. After incubation with gentle agitation for 30–60 mins at 4°C, extracted nuclear proteins were separated by centrifugation at 100,000 *g* for 30 min to pellet other nuclear material. Protein concentration was measured using Bradford reagent (Bio-Rad) and mature SREBP nuclear proteins were analyzed by immunoblotting.

Immunoblotting for SREBPs

Aliquots of nuclear extract (25–75 μg protein) were solubilized in SDS sample buffer, then individual proteins and pre-stained molecular weight markers (Bio-Rad) were separated by SDS-PAGE (8%) and transferred to PVDF membranes (Immobilon-P, Millipore Corp., Bedford, MA). SREBP-1 was identified by immunoblotting with mouse monoclonal anti-SREBP-1 antibody (generously provided by Drs. Michael Brown and Joseph Goldstein, UT Southwestern) against amino acid residues 301–407 of human SREBP-1a (22). This antibody recognizes all splice variants of SREBP-1 as residues 301–407 are common (23). Bound antibody was detected with affinity-purified horseradish peroxidase-conjugated sheep anti-mouse IgG and enhanced chemiluminescence (ECL, Amersham International, UK) as previously detailed (24). SREBP-2 was identified by immunoblotting with rabbit polyclonal anti-SREBP-2 antibody (generously provided by Drs. Michael Brown and Joseph Goldstein, UT Southwestern) against amino acid residues 48–403 of human SREBP-2 (25, 26). Bound antibody was detected with affinity-purified horseradish peroxidase-conjugated donkey anti-rabbit IgG and ECL (Amersham).

Statistical analysis

Statistical analyses were performed with the two-tailed Student's *t*-test for paired (unstimulated versus stimulated) or unpaired (all other comparisons) observations, as appropriate.

RESULTS

LDL receptor mRNA increased ~2.5-fold after PMA and ionomycin stimulation of Jurkat T cells cultured in lipo-

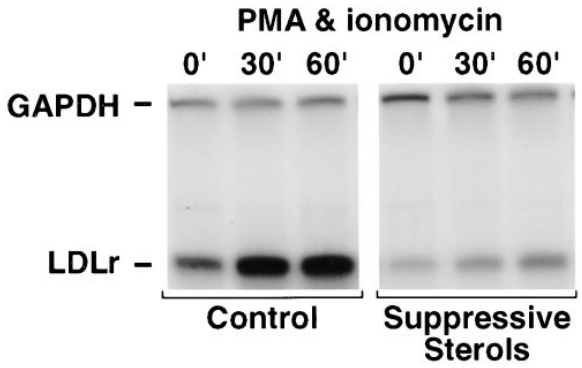

Fig. 2. LDL receptor mRNA increases in Jurkat T cells after stimulation with PMA and ionomycin regardless of sterol regulation. Jurkat T cells cultured in lipoprotein-deficient medium, without (control) or with supplementary suppressive sterols (25-hydroxycholesterol 0.5 μg/ml, cholesterol 10 μg/ml), for 48 h were stimulated with PMA and ionomycin for varying lengths of time. Total RNA was prepared and LDL receptor mRNA levels were quantified by nuclease protection assay as detailed in Materials and Methods.

