Nutrient regulation of gene expression by the sterol regulatory element binding proteins: Increased recruitment of gene-specific coregulatory factors and selective hyperacetylation of histone H3 \textit{in vivo}

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We have evaluated the mechanism for sterol-regulated gene expression by the sterol regulatory element binding proteins (SREBPs) in intact cells. We show that activation of SREBPs by sterol depletion results in the increased binding of Sp1 to a site adjacent to SREBP in the promoter for the low density lipoprotein (LDL) receptor gene \textit{in vivo}. Similarly, sterol depletion resulted in the increased recruitment of two distinct SREBP coregulatory factors, NF-Y and CREB, to the promoter for hydroxymethyl glutaryl CoA reductase, another key gene of intracellular cholesterol homeostasis. Furthermore, increased acetylation of histone H3 but not H4 was also detected in chromatin from both promoters on SREBP activation. Thus, SREBP activation results in the similar selective recruitment of different coregulatory generic transcription factors to two separate cholesterol-regulated promoters. These studies demonstrate the utility of the chromatin immunoprecipitation technique for analyzing the differential action of low-abundance transcription factors in fundamental regulatory events in intact cells. Our results also provide key \textit{in vivo} support for the mechanism proposed from cell-free experiments, where SREBP increased the binding of Sp1 to the LDL receptor promoter. Finally, our findings also indicate that subtle differences in the pattern of core histone acetylation play a role in selective gene activation.

Feedback regulation of mammalian cholesterol homeostasis is mediated by a positive regulatory mechanism through the sterol regulatory element binding proteins (SREBPs) (1). The SREBPs also regulate key genes of fatty acid metabolism so they control flux into the two major lipid classes in mammalian cells (1, 2). When sterol levels fall in cultured cells, the SREBPs are released from membranes of the endoplasmic reticulum and nuclear envelope through the action of two sequential proteolytic activities (3). The resulting soluble mature transcription factor enters the nucleus, where it activates a set of target genes that are involved in lipid accumulation.

In all promoters for SREBP target genes that have been carefully studied thus far, SREBP-dependent regulation requires an additional generic coregulatory DNA binding factor(s) for efficient expression (4). The identity of the coregulatory factor and the position of its binding site relative to the binding site(s) for the SREBPs differs from promoter to promoter. The common use of SREBP provides a mechanism for coordinate regulation. However, the unique coregulatory factors and subtle differences in promoter architecture provide the opportunity for more subtle promoter-specific regulatory effects that integrate other cellular signaling pathways with simple nutrient sensing to provide optimal control of cellular lipid levels.

To understand the mechanism for coordinate and genespecific activation by the SREBPs, we have been investigating how they function to activate transcription synergistically with distinct coregulatory factors in different promoters. The gene that encodes the key protein of cholesterol uptake, the low density lipoprotein (LDL) receptor, has a promoter that contains a single SREBP site flanked on either side by a coregulatory site for the generic Sp1 protein (see Fig. 2A). SREBP and Sp1 activate the LDL receptor promoter synergistically in transfected cells (5) and from reconstituted chromatin templates in experiments performed with cell-free extracts (6).

DNA binding studies with purified proteins \textit{in vitro} demonstrated that SREBP stimulated the adjacent binding of Sp1 at the LDL receptor promoter approximately 10-fold through stimulating the initial on rate of binding (5, 7). Based on this and other key observations, we proposed a model for sterol regulation of the LDL receptor promoter that is presented in Fig. 1. We wanted to evaluate this model further and address other aspects of SREBP-dependent gene activation in intact cells to establish a working model to account for the ability of SREBPs to function with different coregulatory proteins in stimulating different promoters. Toward this goal, we have used the chromatin immunoprecipitation (CHIP) technique to analyze SREBP coregulatory factor binding to promoters of endogenous sterol-regulated genes in intact cells. The experiments demonstrate that SREBP does indeed recruit Sp1 to the endogenous LDL receptor promoter and further show that a similar mechanism for coregulatory factor recruitment occurs in another cholesterol-regulated promoter, where different generic coregulatory factors function synergistically with SREBP. Additionally, we show that SREBP activation results in an increased level of acetylation of histone H3, but not H4, in chromatin at sterol-regulated promoters.

