SREBP transcriptional activity is mediated through an interaction with the CREB-binding protein

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The sterol regulatory element binding proteins (SREBP-1 and -2) activate transcription of genes whose products are involved in the cellular uptake and synthesis of cholesterol. Although considerable effort has been exerted to define the events regulating the levels of active SREBP, little is known about the transcriptional cofactors mediating SREBP function. In an unbiased search for potential coactivators of SREBP, we isolated a protein of 265 kD from HeLa cells that directly bound SREBP-1 and SREBP-2. Peptide sequencing and Western blot analysis established that the 265-kD protein was CBP (CREB-binding protein), a recently identified transcriptional coactivator. The putative activation domain of SREBP was shown to bind specifically to amino-terminal domains of recombinant CBP and p300 (a CBP-related protein). Moreover, transfection studies demonstrated that CBP enhances the ability of SREBP to activate transcription of reporter genes in HeLa cells. Together, these data suggest that CBP mediates SREBP transcriptional activity, thus revealing a new step in the biochemical pathway regulating cholesterol metabolism.

[*Key Words:* SREBP; CBP; transcriptional coactivators; cholesterol metabolism]

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Cholesterol is essential to humans and other animals both as a component of cellular membranes and as a biosynthetic precursor to molecules such as vitamin D and steroid hormones (Goldstein and Brown 1977). Amounts of cellular cholesterol are strictly regulated in higher eukaryotes through a negative feedback mechanism (Brown and Goldstein 1986). Under circumstances of intracellular cholesterol depletion, the cell responds by stimulating transcription of genes whose products are involved in the uptake (LDL receptor gene) and synthesis (HMG-CoA synthase and HMG-CoA reductase genes) of cholesterol (Brown and Goldstein 1980; Goldstein and Brown 1984). Conversely, when cellular cholesterol levels are high, transcription of these genes is down-regulated. Cholesterol uptake occurs via binding of plasma low-density lipoprotein (LDL) to LDL receptors that reside on the surface of all cells. The LDL-bound receptor is internalized, and the cholesterol component of LDL is liberated for cellular use by the action of lysosomal enzymes (Goldstein et al. 1985).

Transcriptional regulation of the HMG-CoA synthase, HMG-CoA reductase, and LDL receptor genes is mediated through a DNA element common to the promoters of each of these genes (Goldstein and Brown 1990). Deletion of this sequence, known as the SRE (sterol regulatory element), specifically results in loss of

feedback responsiveness to cellular sterol depletion. A synthetic SRE DNA fragment was used as an affinity probe to isolate two SRE-binding proteins, SREBP-1 and SREBP-2 (Hua et al. 1993; Wang et al. 1993; Yokoyama et al. 1993). The SREBPs, which share considerable amino acid sequence, have been shown in transfection experiments to act as transcription factors, increasing the expression of reporter genes containing SREs (Hua et al. 1993; Sato et al. 1994). Because the SREBP-1 and SREBP-2 proteins are extremely similar, for purposes of simplicity we will refer to them generically as SREBP when describing their shared characteristics.

Recent evidence indicates that SREBP itself is regulated by a proteolytic process whose activity is responsive to cellular sterol levels (Wang et al. 1994; Sakai et al. 1996). Nascent SREBP exists in a transcriptionally inactive form bound to the membranes of the endoplasmic reticulum and the nucleus. Under circumstances of sterol deprivation, SREBP is proteolytically cleaved, resulting in release of a soluble SREBP peptide into the cytoplasm. Following its subsequent migration into the nucleus, this peptide binds the SRE and up-regulates gene expression through either direct or indirect communication with as yet unidentified components of the transcription machinery.

In an initial search for proteins within the transcription apparatus that might serve such a coactivator role, we tested the RNA polymerase II (Pol II) TAFs (TBP-

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associated factors} (Dynlacht et al. 1991) for a potential interaction with SREBP-1. The Pol II TAFs, along with TBP (TATA box-binding protein), constitute the general transcription factor TFIID, which is an integral component of the transcription machinery. Several of these TAFs have been shown to act as transcriptional coactivators, binding directly to the activation domains of sequence-specific transcription factors such as Sp1 (Chen et al. 1994; Gill et al. 1994), VP16 (Goodrich et al. 1993; Klemm et al. 1995), p53 (Lu and Levine 1995; Thut et al. 1995), bicoid (Sauer et al. 1995), and hunchback (Sauer et al. 1995). Through these interactions with Pol II TAFs, it is thought that enhancer-binding factors can recruit the transcription apparatus to specific promoters to stimulate the production of messenger RNA (Goodrich and Tijan 1994; Sauer et al. 1995). In contrast to results obtained with many other activators, SREBP immobilized on beads failed to interact specifically with the Pol II TAFs (data not shown). This result suggested that SREBP might direct transcriptional activation by contacting other targets in the transcription apparatus, including factors that are not intrinsic components of the preinitiation complex.

