Selective Association of Sterol Regulatory Element-binding Protein Isoforms with Target Promoters in Vivo*

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The mRNAs for all three members of the sterol regulatory element-binding protein (SREBP) family are widely expressed, and the proteins are highly similar. They have potential to both hetero- and homodimerize through their bHLHHLZ domains, so it has been difficult to definitively study the role of each one apart from the other two. In the current study, we have utilized cell lines that express only one functional SREBP and the chromatin immunoprecipitation technique to analyze individual SREBP binding to three specific target genes: hydroxymethylglutaryl-CoA reductase (Red), fatty acid synthase (FAS), and squalene synthase (SQS). Our studies show that SREBP-2 binds to promoters for all three genes, and in agreement with the original report using these cells, all three mRNAs are also induced. In the line expressing only SREBP-1a, mRNAs for Red and FAS are induced, but SQS is not. Chromatin immunoprecipitation shows that SREBP-1a is recruited efficiently to Red and FAS promoters but not to SQS. This observation indicates SREBP-2 selectively binds the SQS promoter and is sufficient to explain the lack of SQS mRNA induction in the SREBP-1a-expressing cells. SREBP-1c protein was not stably recruited to any SREBP target promoter despite being fully active in DNA binding when purified from extracts of the corresponding cells. This is also sufficient to explain the lack of SREBP target gene induction by the singular expression of SREBP-1c. We also show that whereas SREBP-1a and -2 proteins interact efficiently with transcriptional co-activators that modify cellular chromatin, SREBP-1c does not. Taken together, our data support a model suggesting that chromatin modification is required during the initial stage of specific site recognition by SREBPs in native chromatin in vivo.

The sterol regulatory element-binding protein (SREBP)1 family controls flux of cellular metabolites into the major lipid pathways in mammalian cells. There are three very similar proteins in this family; SREBP-1a and -1c are encoded by overlapping mRNAs from a single gene, and SREBP-2 is the product of a distinct genetic locus (1, 2). Primary transcripts for SREBP-1a and -1c mRNAs initiate from different promoters; each has a unique first exon that is spliced to the same second exon, and the remaining coding sequences are indistinguishable. Thus, the two corresponding proteins are identical except for the amino acids encoded in the first exon. In SREBP-1a there are 28 unique amino acids, whereas in SREBP-1c there are only four specific residues in addition to the initiator methionine. Because the transcriptional activation domain is located at the extreme amino terminus (3), the two proteins differ in their ability to stimulate transcription. 1a is a relatively potent transcriptional activator that efficiently interacts with the twin coactivators CBP and p300 (4) as well as the multisubunit mammalian mediator complex (5, 6). In contrast, 1c is a very poor transcriptional activator. The activation domain of SREBP-2 is similar to 1a, and it is also a potent transcriptional activator.

A major goal in the study of any gene family is to distinguish the separate and overlapping functions for the individual members. Studies to tease out the individual roles for the different SREBPs have included analyzing expression patterns in different tissues, responses to specific regulatory cues, and overexpression as well as genetic studies in cultured cells and gene-targeted mice (1). When taken together, these studies indicate that SREBP-1 is selectively associated with fatty acid regulation, and SREBP-2 is preferentially involved in sterol regulation. However, there is significant overlap, and because all three SREBPs are widely expressed and have the potential to homo- and heterodimerize, there is even the possibility of selective roles for specific homo- as well as heterodimeric SREBP complexes. This has complicated the conclusions from regulatory and most overexpression studies, because they have been performed under conditions where the other two SREBP isoforms are also expressed.

To specifically evaluate target gene selectivity in the SREBP family, Pai et al. (7) took advantage of a mutant CHO cell line that cannot express any active SREBPs from endogenously expressed proteins. This mutant cell line has a deletion in the gene encoding the site 2 protease, which is absolutely required for releasing the precursor SREBPs from their membrane tether (8). Without this cleavage, SREBP isoforms remain anchored in the endoplasmic reticulum membrane and fail to accumulate in the nucleus. Pai et al. (7) established transfected cell lines that stably express the mature, nuclear targeted form of each individual SREBP isoform in an edysone-inducible expression cassette in the background of the site 2 protease mutant cell line. In the original report using these cell lines, expression of either SREBP-1a or SREBP-2 proteins induced mRNAs from most SREBP target genes roughly equivalently. There were two notable exceptions. Squalene synthase (SQS) was activated.