Materials and Methods

Cell Cultures and Media. Stock flasks of Chinese hamster ovary (CHO)-7 cells (8) were grown in a 50/50 mixture of Ham's F-12 and DMEM (Irvine Scientific) containing 10% (vol/vol) FBS at 37°C and 8% CO₂. Twenty to twenty-four 15-cm tissue culture dishes were plated at 2,000,000 cells per dish on day 0 in the above medium. On day 1, the dishes were rinsed twice with 1× PBS, and half of the dishes were fed with either induced media.

This paper was submitted directly (Track II) to the PNAS office. Abbreviations: SREBP, sterol regulatory element binding protein; LDL, low density lipoprotein; HMG, hydroxymethyl glutaryl; CHIP, chromatin immunoprecipitation; CHO, Chinese hamster ovary; HAT, histone acetylase; CREB, cAMP response element binding protein; CBP, CREB binding protein.

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(Ham's F-12/DMEM containing lipoprotein-depleted serum instead of FBS). The other half were fed suppressed media (Ham's F-12/DMEM containing lipoprotein-depleted serum with 10 μg/ml cholesterol and 1 μg/ml of 25-OH-cholesterol). Cells were processed for the CHIP procedure (described below) after an additional 24-h incubation. Lipoprotein-deficient serum was prepared by ultracentrifugation as described (9). Stock solutions of cholesterol and 25-OH-cholesterol (Steraloids, Wilton, NH) were prepared in ethanol.

CHIP Assay. We used a modification of the technique described by P. Farnham and colleagues (10). Dishes of CHO-7 cells were placed in a fume hood, and formaldehyde was added to the culture medium to a final concentration of 1% followed by a room temperature incubation for 4, 6, 8, or 10 min. Glycine was added to a final concentration of 125 mM, and the dishes were incubated for an additional 5 min at room temperature, medium was removed followed by three rinses with cold 1X PBS. Cells were scraped into 1X PBS and collected by centrifugation, washed once with 1X PBS containing 1 mM PMSF, and the cell pellets were resuspended by gentle pipetting in 5 ml of cell lysis buffer [5 mM Pipes (KOH), pH 8.0/85 mM KCl/0.5% (vol/vol) NP-40] with the protease inhibitors leupeptin, pepstatin, and PMSF all added at 0.2 mM. Samples were incubated at 4°C overnight on a rotating wheel. The samples were centrifuged at 4°C overnight on a rotating wheel. The samples were centrifuged for 10 min at 10,000 rpm, the supernatant was transferred to a new tube, and 60 μl of protein A, G, or a mixture of A and G beads were added and samples were incubated at 4°C overnight on a rotating wheel. The samples were centrifuged for 10 min at 10,000 rpm, the supernatant was transferred to a new tube, and 60 μl of protein A, G, or a mixture of A and G beads were added and samples were incubated 1–2 h at 4°C on a rotating wheel. The samples were washed four times with 1 ml wash buffer [0.1% (vol/vol) Triton X-100/20 mM Tris, pH 8.0/150 mM NaCl/2 mM EDTA] and eluted by three successive 5-min incubations with 150 μl elution buffer [1% (wt/vol) SDS/50 mM NaHCO3]. The eluates were pooled, 1 μl of RNase (10 mg/ml) was added and NaCl was adjusted to 0.3 M, and the samples were incubated at 65°C for 4 h to reverse the formaldehyde crosslinking. Digestion buffer was added [10 μl 2 M Tris, pH 6.8/10 μl 0.5 M EDTA/2 μl of proteinase K (20 mg/ml)], and the samples were placed at 45°C for 2 h. Samples were extracted with phenol/CHCl3 then CHCl3 only followed by precipitation with ethanol. Samples were resuspended in 50 μl sterile H2O, and 2–4 μl were used in each PCR.