To address these possibilities in a systematic manner, we used an SREBP protein probe in Far Western blot analysis to search for potential SREBP-interacting proteins that might be present in crude human and *Drosophila* cell nuclear extracts. Remarkably, this screen yielded a single protein of 265 kD (p265) from both species that bound specifically to the activation domains of both SREBP-1 and SREBP-2. The p265 protein was purified on a preparative scale, and its identity was determined by peptide microsequencing. Subsequent binding studies performed with recombinant fragments of p265 confirmed the interaction and permitted mapping of the SREBP-binding domain on p265. Finally, cotransfection experiments in HeLa cells revealed that p265 acts to stimulate SREBP-dependent transcriptional activation. These results implicate p265 as a candidate coactivator for SREBP and define a new step in the cholesterol regulatory pathway.

Results

Biochemical screen for SREBP-binding proteins

It was shown previously that a 90-amino-acid amino-terminal deletion of hamster SREBP-1 abolished the protein's ability to activate transcription in mammalian cell transfection experiments (Sato et al. 1994). Moreover, the truncation imparted dominant-negative activity, as cotransfection of this shorter SREBP-1 form along with nonamino-terminally truncated SREBP-1 inhibited the latter's transcriptional activity. These data suggested that the 90-amino-acid deletion crippled the activation domain of SREBP-1 without adversely affecting its ability to bind DNA (the DNA-binding domain of SREBP-1 is not contained within this deletion).

On the basis of these results, we designed a screen to isolate proteins that might contact the SREBP activation

domain and thereby mediate its function. Recombinant proteins containing amino acids 1–323 or 91–323 of human SREBP-1 fused to the carboxyl terminus of glutathione *S*-transferase (GST) were produced in *Escherichia coli* (referred to as GS1 and GS1Δ, respectively). GST fusion proteins incorporating homologous regions of human SREBP-2 (amino acids 1–330 and 94–330) were also produced (called GS2 and GS2Δ, respectively). As an initial experiment, both SREBP-2 proteins were radioactively labeled and used individually to probe Far Western blots containing proteins derived from crude HeLa cell nuclear extracts. The GS2 probe recognized a single band migrating at 265 kD (p265), whereas GS2Δ failed to bind any of the proteins on the membrane (Fig. 1A).

To determine whether SREBP-2 could also associate with p265 in solution, glutathione–agarose beads prebound to the GST fusion proteins were incubated with crude HeLa nuclear extracts. Bound HeLa cell proteins were eluted with SDS sample buffer, separated by PAGE, transferred to nitrocellulose membranes, and probed with labeled GS2. The p265 band was apparent in the lane corresponding to the GS2 pulldown but not in the adjacent lane in which GS2Δ was used as the affinity resin (Fig. 1B, lanes 3,4). Strikingly, GS1 and GS1Δ behaved identically to their SREBP-2 counterparts in this assay, suggesting that the activation domains of both SREBP family members interact with the same 265-kD protein (Fig. 1B, lanes 1,2). This notion is further supported by the finding that the SREBP-2 Far Western probe recognized the band precipitated by the SREBP-1 affinity beads.

Transfection of mammalian SREBP-1 and SREBP-2 expression vectors into both mammalian (Hua et al. 1993; Sato et al. 1994) and *Drosophila* (Sanchez et al. 1995) cells (Schneider cells) has been shown to result in transcriptional activation of SRE-containing reporter genes, indicating that the mechanisms used by flies and mammals to support SREBP-induced transcription are functionally conserved. Thus, if the role of p265 in binding both SREBPs is to serve as a transcriptional coactivator, one might expect that a functional equivalent to p265 would also exist in *Drosophila*. To test this possibility, we performed an identical assay to that shown in Figure 1B, except that Schneider cell nuclear extracts were substituted for HeLa cell nuclear extracts. Consistent with our prediction, the activation domains of both SREBPs interacted specifically with a protein present in the *Drosophila* extract (Fig. 1C, lanes 1,2,4). As this band migrates at virtually the same size as a human p265, it may represent a *Drosophila* p265 homolog.