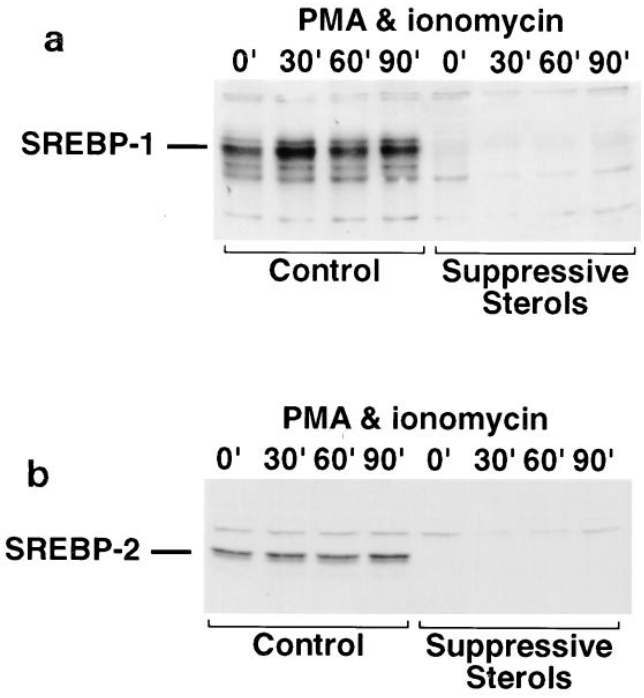

Fig. 3. Stimulation with PMA and ionomycin induces an increase in the abundance of nuclear SREBP-1 but not SREBP-2. Jurkat T cells cultured in lipoprotein-deficient medium, without (control) or with supplementary suppressive sterols (25-hydroxycholesterol 0.5 μg/ml, cholesterol 10 μg/ml), for 48 h were stimulated with PMA and ionomycin for varying lengths of time (zero to 90 minutes). Nuclear extracts were prepared and SREBPs were detected by immunoblotting as detailed in Materials and Methods. a: Detection of SREBP-1. Similar results were obtained in two other experiments (data not shown). b: Detection of SREBP-2. Similar results were obtained in two other experiments (data not shown).

protein-depleted medium (**Fig. 2**). LDL receptor mRNA levels in unstimulated Jurkat T cells were decreased by 80% after incubation with suppressive sterols (25-hydroxycholesterol 0.5 μg/ml and cholesterol 10 μg/ml). Despite this diminution, stimulation with PMA and ionomycin increased LDL receptor mRNA levels by 1.7-fold.

To examine the impact of mitogen stimulation on nuclear localization of SREBPs, Jurkat T cells, incubated with or without suppressive sterols, were harvested 30, 60, or 90 min after stimulation with PMA and ionomycin and nuclear extracts were prepared. As seen in **Fig. 3a**, SREBP-1 was detected in unstimulated cells incubated in lipoprotein-depleted medium. There was a modest increase (<2-fold) in the abundance of SREBP-1 after stimulation with PMA and ionomycin. When nuclear extracts from cells incubated with suppressive sterols were assayed, there was no detectable SREBP-1 regardless of mitogenic stimulation (Figure 3a). Increasing the amount of nuclear extract protein analyzed (to 75 μg) did not reveal SREBP-1 (data not shown).

SREBP-2 was readily detected in nuclear extracts from control, unstimulated Jurkat T cells (Fig. 3b). There was no appreciable increase in the abundance of SREBP-2 with mitogenic stimulation. In cells cultured in medium supplemented with suppressive sterols, SREBP-2 was markedly diminished or undetectable and did not change with mitogenic stimulation. Thus, neither SREBP-1 nor SREBP-2 was detectable in Jurkat T cells cultured in medium supplemented with suppressive sterols. These results suggest that the increase in LDL receptor mRNA levels in such cells after mitogenic stimulation was independent of SREBPs and sterol regulation.

In order to identify the sequences in the LDL receptor promoter that were required for sterol-independent regulation, the next series of experiments used Jurkat T cells transfected with fragments of the LDL receptor promoter fused to the reporter gene CAT. Two different sized promoter fragments sharing identical 3′ termini, but extending either 1472 or 142 base pairs 5′ of the transcription start site, were analyzed. The behavior of each LDL receptor promoter fragment was studied in the presence of a wild-type or mutant SRE-1 sequence (Fig. 1). For each promoter–CAT construct, several stable transfectants were independently generated and analyzed in these studies. Initial experiments were conducted to verify that the SRE-1 mutation completely disrupted sterol regulation. Transfectants containing either a wild-type or mutant SRE-1 LDL receptor promoter–CAT construct were initially incubated in medium supplemented with 10% v/v human serum (200 μg cholesterol/ml) to down-regulate CAT expression. Cells were next washed extensively to remove lipoprotein cholesterol and then cultured in medium supplemented with 10% v/v lipoprotein-deficient serum, with or without exogenous sterols (25-hydroxycholesterol 0.5 μg/ml and cholesterol 10 μg/ml). Cells were harvested at different time points and CAT activity was assayed in whole cell lysates.

As shown in **Fig. 4a**, removal of exogenous sterols resulted in a time-dependent induction of CAT activity in Jurkat T cells transfected with the wild-type SRE-1 1472–CAT construct. When these wild-type transfectants were