Standard PCR for hamster hydroxymethyl glutaryl (HMG) CoA reductase or LDL receptor promoters were performed with 32P-kinased oligonucleotides and AmpliTaq Gold (Perkin–Elmer). The primers for the hamster LDL receptor promoter were designed to hybridize and amplify a ~230-bp fragment encompassing the region displayed in Fig. 24. The HMG CoA reductase primers were designed to hybridize and amplify a ~230-bp product encompassing the region displayed in Fig. 24. To provide reactions that were in the linear dose response for the individual samples, we performed test PCR and varied the number of cycles for each promoter and antibody combination, as indicated in the figure legends. Additionally, for each immunoprecipitation experiment, a dilution curve of the starting DNA was performed to ensure that the final quantity of PCR product was proportional to the input. An example of this is shown for Sp1 binding to the LDL receptor promoter in Fig. 2C. All of the data presented in the figures are representative samples of several different experiments performed for each antibody and PCR combination.

The amounts of primary antibodies and the use of protein A or G beads varied as follows: for acetylated H3 and H4 (Upstate Biotechnology 06-599 or 06-866, respectively), 5 μg of antibody and protein A beads were used; for anti-NF-κB (Santa Cruz Biotechnology sc-7712), 10 μg of antibody and protein G beads were used; for CREB-1 (Santa Cruz Biotechnology sc-186), 10 μg and a mixture of protein A and G were used; for Sp1 (Santa Cruz Biotechnology sc-59), 30 μg of antibody and a mixture of protein A and G were used.

Fig. 1. Model for SREBP-dependent gene activation. A schematic representation of key steps in activation of the LDL receptor promoter by SREBP and Sp1. The model depicts full-length SREBP tethered to the endoplasmic reticulum membrane through its two-pass membrane spanning domain. On sterol depletion, the protein is clipped out of the membrane and migrates to the nucleus, where it binds to the SREBP site in the LDL receptor promoter adjacent to an Sp1 site. This stimulates Sp1 to bind to the adjacent site, whereon the DNA bound Sp1 leads to a destabilization of DNA-bound SREBP (5). After it falls off the DNA, SREBP is rapidly degraded by a nuclear calpain type of protease activity (27). Under this model, the activation of the LDL receptor promoter by SREBP is transient and rapidly reversible, unless processing continues and SREBP levels continue to increase because of a chronic low cellular sterol level.
PAGE and Immunoblot Analysis. Equivalent amounts (normalized for A_{260}) of chromatin extracts were analyzed by SDS/PAGE, transferred onto nitrocellulose, and incubated in Tris-buffered saline (TBS; 20 mM Tris, pH 7.6) containing 5% (wt/vol) nonfat dry milk overnight at 4°C. The next day, the filters were washed in TBS with 0.05% (vol/vol) Tween 20 and incubated with the indicated primary antibody for 1 h, washed three times with TBS, and incubated with secondary antibody for 1 h. After three TBS washes, bound antibody was detected by using the Super Signal Kit (Pierce) and autoradiography.