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efficiently by SREBP-2 but was not stimulated above baseline by SREBP-1a, and HMG-CoA reductase was efficiently stimulated by SREBP-2 but was stimulated to a lesser degree by SREBP-1a.

Importantly, the singular expression of SREBP-1c was insufficient to activate any SREBP target gene above base-line levels despite being expressed at the same level as SREBP-1a or -2 protein in the companion cell lines. The reasons for the selective activation of SQS and to a lesser degree HMG-CoA reductase by SREBP-2 and the complete absence of activation of any SREBP target gene by SREBP-1c have not been established.

To address these questions, we have utilized the chromatin immunoprecipitation technique (ChIP) to evaluate SREBP binding to selective promoters in these mutant CHO cell lines. In the experiments reported here, we show that the promoters for HMG-CoA reductase and SQS selectively bind SREBP-2, and the promoter for fatty acid synthase (FAS) binds both SREBP-1a and -2 equivalently. In contrast, we show that SREBP-1c does not stably associate with the promoters of any of these three genes, although when the protein is purified from the corresponding cells it efficiently binds to an SREBP target site in vitro. We also show that although SREBP-1a and -2 both interact efficiently with the co-activators CBP/p300, SREBP-1c is defective in this crucial function. Thus, our results for promoter binding in vitro and coactivator binding in vitro are sufficient to explain the differential patterns of gene expression observed previously (7). When integrated together, our new data form the basis of a model to explain the lack of SREBP-1c binding to its target DNA sites in cellular chromatin.

MATERIALS AND METHODS

Cell Cultures and Media—Stock flasks of M19-transfected cells expressing SREBP-1a, -1c, or -2 (7) were cultured in a 50/50 mixture of Ham’s F-12 and Dulbecco’s modified Eagle’s medium high glucose (Irvin Scientific) containing penicillin and streptomycin, glutamine, Hepes, pH 7.2, 500 μg/ml G418 sulfate, 1 mM mevalonic acid, 5% (v/v) fetal bovine serum, 5 μg/ml cholesterol, 20 μM dea, 500 μg/ml Zeocin. Incubation was at 37 °C and 5% CO₂. For each time course, 30 dishes (15 cm) were plated at 8,000,000 cells/dish on zero in the medium with Zeocin. After 24 h, the cells were cultured with ponasterone A. The ponasterone A concentrations were 3 μM for the cells expressing either SREBP-1c or SREBP-2 and 0.3 μM for the SREBP-1a cells. There were three dishes used for each time point, and cells were induced for 0, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, or 8 h. Ponasterone additions were staggered so that all dishes were harvested at the same time. Chromatin Collection and Cross-linking—A modification of our earlier protocol was used (9). After ponasterone induction, formaldehyde was added directly to the culture medium to a final concentration of 1% (v/v), and the dishes were gently shaken on a platform shaker at room temperature for 5 min. Glycine was added to 125 mM, and the dishes were returned to the shaker for an additional 5 min. Dishes were washed three times with ice-cold PBS and harvested by scraping into 3 ml of ice-cold phosphate buffered saline (PBS). Cells from triplicate dishes were pooled and collected by centrifugation in a Sorvall RC3B at 2000 rpm for 5 min at 4 °C, and the supernatants were discarded. Cell pellets were resuspended in 10 ml of ice-cold PBS plus 200 μM phenylmethylsulfonyl fluoride. Cells were collected by centrifugation above and resuspended in 5 ml of an ice-cold solution containing 5 mM PIPES, pH 8.0, 85 mM KCl, 0.5% (v/v) Nonidet P-40 plus protease inhibitors (200 μM phenylmethylsulfonyl fluoride, 1.4 μg/ml pepstatin, 1 μg/ml leupeptin). Samples were allowed to swell on ice for 10 min before sonication with three strokes of a glass Dounce homogenizer with a Teflon pestle to release nuclei. nuclei were collected by centrifugation as above, and the supernatants were discarded. Nuclei were resuspended in 50 mM Tris, pH 7.6, 10 mM EDTA, 1% SDS plus protease inhibitors as above (50 μl/15-cm dish of cells). Samples were incubated on ice for 10 min, and immunoprecipitation dilution buffer A (0.01% SDS, 1% (v/v) Triton X-100, 1.2 mM EDTA, 16.7 mM Tris, pH 7.6, 167 mM NaCl) was added to a final volume to 750 μl. Nuclei were sonicated at 10-s bursts from a Branson sonifier model 450 with a microtip on a setting of 4. Routinely, seven repetitions of the sonication were performed, and the formaldehyde cross-link was reversed on a small aliquot of the material, and the degree of DNA fragmentation was verified by gel electrophoresis before proceeding. We routinely sonicate the chromatin so that the DNA is fragmented to an average size of 300–500 bp. If needed, sonication is repeated until this size distribution is achieved. Samples are diluted a total of 20-fold by the addition of more immunoprecipitation dilution buffer and then normalized by A₂₆₀ and stored in 0.5-ml aliquots at −80 °C.