Purification of p265

In an effort to purify p265, we sought initially to determine whether the protein was sufficiently abundant to directly visualize in gels. Crude HeLa cell nuclear extracts were bound to each of the four GST–SREBP affinity resins, eluted with SDS sample buffer, separated by PAGE, and stained with silver. Remarkably, both GS1 and GS2 precipitated the same set of four high-molecu-

Figure 1. Screen for proteins that interact with the SREBP activation domain. (A–C) 32 P-labeled GST fusion proteins were used as Far Western blotting probes to detect SREBP-interacting proteins in crude HeLa nuclear extracts (A) and affinity bead precipitations from crude HeLa (B) and Schneider (C) nuclear extracts. The size of p265 was determined by extrapolation from a plot of marker values vs. gel migration distance (data not shown). (D) Proteins were precipitated as in B and stained with silver.

lar-weight bands after only this one purification step (Fig. 1D, lanes 1,3). Furthermore, the association appeared to be specific, as GS1 Δ and GS2 Δ failed to bind any proteins in the HeLa nuclear extract (Fig. 1D, lanes 2,4).

The highest molecular weight band detected by silver staining comigrated with the 265-kD protein identified by Far Western blotting, prompting us to focus our attention on this band. To determine whether the 265-kD signal observed in each of these assays represented the same protein, crude HeLa nuclear extracts were further fractionated and reassayed by both methods. Upon fractionation by phosphocellulose chromatography, the p265 Far Western signal was shown to peak at a salt elution step of 0.3 M KCl (Fig. 2A, lane 4). The 0.3 M KCl eluate was dialyzed to 0.1 M KCl and subsequently fractionated by anion exchange chromatography (Poros Q resin). Both Far Western blotting and silver staining of the resultant fractions revealed superimposable patterns of gradient elution activity with respect to the 265-kD signal (Fig. 2B, middle and upper panels). By both assays, the abundance of p265 peaked in fractions 5 and 6 (to avoid confusion, it should be noted that in this experiment p265 was not the lowest mobility band seen on silver staining; a band of $\sim$ 300 kD, which was not reactive by Far Western blotting and which we had not observed previously, eluted in fractions 6–11). Taken together, these results suggest strongly that both assays detected the same SREBP-binding activity.

As p265 appeared to be relatively abundant, we chose to purify it on a preparative scale to determine its identity by peptide sequence analysis. GS2 affinity resin was

incubated with phosphocellulose-fractionated HeLa nuclear extracts, followed by high-salt elution of bound proteins. The eluted proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane, and the p265 band was excised for microsequencing. Using the resultant peptide sequences (Fig. 2C) as templates to search protein data bases, p265 was unambiguously identified as the human CREB-binding protein (CBP), a recently isolated 265-kD protein that exhibits properties of a transcriptional coactivator (Arias et al. 1994; Kwok et al. 1994; Bannister and Kouzarides 1995; Dai et al. 1996; Kamei et al. 1996).

To further ensure that CBP was the SREBP-binding protein, Western blots derived from the Poros Q-fractionated HeLa nuclear extracts described above were probed with antibodies directed against mouse CBP. This antiserum recognized a polypeptide of 265 kD whose elution pattern exactly matched that observed by silver staining and Far Western blotting (Fig. 2B, lower panel), confirming the equivalence of p265 and CBP.