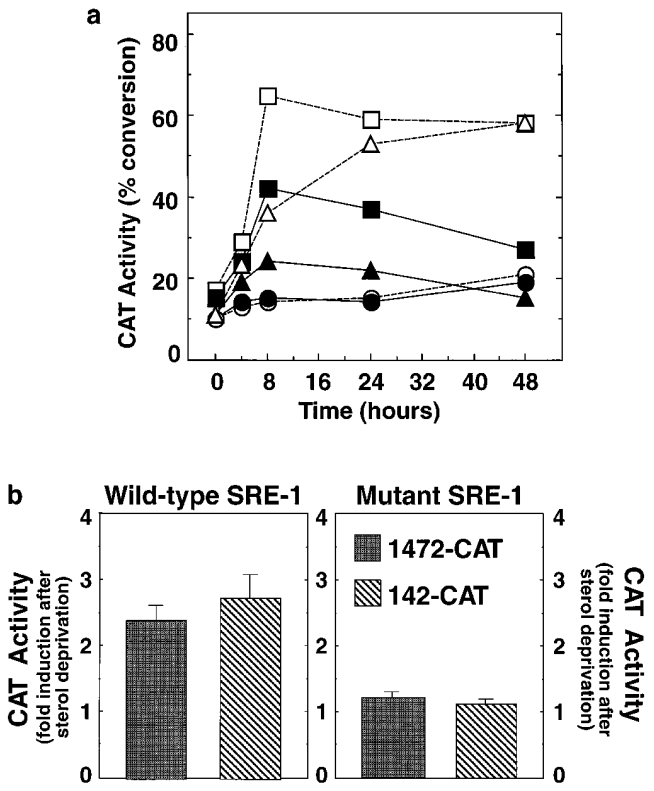

Fig. 4. SRE-1 mutation prevents induction of CAT activity by sterol depletion. a: Stable 1472–CAT transfectants containing either a wild-type (□, ■ and △, ▲) or mutant (○, ●) SRE-1 construct were cultured for 3 days in medium containing 10% human serum (200 μg cholesterol/ml). The cells were then washed and cultured in lipoprotein-deficient medium in the presence (solid symbols) or absence (open symbols) of 25-hydroxycholesterol (0.5 μg/ml) and molecular cholesterol (10 μg/ml). Cells were harvested after the indicated periods of incubation, whole cell lysates were prepared and CAT activity in a fixed amount of total protein was determined as described in Materials and Methods. The results for the transfectant expressing the mutant promoter are representative of three independent transfectants. b: Jurkat T cells transfected with 142–CAT or 1472–CAT constructs containing either a wild-type or mutant SRE-1 were cultured in lipoprotein-deficient medium with or without 25-hydroxycholesterol and cholesterol for >24 h before assay. Results are expressed as mean ± SEM; wild-type SRE-1 1472–CAT: n = 8 experiments using 3 transfectants; wild-type SRE-1 142–CAT: n = 4, 2 transfectants; mutant SRE-1 1472–CAT: n = 12, 5 transfectants; and mutant SRE-1 142–CAT construct: n = 6, 4 transfectants.

cultured in the presence of sterols, there was a transient increase in CAT activity followed by a progressive decline. In contrast, CAT activity in cells transfected with the mutant SRE-1 1472-CAT construct was not induced by the removal of exogenous sterols. Furthermore, the transient increase in CAT activity noted in wild-type transfectants cultured in the presence of exogenous sterols was not observed in cells transfected with the SRE-1 mutant. Similar results were noted with the 142 base pair promoter–CAT construct (Fig. 4b). Thus, deprivation of exogenous sterols for ~48 h induced wild-type SRE-1 1472–CAT activity by 2.4 ± 0.2-fold (mean ± SEM, n = 8 experiments using 3 transfectants) and wild-type 142-CAT activity by 2.7 ± 0.3-fold (n = 4, 2 transfectants). In contrast, an irrelevant promoter construct, the thymidine kinase minimal promoter linked to CAT was not induced by sterol deprivation (1.1 ± 0.1-fold, n = 4, 4 transfectants). Mutation of the SRE-1 sequence in either the 1472–CAT or 142–CAT constructs abolished induction of CAT activity after sterol deprivation. Thus, CAT activity was increased 1.2 ± 0.1-fold (n = 12, 5 transfectants) in Jurkat T cells expressing the mutant SRE-1 1472–CAT construct and 1.1 ± 0.1-fold (n = 6, 4 transfectants) in cells containing the mutant SRE-1 142–CAT construct. These data demonstrate that mutation of the SRE-1 completely abolished sterol-dependent transcription regulated by either of the LDL receptor promoter fragments studied.