FLAG Immunoprecipitation—Chromatin samples (500 μl) were first precipitated with mouse monoclonal serum (20 μl) and 1 μg of protein G-Sepharose (Roche Applied Science) bead slurry (60 μl of a 50/50 slurry of beads in TBS (16.7 mM Tris, pH 7.6, 167 mM NaCl), 1 mg/ml bovine serum albumin, 200 μg/ml salmon sperm DNA). Samples were incubated for 2 h at 4 °C on a rotator, and beads were collected by centrifugation in a microcentrifuge at 2000 × g. The unbound material (chromatin) was transferred to a new tube, and 25 μg of anti-FLAG M2 antibody (Sigma) were added. Samples incubated for 2 h at 4 °C on a rotator, 60 μl of blocked protein G slurry were added, and incubation was continued on the rotator for an additional 2 h at 4 °C. Samples were transferred into microspin columns (Bio-Rad) to collect the beads after the washing steps. Beads were first washed twice with 500 μl of ice-cold buffer B (0.05% (w/v) SDS, 1% (v/v) Triton X-100, 20 mM Tris, pH 7.6, 2 mM EDTA, 500 mM NaCl), washed once sequentially with buffer D (0.05% (w/v) SDS, 1% (v/v) Triton X-100, 20 mM Tris, pH 7.6, 2 mM EDTA, 500 mM NaCl), buffer E (0.25 mM LiCl, 1.0% (v/v) Nonidet P-40, 1.0% deoxycholate, 10 mM Tris, pH 7.6, 1 mM EDTA), and buffer C (0.1% (v/v) Triton X-100, 150 mM NaCl, 20 mM Tris, pH 7.6, 2 mM EDTA). Beads were transferred to a siliconized microcentrifuge tube, and bound material was eluted by incubating the beads with 75 μl of ice-cold buffer (0.1 mM EDTA, 0.5% (v/v) NaCl, and 1 μM bicine, 1.0% (w/v) SDS) while vortexing for 10 min. This was repeated a total of four times. The four eluates were pooled, and NaCl and RNAse were added to 300 mM and 10 μg, respectively, and samples were heated at 65 °C for 6 h to reverse the Schiff’s base linkage. DNA was collected using a PCR purification kit (Qiagen).

Polymerase Chain Reaction Analysis—For each reaction, 1 μl of the DNA eluate from the FLAG precipitation was used. All PCRs were analyzed in triplicate, and 30–40 cycles were used, depending on which conditions were established for the linear phase of the amplification protocol for each primer pair using the input DNA as a control. The amounts of DNA eluate used from the immunoprecipitation were chosen so that the signals achieved were in the linear range of the input PCR curve. Separate input curves for each oligonucleotide pair were included in each separate PCR experiment for accuracy. This ensures that the PCRs are within the linear range of the amplification cycle and that comparisons can be made between different promoters.

The DNA sequences for the primers are as follows. For HMG-CoA reductase oligonucleotides (−230 to +13 was amplified) the sequences are 5′-AGTACGCGGACGCTCAGGAG-3′ and 5′-AAGGAGACAGCCTT-ACGCAC-3′. For squalene synthase oligonucleotides (−237 to −3 was amplified), the sequences are 5′-ATCCAGCCGAGACTCCTCCGGCTGGCCTC-3′ and 5′-TATCGTCCACGGGGTGTCCACGATTATA-3′. For fatty acid synthase oligonucleotides (−115 to −20 was amplified), the sequences are 5′-GGCGACCGCCGCACCTTATT-3′ and 5′-GGCGGCTA-TTAACCACCGGCG-3′.