Mapping CBP's SREBP-interacting domain

We then sought to map the SREBP-binding domain on CBP. Crude *E. coli* protein lysates containing recombinant mouse CBP fusion proteins (CBP amino acids 1–682, 451–682, or 1680–1891) were electrophoretically separated, blotted, and probed with GS2. As shown in Figure 3, the probe bound avidly to amino-terminal fragments of CBP (amino acids 1–682) and p300 (amino acids 1–663) (Yee and Branton 1985; Harlow et al. 1986), a paralog of CBP. To a somewhat lesser extent, GS2 also

Figure 2. Purification and identification of p265. (A) Far Western blot to detect p265 in HeLa nuclear extracts fractionated by phosphocellulose chromatography. The eluate containing peak p265 activity (lane 4) was further fractionated (B) by anion exchange chromatography (Poros Q) with column loading at 0.1 M KCl HEMG and elution by salt gradient from 0.1 M to 1 M KCl HEMG. The 12 fractions shown represent eluates from the initial portion of the gradient (0.1–0.37 M KCl HEMG). Each of the resulting fractions was purified in batch with GST-SREBP-2 (amino acids 1–330) affinity beads and assayed by silver staining, Western blotting (polyclonal anti-CBP), and Far Western blotting [GST-SREBP-2 (amino acids 1–330)] using the probes indicated in parentheses. (C) Alignment of p265 peptide sequences with human CBP and p300. Areas in which p300 fails to match the peptide sequences are shown in gray.

interacted with a smaller region of CBP (amino acids 451–682), containing the CREB-binding domain (Lundblad et al. 1995). By comparison, GS2 interacted about 50-fold less efficiently with more carboxy-terminal portions of both CBP and p300 [the E1A-binding domains of both proteins (Arany et al. 1995; Lundblad et al. 1995)].

Figure 3. Interaction between the SREBP-2 activation domain and recombinant versions of CBP and p300. Crude *E. coli* lysates containing overexpressed CBP and p300 proteins were assayed either by Coomassie staining (left) or Far Western blotting (right; GST-SREBP-2 [amino acids 1–330] probe). Proteins CBP (amino acids 1–682), CBP (amino acids 451–682), and p300 (amino acids 1–663) were produced as fusions to GST. The remaining proteins were expressed as fusions to a six-histidine affinity tag. The relative intensities of Far Western blotting signals were determined by PhosphorImager (Fuji Bas 1500), and values represent the average of duplicate blots.

Moreover, the probe had no discernible affinity for any native *E. coli* proteins, providing further support for the specificity of the SREBP–CBP interaction. These results suggest that the activation domains of SREBP-2 and CREB may share a common or overlapping target binding region on CBP (Lundblad et al. 1995) (amino acids 451–682). The data also demonstrate that eukaryotic protein modifications are unnecessary for CBP–SREBP interaction, as all mammalian proteins used in these assays were produced in *E. coli*. In contrast, it has been shown previously that CREB must be phosphorylated to bind CBP (Chrivia et al. 1993).

Functional characterization of the SREBP–CBP interaction

To determine the functional implications of the SREBP–CBP interaction, we performed transfection experiments in HeLa cells. Transfection of expression vectors encoding either hamster SREBP-1 (amino acids 1–410) or human SREBP-2 (amino acids 1–462) stimulated the activity of a reporter construct containing a single copy of the SRE in its native context within the LDL receptor promoter (Fig. 4A, bars 7,11) but failed to stimulate a reporter lacking the LDL receptor promoter sequences (Fig. 4A, bars 1–3). Furthermore, the addition of increasing amounts of CBP expression construct resulted in a progressive enhancement of activator-dependent reporter activity (Fig. 4A, bars 8,9,12–14). The CBP titrations had little effect on basal reporter activity (Fig. 4A, bars 4–6).

To rule out any indirect effects of CBP on SREBP that might be unrelated to the SREBP activation domain and to allow for the controlled comparison of several activators on a single promoter, we constructed mammalian