Subsequent experiments focused on the response of the two LDL receptor-promoter fragments to mitogenic stimuli. The mitogenic combination of PMA and ionomycin induced CAT mRNA levels 2.0 ± 0.3-fold (n = 6, 4 transfectants; $P < 0.02$) in Jurkat T cells transfected with the wild-type SRE-1 1472–CAT construct and cultured in lipoprotein-deficient medium (**Fig. 5**, left panel). The magnitude of this response was equivalent to that previously observed with the ~6.5 kb fragment of the LDL receptor promoter (10). Similarly, CAT mRNA levels were induced 1.8 ± 0.1-fold (n = 6, 4 transfectants; $P < 0.002$) by PMA and ionomycin stimulation of Jurkat T cells transfected with the wild-type SRE-1 142–CAT construct. When the transfected Jurkat T cells were cultured in 10% v/v bovine calf serum (BCS, 130 μg cholesterol/ml) mitogenic induction of CAT mRNA levels was also demonstrated (Fig. 5, center panel). Thus, PMA and ionomycin induced 1472–CAT mRNA levels by 2.3 ± 0.3-fold (n = 4, 2 transfectants). PMA and ionomycin responses of the wild-type SRE-1 142–CAT construct were modestly but not significantly lower (1.6 ± 0.2-fold induc-

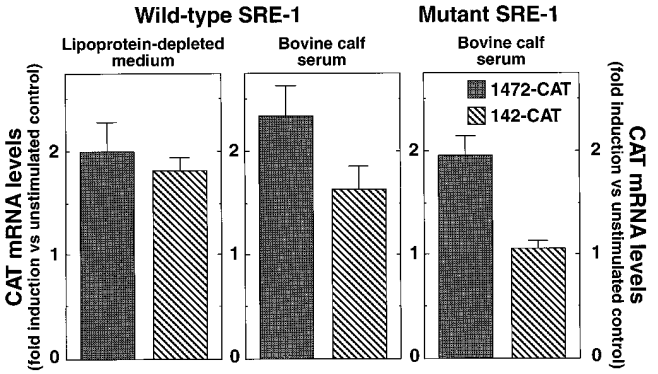

Fig. 5. Mutation of SRE-1 abolishes responses to stimulation with PMA and ionomycin in 142–CAT but not 1472–CAT. Jurkat T cells expressing 1472–CAT or 142–CAT with wild-type SRE-1 (left and center panels) or mutant SRE-1 (right panel) were incubated in lipoprotein-deficient (LPP, left panel) or lipoprotein-containing (BCS, center and right panels) medium and stimulated with the mitogenic combination of PMA and ionomycin for 2 h before cells were harvested and total RNA was extracted. Specific mRNA species were quantified by nuclease protection assay as detailed in Materials and Methods. Results are expressed as mean fold induction compared with unstimulated control cells ± SEM; left panel: n = 6 experiments using 4 transfectants for each promoter; center panel: n = 4, 2 transfectants for each promoter; mutant SRE-1 1472–CAT: n = 9, 3 transfectants; mutant SRE-1 142–CAT: n = 11, 3 transfectants.

tion, n = 4, 2 transfectants; $P > 0.5$ vs. 142–CAT response in lipoprotein-deficient medium) when cells were cultured in cholesterol-containing medium.

Mutation of the SRE-1 in the 1472 base pair promoter fragment did not alter induction of CAT mRNA levels stimulated by PMA and ionomycin. This combination of mitogens induced CAT mRNA levels 2.0 ± 0.2-fold (n = 9, 3 transfectants; $P < 0.001$ compared to unstimulated, and $P > 0.3$ compared to induction by the wild-type SRE-1 1472 promoter construct). In contrast, mutation of the SRE-1 sequence in the 142-CAT construct abolished the increase in CAT mRNA levels after stimulation by PMA and ionomycin (1.1 ± 0.1-fold, n = 11, 3 transfectants; $P > 0.5$) as shown in Fig. 5 (right panel). Endogenous LDL receptor mRNA levels increased by a mean of 2.0 ± 0.2-fold after PMA and ionomycin stimulation of cells expressing mutant SRE-1 142–CAT, identical to responses observed previously (10), demonstrating that signal transduction was intact in the these cells.

In the final studies, the ability of mitogenic stimulation to increase reporter gene expression in cells incubated with suppressive sterols was determined. As shown in **Fig. 6**, transfected 142–CAT mRNA levels were decreased when Jurkat T cells were cultured in lipoprotein-deficient medium supplemented with 25-hydroxycholesterol and cholesterol. Native LDL receptor mRNA levels were similarly decreased (data not shown). When these cells were stimulated with PMA and ionomycin, however, there was an increase in the levels of 142–CAT mRNA (Fig. 6) and LDL receptor mRNA levels (data not shown). These findings indicate that the combination of PMA and ionomycin stimulates transcription driven by the 142 base pair proximal LDL receptor promoter in the presence of suppressive sterols that prevent nuclear appearance of SREBP-1 and -2, despite the dependence of this construct on an intact SRE-1 for up-regulation of activity.