The ethidium bromide band intensities were measured using BioRad gel imaging software. Background values (samples where primary antibody was omitted from the immunoprecipitation) were subtracted, and the intensities were calculated as a percentage of the input DNA signal.

Immunoblotting—Aliquots of each normalized (by A₂₆₀) chromatin preparation (10 μl) were analyzed by SDS-PAGE. An aliquot of the same sample expressing SREBP-1a was used as a standard reference for comparison and normalization on every gel. Proteins were electroblotted onto nitrocellulose at 4 °C for 2 h. The filters were blocked overnight in TBS plus 5% (w/v) nonfat dry milk at 4 °C and reacted with a FLAG primary antibody (M2; Sigma) at 1:2000 dilution in TBS plus 0.1% (v/v) nonfat dry milk. An anti-native mouse IgG (with streptavidin secondary antibody was used (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a 1:1000 dilution in TBS with 1% (w/v) nonfat dry milk for 1 h. Filters were then developed with the SuperSignal kit (Pierce). For immunoblotting of the samples to analyze the efficiency of the FLAG immunoprecipitation, 10-μl equivalents (normalized for eluted volume) of each eluate were analyzed by this same protocol.

RNA Analysis—RNA was extracted (Trizol; Invitrogen) from cells that were cultured in companion dishes induced with ponasterone as above. Total RNA (25 μg) from each time point was analyzed by electrophoresis on a 1% agarose gel containing 2 μM formaldehyde. The gels were treated by standard methods for RNA transfer and hybridization.
with probes for HMG-CoA reductase, squalene synthase, and fatty acid synthase as described (10).

**Electrophoretic Mobility Shift Assay (EMSA)**—Cells expressing SREBP-1a or -1c were plated at 2,000,000 cells/15-cm dish and cultured for 48 h prior to the addition of ponasterone A (3 μM ponasterone A for 4 h for the cells expressing SREBP-1a and 10 μM ponasterone A for 24 h for the cells expressing SREBP-1c). Cells were harvested by scraping into 1 ml of PBS and collected by centrifugation in an RC3B (5 min at 4000 rpm). Cells were washed two times with ice-cold PBS and resuspended in 25 mM Tris, pH 8.0, 2 mM MgCl₂, 1 mM dithiothreitol, 200 μM phenylmethylsulfonyl fluoride, 0.05% (v/v) Nonidet P-40 and incubated at room temperature for 10 min. The resulting nuclei were collected by centrifugation at 2000 rpm in a microcentrifuge, and the cytoplasmic extract supernatants were discarded. The nuclei were resuspended in 2 ml of extraction buffer (0.5% (v/v) Triton X-100, 100 mM NaCl, 20 mM Tris, pH 7.6, plus protease inhibitors as above). This was incubated for 30 min on ice, and then insoluble nuclear debris was removed after centrifugation at 12,000 rpm at 4 °C. The nuclear extract supernatant was removed to a new tube, and glycerol was added to 10% before storage at −80 °C. SREBP were purified using 800 μl of FLAG-agarose bead slurry 50/50 (Sigma). Proteins were incubated with beads for 2 h at 4 °C on a rotator followed by washing with 5 column volumes of buffer A and 5 column volumes of buffer Z 0.1 (25 mM HEPES, 2 mM MgCl₂, 0.1 mM KCl, 10% (v/v) glycerol). Bound proteins were eluted with successive 50-μl elutions in Z 0.1 buffer containing a peptide with three copies of the FLAG epitope sequence (100 μg/ml). Fractions containing SREBP (detected by a FLAG immunoblot) were pooled and normalized for SREBP levels again by immunoblotting. Equal amounts of SREBP-1a- and SREBP-1c-immunoreactive proteins were used in a standard EMSA reaction (11) with 5 ng of a probe containing an SREBP binding site that was labeled with [32P]T4 kinase. The sequence of one strand of the oligonucleotide is 5′-CTAGGGGGCATCACCCACCGCCCCGGGCCCAGGCCCAGGCGGCT-3′.