Figure 4. Functional characterization of the SREBP-CBP interaction by transfection. (A) HeLa cells were transfected with CMV promoter-driven expression constructs encoding hamster SREBP-1 (amino acids 1–410) or human SREBP-2 (amino acids 1–462) (250 ng and 400 ng per well, respectively) in six-well plates by calcium phosphate precipitation. pCMV-CBP was cotransfected in the indicated amounts (μg). Five hundred nanograms each of reporter plasmid (pLDL-Syn-TATA-luc or pSyn-TATA-luc; described in Materials and Methods) and transfection control vector (pCMV-lacZ) were used in each transfection. Where necessary, CMV vector lacking an insert was added to equalize the total amount of CMV expression plasmid present in each transfection. All luciferase values (mean ± s.d.) have been corrected for variations in β-galactosidase activity (a measure of transfection efficiency). Values represented by bars 1–9 and 10–14 have been normalized to basal reporter activity (bars 4 and 10, respectively). (B) Transfections were performed and analyzed as above. A plus sign indicates the inclusion of 5 μg of pCMV-CBP in the transfection. The reporter used in all transfections was pG2-TK-luc, a luciferase reporter containing two tandem Gal4 DNA-binding sites upstream of the herpes simplex virus thymidine kinase promoter. The activator constructs (described in Materials and Methods) were used in the following amounts (ng per transfection): pCMV-Gal4, 150 ng; pCMV-Gal4-SREBP-2 (amino acids 1–330), 50 ng; pCMV-Gal4-CREB (amino acids 1–283), 90 ng; pCMV-Gal4-p54 (amino acids 1–42 duplicated), 500 ng. Because the CREB activation domain must be phosphorylated to interact with CBP, a construct expressing the catalytic domain of protein kinase A (Maurer 1989) was included in the Gal4-CREB transfections (1 μg).

expression vectors encoding the yeast Gal4 DNA-binding domain fused to the activation domains of three different transcription factors: SREBP-2 (amino acids 1–330), CREB (amino acids 1–283), and p53 (amino acids 1–42 tandemly duplicated). Gal4-CREB served as a positive control in these experiments, having been shown previously to be responsive to the effects of CBP in transfections (Arany et al. 1995). In contrast, no physical or functional relationship has been demonstrated between p53 and CBP. p53 has been shown to activate transcription via interaction with the TAF_{II}40 and TAF_{II}60 coactivators (Lu and Levine 1995; Thut et al. 1995). When transfected into HeLa cells, all three of these Gal4 fusion constructs directed transcriptional activation of a reporter containing two Gal4 DNA-binding sites upstream of a herpes simplex virus thymidine kinase core promoter sequence (Fig. 4B, bars 5,7,9). In contrast, expression of the Gal4 DNA-binding domain alone had no effect on reporter activity (Fig. 4B, bar 3). Coexpression of CBP markedly enhanced the Gal4-CREB and Gal4-SREBP-2 reporter stimulation (Fig. 4B, bars 6,8). CBP expression also had a slight effect on reporter activity in the presence of Gal4-p53, but this marginal relative induction was also observed in the presence of Gal4 only and in the absence of activators, suggesting that this reporter stimulation was p53 independent (Fig. 4B, bars 2,4,10). Together, these transfection data provide functional support for the hypothesis that CBP plays a role in transducing the SREBP transcriptional activation signal.

Discussion

Searching in an unbiased manner for factors that might mediate the activating signal of SREBP, we isolated the recently identified transcriptional coactivator known as CBP. By Far Western blotting, as well as interaction assays in solution, human CBP derived from a cell extract was shown to bind specifically to the activation domains of both SREBP-1 and SREBP-2. This interaction was confirmed using recombinant versions of CBP, and the region on CBP that contacts SREBP was mapped to its amino-terminal domain. The amino terminus of p300, a protein whose functional properties are indistinguishable from those of CBP, was also shown to interact with SREBP. Furthermore, these experiments revealed that a *Drosophila* protein of similar size to human CBP specifically bound both SREBP activation domains. Finally, transfection studies demonstrated that CBP enhances the ability of SREBP to activate transcription of reporter genes in HeLa cells, thus suggesting that CBP may serve as a coactivator role for SREBP similar to that shown for other transcriptional activators (Janknecht and Hunter 1996).

CBP was first isolated in a systematic screen for CREB-interacting proteins (Chrivia et al. 1993). Using candidate approaches, CBP and/or p300 has been shown to bind also to c-Jun (Arias et al. 1994), c-Fos (Bannister and Kouzarides 1995), YY1 (Lee et al. 1995), c-Myb (Dai et al. 1996; Oelgeschlager et al. 1996), MyoD (Yuan et al. 1996), and nuclear receptors (Kamei et al. 1996). The

characteristics of each of these activator-coactivator interactions have unique features. For example, whereas SREBP, CREB, c-Jun, and c-Myb appear to bind a common region of CBP (amino acids 461–682), the nuclear receptors bind the extreme amino terminus (CBP amino acids 1–101), and c-Fos binds amino acids 1621–1877. Additionally, the activator-coactivator contacts differ in their requirements for protein modification. For example, CREB requires phosphorylation by the cAMP-dependent protein kinase (PKA) to interact with CBP and p300. This modification appears to be an intrinsic regulatory feature of this biochemical pathway. For several other transcription factor–CBP/p300 interactions, however, the binding does not appear to require eukaryotic protein modification. From a teleological standpoint, it is understandable that such modifications would be dispensable for those activators whose activity is modulated by other mechanisms. The function of SREBP, for example, is already tightly controlled by proteolytic cleavage, whereas the activities of the nuclear receptors are regulated by ligand binding.