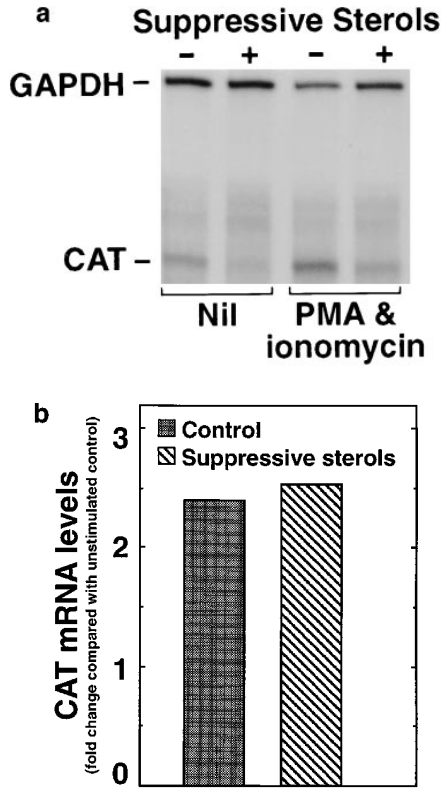

Fig. 6 142–CAT mRNA increases in Jurkat T cells following stimulation with PMA and ionomycin in the presence of suppressive sterols. Jurkat T cells cultured in lipoprotein-deficient medium without (−) or with (+) supplementary suppressive sterols (25-hydroxycholesterol 0.5 μg/ml, cholesterol 10 μg/ml), for 48 h were stimulated with PMA and ionomycin for 2 h. Total RNA was prepared and CAT mRNA levels were quantified by nuclease protection assay as detailed in Materials and Methods. a: Autoradiogram of nuclease protection assay gel; b: Relative abundance of CAT mRNA.

DISCUSSION

The current studies demonstrate that, within the LDL receptor promoter, there are several distinct regulatory regions involved in mitogen induction of LDL receptor gene transcription. There is at least one element regulating transcription that is completely independent of sterol regulation and SRE-1. This sterol-independent element is located between −142 and −1472 of the LDL receptor promoter and is induced by the combination of PMA and ionomycin. In addition to this element, the data also indicate that the SRE-1 participates in transcription induced by mitogenic signals. Thus, the proximal 142 base pair promoter fragment requires an intact SRE-1 sequence for induction by PMA and ionomycin. Of note, however, this promoter element is not regulated by classic sterol-dependent mechanisms as it remains transcriptionally active in the presence of suppressive concentrations of sterols that prevent nuclear localization of SREBP-1 and -2.

Many previous studies of the regulation of LDL receptor gene expression have focused on the mechanism of sterol-mediated repression of gene transcription (1–3 and references therein). The sequence contained within the 10 base pairs constituting SRE-1 was identified as the essential *cis*-acting regulatory element for sterol-mediated suppression of LDL receptor gene expression (14). However, mitogenic stimulation of Jurkat T cells activated LDL receptor gene transcription independently of ambient sterols (10), suggesting that there was an additional mechanism of LDL receptor gene regulation that did not involve SRE-1. Thus, the ~6.5 kb fragment immediately 5′ of the transcription start site of the LDL receptor promoter was sufficient to induce LDL receptor gene transcription in response to stimulation by PMA and ionomycin (10). To identify the regions of this promoter required for mitogenic stimulation of the LDL receptor gene, two smaller promoter fragments from the 3′ end of the ~6.5 kb fragment were analyzed in the current studies. All three promoter fragments (~6.5 kb, 1472 base pairs, and 142 base pairs) share identical 3′ sequences, including the SRE-1.

To permit evaluation of the role of the SRE-1, mutations of the core sequence were produced. In preliminary experiments, two point mutations (ATCACCCCTCT and ATCACCCCGCT; see Fig. 1 and reference 14) failed to disrupt sterol regulation of CAT gene expression in Jurkat T

cells (unpublished data), although these mutations prevented sterol-mediated repression in Chinese hamster ovary cells (14). Therefore, a more extensive mutation was created in which 9 of the 10 nucleotides that constitute the core of the SRE-1 sequence were altered. This scramble mutation effectively abolished induction of transcriptional activation after sterol deprivation regardless of promoter length.