**Protein-Protein Interaction Assay**—Expression and preparation of GST fusion proteins have been described (12). To 25 μl of glutathione-agarose beads (Sigma), 500 μg of the indicated crude GST fusion protein extract and 1 ml of Z 0.1 buffer (50 mM HEPES, pH 7.5, 2 mM MgCl₂, 100 mM KCl, 10% (v/v) glycerol, 0.1% (v/v) Nonidet P-40) plus 0.5% (v/v) nonfat dry milk and 5 μM dithiothreitol) were added followed by an incubation on a rotating wheel at 4 °C for 2 h. The bound GST fusion proteins were pelleted in a microcentrifuge, and nonspecifically bound proteins were removed by washing the pelleted three times with 1 ml of Z 0.1 buffer. Then 150 μg of total protein from HeLa nuclear extract were added to the bound proteins along with 1 ml of Z 0.1 buffer, and samples were incubated at 4 °C for 2 h on a rotating wheel. The reactions were pelleted again and washed three times with 1 ml of Z 0.1 buffer, and the specifically bound proteins were sedimented along with the agarose beads, resuspended in sample buffer and analyzed by SDS-PAGE followed by immunoblotting using either anti-nuclear factor Y-A antibody (Rockland), or anti-p300 antibody (Santa Cruz Biotechnology) as the primary antibody.

**RESULTS**

Pai et al. (7) inserted DNA encoding the mature forms for each of the three SREBP isoforms into an edysine-inducible expression cassette. Two copies of the FLAG peptide epitope were added at the carboxyl terminus to allow relative protein expression levels to be estimated by immunoblotting. These constructs were transfected into a mutant CHO line that can not express endogenously derived SREBPs due to a deletion in the gene encoding the site 2 protease, which is absolutely required for SREBP to traffic to the nucleus (8). The resulting cell lines were shown to induce nuclear SREBP expression in a hormone-inducible fashion, and the concentration of the edysine analog, ponasterone, was independently adjusted in each cell line to provide relatively similar levels of the different SREBP proteins in the nucleus at 24 h postinduction.

When SREBP target gene activation was evaluated in this original study, most SREBP target genes were induced efficiently by either SREBP-1a or -2. In contrast, SREBP-1c did not enhance expression of any target gene above the base-line level. Additionally, HMG-CoA reductase was activated more efficiently by SREBP-2 than SREBP-1a, and SQS mRNA induction was only observed in the line expressing SREBP-2. The reasons for the different patterns of activation were not totally clear, because SREBP-1a and -1c contain the same DNA binding domain. Also, there were some indications from transfection and transgenic overexpression studies that genes of cholesterol and fatty acid metabolism may be selectively activated by SREBP-2 (13) and SREBP-1a (14) respectively, but the absolute preference for SREBP-2 activation of SQS was surprising.

We were interested in evaluating whether the results for SREBP target gene activation in these cell lines were due to the selective association of the different SREBPs to the respective promoters in vivo using the ChIP technique. To analyze the binding of SREBPs to specific target gene promoters, we used an antibody to the FLAG peptide, which was common to all three SREBPs. Because we wanted to evaluate relatively early events in the activation process, we performed a time course for ponasterone-dependent induction of SREBP nuclear accumulation in the different cell lines, and we adjusted the ponasterone concentration to achieve similar levels of SREBP proteins over the time course period (data not shown). When the appropriate conditions were established, we performed the experiment in Fig. 1 and measured the accumulation of SREBP protein over time up to 8 h postinduction.

Representative immunoblots are presented in Fig. 1A, and the different signal intensities were measured and plotted as shown at the bottom of Fig. 1. For purposes of comparison, a control sample from one preparation of protein from the SREBP-1a-expressing cell line was analyzed on every gel for normalization of different films. The level of each protein is plotted relative to the signal intensity for SREBP-1a at 4 h, set at 100%. SREBP-1a was induced the earliest, and it reached a maximal level by 4 h and started to decline by 8 h. The kinetics for SREBP-1c and -2 induction were slightly delayed, but they accumulated to similar levels as SREBP-1a (Fig. 1).

As a control, an aliquot of the isolated chromatin from each time point was tested in a PCR to confirm that equal DNA recoveries were achieved in the different cell lines and at the different time points analyzed (Fig. 2). The data in Figs. 2, B–D, also demonstrate that the PCR signal intensities used throughout this report with all three promoters reside within the linear phase of PCR.