**Results**

Sterol Depletion Results in Sp1 Recruitment to the LDL Receptor Promoter in Vivo. We adapted the CHIP assay to evaluate the mechanism for synergistic activation of the LDL receptor promoter by SREBP and Sp1 in intact cells. CHO-7 cells were cultured in the absence (induced, I) or presence (suppressed, S) of regulatory sterols and harvested after a brief treatment with formaldehyde. Isolated chromatin from each preparation was diluted 1:1,000, and 2 μl were analyzed in lanes 1 and 2. Samples were processed through the CHIP protocol, and the primary antibody was left out (lanes 3 and 4) or an Sp1 antibody was included (lanes 5 and 6). Twenty-eight cycles of PCR were used. (C) The input material from the induced (I) or suppressed (S) was serially diluted in 3-fold steps in lanes 1–3 to document that the amount of PCR product accurately reflects the amount of template DNA added to the PCR. (D) Equal aliquots of starting chromatin from each sample were subjected to SDS/PAGE and immunoblot analysis with the Sp1- or SREBP-2-specific primary antibodies, as indicated. P and M denote the positions of putative precursor and mature forms, respectively, of SREBP-2.
of PCR product accurately reflects a difference in starting material was documented by performing a serial dilution on the input samples (Fig. 2C). A control in which the Sp1 antibody was omitted from the immunoprecipitation reaction resulted in no detectable PCR products (Fig. 2B, lanes 3 and 4). Additionally, the immunoblot in Fig. 2D shows that equal levels of Sp1 were present in the starting chromatin preparations (lanes 1 and 2) and that SREBP-2 processing occurred normally in response to sterol depletion (lanes 3 and 4). Thus, Sp1 was recruited to the LDL receptor in vivo in response to sterol depletion, indicating that it is a key SREBP coregulatory factor and that its DNA binding is stimulated by SREBPs in living cells.

**Increased Recruitment of Coregulatory Factors by SREBP at Other Sterol-Regulated Promoters.** Because the SREBP coregulatory protein(s) in different promoters vary, we wanted to evaluate another promoter where SREBP functions synergistically with different coregulators. HMG CoA reductase is the rate-controlling enzyme of cholesterol biosynthesis. The promoter for its gene contains multiple SREBP recognition elements and two different coregulatory sites; one for CAAT box binding factor/nuclear factor Y (CBF/NF-Y; herein referred to as NF-Y) and one for a CREB/ATF family member (Fig. 3A) (11, 12). We used the CHIP technique to evaluate the association of both NF-Y and CREB with the HMG CoA reductase promoter in response to changes in cell cholesterol (Fig. 3B). The PCR results demonstrated that both CREB and NF-Y were bound to the HMG CoA reductase promoter in chromatin prepared from sterol-depleted cells (Fig. 3B, lanes 5 and 11), but the amount of DNA immunoprecipitated from chromatin prepared from sterol-replete cells was significantly lower (Fig. 3B, lanes 6 and 12). Immunoblotting experiments revealed that starting levels for both CREB and NF-Y were similar in both chromatin preparations (Fig. 3C). Thus, analogous to Sp1 in the LDL receptor promoter, these two distinct coregulatory proteins are recruited to the HMG CoA reductase promoter when SREBPs are activated in response to sterol depletion.

**SREBP Activation Leads to Increased Acetylation of Chromatin-Associated Histone H3 but Not Histone H4.** Several recently described transcriptional coactivator proteins, such as CREB binding protein (CBP) and P300, have been shown to possess intrinsic histone acetylase (HAT) activity (13) and they interact with P/CAF, which has a distinct HAT activity (14). Because the activation domain of SREBP-1α interacts with CBP (6, 15) and P300 (unpublished observations), we evaluated whether nutrient regulation by the SREBPs also was accompanied by increased acetylation of specific histones in chromatin at sterol-regulated promoters. We used the CHIP technique and antibodies directed against acetylated forms of either histone H3 or H4 to search for cholesterol-dependent changes in the levels of acetylation of these histones at the LDL receptor (Fig. 4A) and HMG CoA reductase (Fig. 4B) promoters. In both cases, acetylated H3 containing DNA was enriched in samples recovered from cholesterol-depleted cells (Fig. 4A and B, lanes 5 and 6). However, in contrast, the level of histone H4 acetylation was the same under both culture conditions for both promoters (Fig. 4A and B, lanes 7 and 8). It should be noted that others have used the same H4 antibody used in our studies and showed a preferential association of H4 to the active FMR1 gene (16).