CBP and p300 augment activation of all transcription factors that they have been shown to bind, suggesting that CBP and p300 may serve a general role in coactivating transcription by a large number of sequence-specific activators. The mechanism by which CBP and p300 transmit their signals to up-regulate transcription is therefore of considerable interest, and several studies have attempted to address this question. Kwok et al. (1994) showed that bringing CBP to a promoter is sufficient to activate transcription, even in the absence of an added enhancer factor. In this study a transfected construct expressing a carboxy-terminal portion of CBP (lacking the CREB- and SREBP-interaction domains) fused to the yeast Gal4 DNA-binding domain was observed to stimulate expression of a reporter gene bearing Gal4 DNA-binding sites [a similar result was subsequently obtained using Gal4–p300 fusions (Yuan et al. 1996)]. They further showed that this region of CBP was capable of contacting TFIIB, a general transcription factor for RNA Pol II transcription. It has been reported additionally that p300 binds either directly or indirectly to TBP (Abraham et al. 1993) and that CBP associates with RNA Pol II (Kee et al. 1996). These data suggest the possibility that CBP and p300 play a role in recruiting the basal transcription apparatus to p300/CBP-responsive promoters. Nonetheless, it is still too early to discern whether these interactions play such a role *in vivo*.

Recently, Yang et al. (1996) reported an interaction between p300/CBP and a novel protein that they called p/CAF (p300/CBP-associated factor). The discovery of this interaction was intriguing for two reasons. First, p/CAF was demonstrated to compete with the adenoviral E1A protein for binding to a carboxy-terminal region on p300 and CBP. E1A had been shown previously to down-regulate both p300 and CBP activity via its interaction with these coactivators (p300 was initially isolated as an E1A-interacting protein) (Yee and Branton 1985; Harlow et al. 1986; Arany et al. 1995; Lundblad et al. 1995). Interestingly, transfected p/CAF and E1A have

antagonistic effects on cell cycle progression, implying that p300/CBP function may be regulated or mediated through the domain targeted by p/CAF and E1A. The other remarkable finding of this study was that p/CAF appeared to exhibit intrinsic histone acetylase activity. Although the function of histone acetylation has not been fully elucidated, it is thought to be involved in the restructuring of chromatin architecture (Turner 1993; Wolffe 1994). Thus, p/CAF might transduce the p300/CBP signal through displacement or repositioning of histones, which might, in turn, indirectly facilitate recruitment of the transcription apparatus to specifically targeted promoters. This is a tantalizing hypothesis in light of recent observations that the synergistic transcriptional activation mediated by SREBP and Sp1 observed *in vivo* (Briggs et al. 1993; Sanchez et al. 1995) can only be reiterated *in vitro* when histones are incorporated into DNA templates (K. Robinson, J. Kadonaga, J. Oliner, and R. Tjian, unpubl.). It is interesting that the cooperative effects of another pair of sequence-specific transcription factors, c-Myb and NF-M, may also be mediated by CBP (Oelgeschlager et al. 1996), although the mechanism underlying synergism in this system is unknown.

Although this work reveals an additional step in the metabolic cascade regulating cellular cholesterol levels, the mechanism through which CBP transmits its signal from SREBP (and other transcription factors) to the transcription machinery remains to be defined in detail. It should be emphasized that the above mechanisms proposed to account for CBP function in transcription are not mutually exclusive nor do they preclude the involvement of other interactions in the transmission of the CBP signal. Other factors, including the nuclear hormone receptor coactivator SRC-1 (Onate et al. 1995; Kamei et al. 1996), are being evaluated in this regard (Jankecht and Hunter 1996). Given the growing number of activators whose effects are mediated through CBP, it is likely that elucidation of the pathway in which CBP acts will have wider implications for the study of transcriptional control.