The remaining portions of the chromatin samples were then subjected to an immunoprecipitation protocol with the FLAG antibody to capture the DNA-bound SREBPs. Aliquots of each sample after the immunoprecipitation were first evaluated by an immunoblotting analysis to test the efficiency and consistency of the immunoprecipitation protocol. At each time point, the FLAG-tagged SREBPs were efficiently and consistently collected by the immunoprecipitation procedure in proportion to their total overall levels (Fig. 1A, compare the gels labeled Pre for direct Western blot with those labeled Post for after immunoprecipitation).

Portions of the remaining samples after the immunoprecipitation were subjected to PCR with primers designed to amplify the SREBP binding regions of the hamster promoters for HMG-CoA reductase (Fig. 3), squalene synthase (Fig. 4), and fatty acid synthase (Fig. 5). These three promoters were chosen.
because they are representative of the three patterns for SREBP target responses in the study of Pai et al. (7). Representative gels for the PCRs are shown with duplicate reactions in A of Figs. 3–5. The signal intensities from the PCR gels were measured and are plotted as a function of time along with RNA levels for each gene under study.

The ChIP analysis shows that the time course for SREBP-1a recruitment to the HMG-CoA reductase promoter lags slightly behind but closely follows the total protein induction curve (compare Figs. 1B and 3B). RNA accumulation, as measured in companion dishes of the same experiment by an RNA blotting protocol, lags slightly behind the protein binding but is robustly induced as well by 8 h. (Fig. 3B, top). Similarly, SREBP-2 binding follows the protein induction curve from Fig. 1, but maximal binding occurs at a lower level of protein induction and represents a higher percentage of the input than for SREBP-1a (Fig. 3D). Also, the SREBP-1a signal was reproducibly lower by 8 h, whereas the SREBP-2 signal was consistently strong throughout the entire time course (Fig. 3, compare B and D). These observations are consistent with SREBP-2 being...
a more efficient activator of HMG-CoA reductase. RNA accumulation in the SREBP-2-expressing cells was also relatively robust by the end of the time course.

In contrast to the results with SREBP-1a and -2, we were unable to detect SREBP-1c protein stably associated with the HMG-CoA reductase promoter above the base line (Fig. 3) although the protein was induced to a level comparable with that observed for SREBP-1a and -2. In agreement with this result, HMG-CoA reductase RNA was also not induced in the SREBP-1c-expressing cells.

SREBP-2 binding to the SQS promoter was robust and followed closely with the corresponding protein induction curve (compare Figs. 1 and 4). Interestingly, neither SREBP-1a nor 1c were recruited to the SQS promoter in any sustained manner (Fig. 4, B and C), and consistent with these ChIP data, SQS RNA was only induced by SREBP-2 as well.

Both SREBP-1a and -2 induced fatty acid synthase RNA in a time-dependent fashion after ponasterone treatment (Fig. 5). In agreement with this, SREBP-1a and -2 were recruited to similar levels on the FAS promoter over the time course with kinetics that both lag slightly behind the protein induction curves of Fig. 1 and slightly precede the RNA accumulation measured in Fig. 5, B and D. Once again, SREBP-1c expression did not result in either stable binding to the promoter or accumulation of FAS RNA (Fig. 5).

The results presented in Fig. 1 demonstrate that SREBP-1c was induced and present in the nucleus, but the protein was not found associated with any of the promoters examined in Figs. 3–5. To determine whether the SREBP-1c protein expressed in these cells possessed the intrinsic capacity to bind DNA specifically, we purified the FLAG-tagged SREBP-1c and SREBP-1a proteins from extracts of the corresponding cells using FLAG antibody immunoaffinity chromatography. When equivalent amounts of the resulting purified SREBP-1a or -1c proteins were analyzed using an in vitro gel mobility shift assay, both proteins bound similarly to an SREBP binding site probe (Fig. 6). Thus, the SREBP-1c protein binds DNA in vitro similarly to SREBP-1a, but it fails to bind to native promoters in cellular chromatin.

The differences between SREBP-1a and -1c are confined to their extreme amino termini. Thus, because the SREBP-1c protein was fully capable of binding DNA in vitro, we reasoned that its inefficient recruitment to target sites in chromatin was
due to a functional defect in its amino terminus. The activation domain of SREBP-1a was previously localized to its extreme amino terminus (3), and this region was shown subsequently to bind the transcriptional coactivators CBP/p300. These twin coactivators are capable of interacting with sequence-specific DNA-binding proteins like SREBP-1a in solution, which could then direct the co-activators to specific target sites in chromatin. If chromatin modification by the SREBP-CBP complex was required for stable DNA site recognition (4), then a deficiency in CBP/p300 binding by SREBP-1c could explain the ChIP results of Figs. 3–5.