**Discussion**

Sterol regulation of the LDL receptor promoter requires the concerted action of two proteins: the sterol-regulated SREBP and the generic coregulator Sp1. We previously have demonstrated that SREBP increases the DNA binding of Sp1 to the LDL receptor promoter in vitro (5), and it requires only the DNA binding domain of SREBP and a region encompassing the DNA binding and conserved buttonhead (btd) domains of Sp1 (17, 18). However, other domains in both proteins were additionally required for efficient activation when synergistic activation by SREBP and Sp1 on the LDL receptor promoter was evaluated by a transient DNA transfection assay in Drosophila SL2 cells (17). Thus, we proposed that synergistic activation of the LDL receptor promoter occurs at two steps in the activation process: initially, at the level of DNA binding and also at a subsequent step in the assembly of a transcriptionally active complex once the two proteins are bound to DNA. Recently, the synergistic activation by SREBP and Sp1 has been recapitulated on artificiably assembled chromatin templates in vitro and requires a number of coactivators in addition to CBP/P300 (6). In the current studies, we demonstrate that, as sterol levels fall, the increased processing and nuclear accumulation of SREBPs results in an increased association of Sp1 with the LDL receptor promoter DNA in vivo (Fig. 2), which is in support of our prior in vitro DNA binding observations.

We also show that the mechanism for SREBP recruitment of its generic coregulatory factor is not specific for Sp1 in the LDL receptor promoter because similar increased recruitment of the different required coregulatory factors for the HMG CoA reductase promoter, NF-Y and CREB, was also observed on depletion of intracellular cholesterol. In other reports, we have demonstrated that SREBPs interact directly with Sp1, NF-Y, and CREB in solution in the absence of DNA (4, 19, 20). Thus, the increased recruitment of generic transcription factors to sterol-regulated promoters by a direct protein–protein interaction is likely to be a common aspect of regulated gene expression mediated by the SREBP proteins.
The N-terminal activation domain of SREBPs interacts with the CBP and P300 coactivator proteins (refs. 15 and 21, and unpublished observations). These multifunctional non-DNA binding coactivator proteins are brought to promoters through interactions with site-specific DNA binding proteins, and they stimulate transcription both actively through their intrinsic HAT activity and passively by providing a scaffold to assemble other required coactivators at specific promoters (13). One of the key passive roles is the recruitment of the P/CAF protein, which is a distinct HAT enzyme (14).

Because the activation domain of SREBPs interact with CBP and P300, we evaluated the importance of histone acetylation in sterol-regulated gene expression. We used antibodies specific for the acetylated forms of either histone H3 or H4 separately in the CHIP protocol and as shown in Fig. 4, increased acetylation of histone H3 was associated with SREBP activation. However, the level of histone H4 acetylation at both the LDL receptor and HMG CoA reductase promoters was constant and independent of SREBP activation. Thus, acetylation of chromatin, specifically at histone H3, is associated with gene activation by SREBPs. Because the intrinsic HAT activity of P300 readily modifies all four core histones, whereas the P/CAF enzyme shows a strong preference for histone H3 in vitro (22), our results are consistent with a role for the histone acetylase activity of P/CAF in gene activation by SREBPs.

In previous studies, the levels of acetylation for core histones H3 and H4 were evaluated during the activation of specific genes by using similar antibodies in the CHIP technique. In the case of gene activation by nuclear receptors and at the active FMR1 locus, there was a preference for H4 hyperacetylation (16, 23), and in the activation of interferon β expression by virus infection, both H3 and H4 were hyperacetylated at proximal promoter sites (24). Along with the preferential acetylation of H3 demonstrated for the LDL receptor and HMG CoA reductase genes in our studies, the selective hyperacetylation of H3 was also recently shown to be associated with the cell cycle regulation of the p21<sup>WAF1</sup> protein gene (25).

It was not immediately obvious why CBP/P300 would recruit the seemingly redundant P/CAF histone acetylase because CBP and P300 possess intrinsic HAT activity. By analyzing CBP