Materials and methods

E. coli expression plasmids

*Bam*HI–*Eco*RI-digested PCR products encoding human SREBP-1 (amino acids 1–323 and 91–323) and human SREBP-2 (amino acids 1–330 and 94–330) were cloned into *Bam*HI–*Eco*RI-digested pGEX-2TK (Pharmacia), a vector designed for the expression of GST fusion proteins in *E. coli* (this vector also encodes the recognition sequence for the catalytic subunit of bovine cAMP-dependent protein kinase, thus permitting radiolabeling of GST fusion proteins). The expression vectors encoding histidine-tagged CBP (amino acids 1–682), CBP (amino acids 451–682), and p300 (amino acids 1–663), as well as those encoding GST-tagged CBP (amino acids 1680–1891) and p300 (amino acids 1642–1856), were gifts of J. Lundblad, R. Kwok, and R. Goodman (Lundblad et al. 1995).

Mammalian expression plasmids and reporters

Cytomegalovirus (CMV) promoter-driven expression vectors encoding human SREBP-2 (amino acids 1–462), Gal4 (amino

acids 1–147), Gal4-rat CREB (amino acids 1–283), Gal4-human SREBP-2 (amino acids 1–330), and full-length mouse CBP were produced in pcDNA3.1 (+) (Invitrogen). The inserts were prepared either by excision or PCR amplification from pXH-4 (Hua et al. 1993), pBS Gal4 (1–147) (Attardi and Tjian 1993), pRcRSV-CREB341 (Loriaux et al. 1993), GST-SREBP-2 (amino acids 1–330) (described above), and pCMX-CBP (R. Kwok and R. Goodman, unpubl.), respectively. Briefly, each construct was produced by a trimolecular ligation involving the above inserts (digested with *NdeI*-*XbaI* or *NdeI*-*XhoI*), a *HindIII*-*NdeI*-digested 110-bp DNA fragment derived from pT β -Stop (Jantzen et al. 1990) containing the β -globin translational leader sequence and a hemagglutinin epitope tag, and *HindIII*-*XbaI*- or *HindIII*-*XhoI*-digested pcDNA3.1 (+). The vector pCMV-Gal4-p53 (a gift of C. Thut, University of California, Berkeley, unpubl.) expresses a Gal4 DNA-binding domain fusion to two tandem copies of human p53 (amino acids 1–42). The vector pRc/CMV7S-hamster SREBP-1 (amino acids 1–410) was generously provided by S. Scheek, M. Brown, and J. Goldstein (Sato et al. 1994). The protein kinase A expression construct pRSV-PKA (Maurer 1989) was kindly given to us by J. Lundblad (Oregon Health Sciences University, Portland).

The pLDL-Syn-TATA-luc reporter (Sanchez et al. 1995) contains an SREBP-responsive enhancer sequence derived from the LDL receptor promoter that has been placed upstream of a heterologous core promoter and the luciferase gene. This construct is derived from pSyn-TATA-luc, which lacks the LDL receptor enhancer sequences (Sanchez et al. 1995). Both vectors were kindly provided by T. Osborne. The pG2-TK-luc plasmid, which was produced by S. Hollenberg and V. Giguere from pBLCAT2 (Luckow and Schutz 1987), is a luciferase reporter containing two tandem Gal4 DNA-binding sites upstream of the herpes simplex virus thymidine kinase promoter.

Purification and identification of p265

Crude HeLa and Schneider cell nuclear extracts were produced according to the method of Dignam et al. (1983) and phosphocellulose (Whatman) fractionation was performed as described (Matsui et al. 1980). Anion exchange chromatography (Poros Q, Poros Corp.) was performed by fast protein liquid chromatography (FPLC; Pharmacia) with column loading at 0.1 M KCl HEMG (25 mM HEPES at pH 7.6, 0.1 mM EDTA, 12.5 mM MgCl₂, and 10% glycerol) and elution by salt gradient from 0.1 M to 1 M KCl HEMG. All HeLa and Schneider cell extracts and fractions were dialyzed to 0.1 M KCl HEMG prior to incubation with GST-SREBP affinity resins. GST fusion proteins were expressed in *E. coli* strain DH5 α and purified as described (Smith and Johnson 1988) using glutathione-agarose beads (Pharmacia). Diagnostic and preparative (see below) scale purifications of p265 using GST affinity resins were performed in essentially the same manner, except that beads used for the former purpose were eluted with Laemmli sample buffer (Laemmli 1970).