Therefore, we compared the binding of SREBP-1a and -1c with p300 and fragments of CBP. The coding sequence for mature SREBP-1a, -1c, or -2 was fused to GST, and the resulting proteins were expressed in E. coli and tested for binding to endogenously expressed native p300 present in nuclear extracts of HeLa cells. As shown in Fig. 7A, SREBP-1a and -2 interacted specifically with p300; however, SREBP-1c did not. In previous studies, we showed that the DNA binding domain of SREBP-1 interacted with the heterotrimeric NF-Y transcription factor (12). Because this region is identical in 1a and 1c, we analyzed binding of NF-Y as a control. Aliquots of the same GST interaction assay used for p300 binding were analyzed for interaction with NF-Y using an immunoblotting protocol for the NF-YA subunit. As expected, SREBP-1c was fully capable of binding NF-Y (Fig. 7B).

We also evaluated the binding of the FLAG-SREBP1 isoforms. The FLAG-SREBP-1a or SREBP-2 bound to the GST-KIX polypeptide fusion, but similarly expressed SREBP-1c failed to interact (Fig. 7C, lanes 1–9). In a separate study, the amino-terminal C/H1 domain of CBP was shown to interact with SREBP-1a (15) and in Fig. 7C (lanes 10–15), we show that both SREBP-1a and -2 interact with this isolated domain of CBP, but once again, SREBP-1c was deficient in this key interaction.

DISCUSSION

The current studies address the mechanism for SREBP recognition and activation of endogenous target genes in native cellular chromatin. We used the chromatin immunoprecipitation method to analyze the recruitment of individual SREBP isoforms to three specific target gene promoters. Using cell lines that express only one of the three SREBPs in a cellular based hormone-inducible expression system, we demonstrated that both SREBP-1a and -2 were recruited to the endogenous HMG-CoA reductase promoter (Fig. 3), but the association of SREBP-2 was quantitatively more efficient and stable over the 8-h time course we performed. Although total levels of SREBP-1a and -2 were comparable over the time course, 1a was induced more quickly and reached a relative plateau level by 3 h. At this time point, SREBP-2 levels were only beginning to accumulate, yet the binding of SREBP-2 to the HMG-CoA reductase promoter had already reached maximal levels (compare Figs. 1 and 3). In contrast, SREBP-1a binding to the HMG-CoA reductase promoter did not reach a maximal level until later, well after maximal protein accumulation occurred. Additionally, the maximal level of binding of SREBP-1a was quantitatively lower and actually declined by 8 h, although the level of total protein remained relatively high. By design, we only evaluated events during the initial 8 h after the addition...
of ponasterone, and it is possible that SREBP-1a protein cycles on and off the promoter. This would be similar to the cyclic binding of estrogen receptor to target promoters observed using the ChIP procedure previously (16, 17). The more efficient and stable association of SREBP-2 with the promoter probably explains the more robust activation of HMG-CoA reductase mRNA observed in these same cells at 24 h after ponasterone addition (7). The stable binding of SREBP-2 is more similar to previous results for the thyroid hormone receptor, which was stably associated with two separate promoters over an extended time period of analysis (18).

The results for the squalene synthase promoter were even more striking (Fig. 4). Here, SREBP-2 was efficiently and stably recruited, whereas SREBP-1a binding was only transiently detected above base-line levels and only at the 3-h time point. Similar to HMG-CoA reductase, binding of SREBP-2 occurred before maximal protein levels were achieved. The absolute differential recruitment of the two SREBPs is sufficient to explain the same clear selectivity observed for SQS RNA accumulation (Fig. 4) (7). These results provide compelling support for the idea that SQS is a highly selective gene target for SREBP-2 (7, 19).

In the analysis of SREBP binding to the fatty acid synthase promoter, both SREBP-1a and -2 were recruited to comparable levels in a similar time frame, and ponasterone induced FAS mRNA similarly in both cell lines as well (Fig. 5). The binding of SREBP-2 did spike and return to a lower level by 8 h. The reason for this may be due to a similar cyclic association with the FAS promoter as presented above for SREBP-1a binding to the HMG-CoA reductase promoter.