The 1472 base pair LDL receptor promoter fragment mediated responses that were equivalent to those previously observed with the ~6.5 kb promoter fragment after stimulation by PMA and ionomycin (10). Mutation of the SRE-1 sequence in the 1472–CAT promoter construct did not alter mitogen-induced LDL receptor gene promoter activity. This finding agrees with earlier data that suggested that mitogens induced LDL receptor gene transcription through mechanisms other than altering cellular sterol balance (6, 10). Whereas these earlier observations provided indirect evidence, the data from the SRE-1 scramble mutation clearly demonstrate that mitogenic signals and sterols can utilize distinct mechanisms to regulate LDL receptor gene transcription. Of note, sequencing of the entire 1472 base pair promoter fragment (unpublished data) has revealed no region that matches the consensus sequence for the element (TRE) implicated in responses to phorbol esters (27). The precise upstream sequences of the LDL receptor gene that control responses to PMA and ionomycin remain to be delineated in detail.

Another element that is SRE-dependent is contained within the proximal 142 base pairs of the promoter. Although SRE-1 is required for the response of the 142 base pair promoter fragment to PMA and ionomycin, it does not appear to function solely by binding SREBPs. Thus, mitogen-induced transcriptional activity of the 142 base pair promoter fragment required SRE-1, but was active in the presence of suppressive sterols, when nuclear localization of SREBPs could not be detected. One possible explanation for the findings is that the SRE-1 itself serves as a mitogen-inducible, *cis*-acting element. Consistent with this hypothesis, the abundance of one transcription factor that binds to the SRE-1, SREBP-1, was modestly increased by stimulation with PMA and ionomycin in the absence of suppressive sterols. In this circumstance, PMA and ionomycin could enhance transcription by directly stimulating activity of SREBP-1. Taken together, these findings suggest that PMA and ionomycin may increase LDL receptor gene transcription via SREBP-1 binding to SRE-1 when SREBP-1 is abundant (in the absence of suppressive sterols). Alternatively, transcription factor(s) other than SREBPs may be responsible for the SRE-dependent increases. This possibility is supported by the observation that 142–CAT mRNA levels increased following mitogenic stimulation in the presence of suppressive sterols, when nuclear localization of SREBPs was not apparent. Changes in the abundance or activity of another putative transcription factor may also account for the mitogen-induced increase in 142–CAT mRNA levels in the absence of suppressive sterols, rather than the modest increase in SREBP-1.

Other investigators have proposed that cellular mitogens and growth factors may increase LDL receptor expression in an SRE-dependent manner (28, 29). Thus, hepatocyte growth factor increased LDL receptor expression in a cultured hepatocellular carcinoma cell line, HepG2, and also increased expression of a reporter gene driven by 3 tandem copies of repeats 2 and 3 (SRE-1 and Sp1, respectively) of the LDL receptor promoter (29). In addition, a point mutation in SRE-1 abolished insulin- and estradiol-induced increases in reporter gene expression driven by LDL receptor promoter fragments in HepG2 cells (28). However, neither insulin nor insulin-like growth factor-I altered the level of SREBP-1 protein or SREBP-1 and -2 mRNAs in HepG2 cells (28). When taken together with the current data, the evidence strongly implies that SRE-dependent increases in LDL receptor expression induced by mitogens and growth factors may not involve the SREBPs and therefore may result from the activity of other transcription factors that use SRE-1 for DNA binding.

The physiologic importance of mitogenic induction of LDL receptor gene transcription remains speculative, but might represent part of a program to prepare cells for growth and division. Thus, LDL receptor activity might be induced, independent of cellular sterol balance, to insure that rapidly growing tissues acquire enough cholesterol for membrane biosynthesis. Additionally, in T cells, the LDL receptor pathway provides certain required unsaturated fatty acids that the T cell cannot synthesize and are necessary for T cell proliferation (30). Regardless of the precise functional significance, these studies demonstrate that non-sterol pathways can regulate LDL receptor gene expression and can be dominant over sterol-mediated down-regulation. Exploitation of these regulatory mechanisms might permit pharmacological induction of LDL receptor gene expression, despite sterol-dependent suppression of transcription. This therapeutic approach could facilitate lowering of plasma LDL cholesterol levels in patients resistant to treatments that are dependent on sterol regulation and thereby decrease the risk of atherosclerosis.

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