Preparative purification of p265 was performed as follows: A crude HeLa cell nuclear extract derived from 290 liters of suspension culture was fractionated by phosphocellulose chromatography, and the protein eluting at 0.3 M KCl HEMG was dialyzed to 0.1 M KCl HEMG, followed by the addition of NP-40 to a final concentration of 0.1%. After ultracentrifugation and filtration (0.22- μ m pore size), this protein was mixed with 7 ml of GST-SREBP-2 (amino acids 1–330) affinity resin and nutated 3 hr at 4°C. The beads were washed four times with 0.1 M KCl HEMG containing 0.1% NP-40, and bound proteins were eluted in batch for 1 hr with 10 ml of 2 M NaCl HEMG containing 0.01% NP-40. The eluate was concentrated by dialysis against PD buffer (1 mM Tris at pH 7.8, 20% PEG 20,000, 0.02% SDS, 0.01% NP-40, and 1 mM DTT), transferred to Eppendorf tubes,

and further concentrated in an evaporation chamber (SpeedVac, Savant) until a final volume of 300 μ l was attained. The sample was separated by SDS-PAGE, transferred to a nitrocellulose membrane, and stained with Ponceau S to permit excision of bands from the membrane. The band corresponding to p265 was digested with Lys-C endoproteinase (Wako), and the resulting peptides were purified by reversed phase chromatography and sequenced. The peptide sequences (shown in Fig. 2C) were used as templates to search protein sequence data bases using the National Center for Biotechnology Instruction (NCBI) BLAST search program.

Western blotting

Western blots were performed as described (Oliner et al. 1992) using rabbit polyclonal antiserum raised against CBP amino acid 455–600 (a gift of M. Montminy, Salk Institute, La Jolla, CA, antiserum #5614) at a 1:5000 dilution.

Far Western blotting

Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and incubated 1 hr at room temperature in blocking buffer (25 mM HEPES at pH 7.6, 25 mM NaCl, 5 mM MgCl₂, 5% nonfat dry milk, 1 mM DTT, and 0.05% NP-40). The membranes were transferred to heat-seal bags and rocked overnight at 4°C in probe buffer (20 mM HEPES at pH 7.6, 75 mM KCl, 2.5 mM MgCl₂, 0.1 mM EDTA, 0.05% NP-40, and 1% nonfat dry milk; 5 ml of probe solution per blot) containing ~ 1 μ g of radiolabeled GST fusion protein (3×10^6 cpm/ml of probe solution), as well as crude lysates from bacteria overexpressing GST-SREBP-1 (amino acids 91–323) or GST-SREBP-2 (amino acids 94–330) (to block nonspecific probe interactions). Membranes were washed (by rocking for 30 min at 4°C with 100 ml of probe buffer lacking GST fusion proteins) and exposed to film or PhosphorImaging screen (Fuji).

GST fusion proteins were radiolabeled (30 min at 30°C) in 30- μ l reactions containing 20 mM Tris (pH 7.5), 100 mM NaCl, 12 mM MgCl₂, 1 mM DTT, 0.4 μ l of glutathione-agarose-bound GST fusion protein (~ 2 μ g of protein), 10 μ l of Sepharose CL4B beads (Pharmacia; inert bead filler), 450 μ Ci [γ -³²P]ATP (Amersham PB 15068), and six units of bovine heart muscle kinase (Sigma P-2645). After washing the beads four times with 1 ml of GE buffer (100 mM Tris at pH 8.0, 120 mM NaCl, 0.1% NP-40), the radiolabeled proteins were eluted in GE buffer containing 20 mM reduced glutathione (Sigma G-4251).

Transfections

HeLa cell monolayers were seeded at 1×10^5 cells per well in six-well tissue culture plates (Falcon) 16–24 hr before CaPO₄-mediated transfection (Sambrook et al. 1989). The media (Dulbecco's modified eagle media supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, 100 U/ml of streptomycin, and 2 mM glutamine) were replaced 12–18 hr after transfection; 30–36 hr later, the cells were harvested, lysed, and assayed for luciferase and β -galactosidase activity, as described previously (Wang and Tjian 1994).

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