In contrast to the detectable and selective binding patterns observed for SREBP-1a and -2, we were unable to detect association of SREBP-1c above base-line levels at any of the promoters analyzed in this study. This is despite the fact that SREBP-1c protein was efficiently induced, accumulated to similar levels as the other SREBPs, and was efficiently immunoprecipitated by the FLAG antibody (Fig. 1). Additionally, when the proteins were purified from the corresponding cells by affinity chromatography, both SREBP-1a and -1c interacted efficiently with an SREBP binding site probe in an EMSA (Fig. 6). Thus, the lack of SREBP-1c binding to native promoters in cellular chromatin and the lack of SREBP target gene activation are not due to the expressed protein having a diminished capacity to bind DNA. Two FLAG epitope tags were added to the carboxyl terminus, so we could accurately compare expression of all three SREBPs. It is unlikely that this small epitope alters activation by 1c, because we have added a bulkier green fluorescent protein tag to the carboxyl terminus and multiple FLAG epitopes to the amino terminus of both SREBP-1a and -1c, and the resulting fusion proteins activate SREBP target promoters to levels comparable with their nonmodified counterparts in transient assays.

In these transfection studies in cultured cells and also in mouse lines that overexpress the SREBPs, 1a activates gene expression much more robustly than 1c (20). The 1a amino-terminal domain interacts efficiently with transcriptional co-activators CBP/p300 and the mammalian mediator complex also known as ARC/DRIP (4, 6). Because the only differences between SREBP-1a and -1c are in their amino termini (20), we reasoned that SREBP-1c might be defective for interacting with CBP/p300. The studies in Fig. 7 confirm this hypothesis and strongly suggest that the weak association with co-activators is at least one reason why SREBP-1c is a relatively poor transcriptional activator compared with 1a.

CBP and p300 have intrinsic histone acetylase activity (21), and both associate with other coactivators that possess chromatin-modifying activities (22). Because SREBP-1a interacts efficiently with these co-activators and 1c does not, we have proposed the model in Fig. 8 to at least partially explain why SREBP-1c failed to associate with target promoters as a homodimer in native chromatin in our study. This model predicts that DNA binding activators such as SREBP-1a interact with coactivator complexes before binding DNA, which is consistent with all binding studies performed to date. Next, the SREBP-1a coactivator directs the associated chromatin-modifying complexes to local sites in the chromatin where the specific DNA target sites for SREBP-1 are located. In this way,
stable DNA binding and chromatin realignment is a coupled process. This model would also predict that activators with an inherently weak ability to recruit chromatin-modifying co-activators such as SREBP-1c would not support localized chromatin redistribution, thus limiting access to specific DNA target sites by the DNA-binding protein. It also predicts that a distinct class of activator proteins similar to SREBP-1c that do not recruit proteins with chromatin-remodeling activities efficiently probably function in conjunction with additional activators that possess these activities, or they stimulate expression of target genes at sites in chromatin where the requisite remodeling has already occurred and/or is not acutely required.

In animal studies, induction of the lipogenic gene program that occurs in liver during the refeeding phase of a fasting/high carbohydrate refeeding regimen is a classic insulin signaling response. The SREBP-1c mRNA is specifically down-regulated during fasting and is robustly activated during the refeeding phase (23, 24). Several of the key genes activated during the lipogenic response are specifically activated by SREBPs (1, 25). Additionally, several gene targeting and overexpression studies combined with nutrient restriction indicate that SREBP-1c induction is required for the normal lipogenic gene response to insulin (reviewed in Refs. 1 and 26). However, our current studies indicate that when only one SREBP-1 isoform is expressed in the nucleus of CHO cells that 1a activates target genes efficiently, but 1c fails to activate gene expression at all. Moreover, 1c is also not efficiently recruited to target gene promoters. It is possible that if we expressed SREBP-1c at higher levels, it would be recruited to target genes and stimulate gene expression as a homodimer in the CHO cells. This could be analogous to the situation in the mammalian liver, where SREBP-1c is expressed at 5–10 times higher levels than 1a and it is down-regulated by fasting and induced by LXR ligands. It is also possible that there is another transcriptional co-activator that is not expressed in CHO cells or that 1c partners with 1a, SREBP-2 (which is also regulated by fasting and refeeding in the liver (1)), or another bHLHLZ protein, allowing SREBP-1c to stably recognize its target sites in the refeed liver.

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