Regulation of the Human P450scc Gene by Steroidogenic Factor 1 Is Mediated by CBP/p300*

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Regulation of the human CYP11A gene encoding cytochrome P450scc, which catalyzes the first step of steroid synthesis, is regulated by many trans-acting transcription factors including steroidogenic factor 1 (SF-1). Transfection experiments in human adrenal NCI-H295 cells demonstrate regulation of the P450scc gene promoter region that contains several putative SF-1 binding sites. Cotransfection of SF-1 with a luciferase reporter construct containing the P450scc gene 5'-flanking region from nucleotides −1676 to +49 increased promoter activity, and deletion of the nucleotide sequence from position −1676 to −1620, which removes a putative cAMP response element (CRE), did not affect the stimulatory response to SF-1. As well, further deletion of the promoter region to nucleotide −110, which contains only one SF-1 binding site, still retained the ability to respond to exogenous SF-1. However, mutation of the remaining site which abolished SF-1 protein/DNA interaction also abrogated any functional response to the factor. All the P450scc reporter constructs which responded to SF-1 were further stimulated by exogenous p300 and CREB-binding protein (CBP), suggesting interaction between SF-1 and p300/CBP. As well, mutation of the binding site that abrogated the response to SF-1 also abolished the response to p300 and CBP. Cotransfection of the adenovirus E1A oncprotein, which has been shown to interact with p300/CBP and interfere with its function, decreased the stimulatory effect of SF-1 and p300/CBP. Cotransfection of a mutated E1A protein, RG2, which does not interact with p300/CBP, did not alter the stimulatory effect of SF-1 and p300/CBP on the P450scc promoter. Deletion of the region from amino acid residues 2–67 in E1A, which has been postulated to interact with p300/CBP, also abolished the inhibitory effect of E1A, whereas deletion of the region from residues 120 to 140 had no effect. Two regions of CBP from amino acids 1 to 451 and from 1460 to 1891 were demonstrated to interact with SF-1 in vitro. Coexpression of fragments of the p300 protein fused to the VP16 protein in the presence of SF-1 and the −110 P450scc reporter construct indicated in vivo the interaction of two regions of p300 with SF-1, thus confirming the in vitro results. Taken together these results indicate that regulation of the human P450scc gene by SF-1 is mediated by p300/CBP. Due to the many putative roles of SF-1 to regulate many genes, its interaction with p300/CBP is potentially a key component effecting important physiological processes.

Cytochrome P450scc is a mitochondrial enzyme that catalyzes the conversion of cholesterol to pregnenolone and is the first step in the synthesis of all steroid hormones (reviewed in Miller (1)). The CYP11A1 gene that encodes P450scc is expressed in steroidogenic tissues such as the adrenal, ovary, testis, and placenta, and some expression has also been detected in the brain (2). The hormonal regulation and developmental pattern of expression of P450scc transcript in the human adrenal cortex, and similar effects are seen with luteinizing hormone and follicle stimulating hormone in human ovarian granulosa cells, and with luteinizing hormone and human chorionic gonadotropin in human testicular Leydig cells (3–5). In each case, interaction of the tropic hormone with the cell surface receptor activates a G protein (G,) that increases intracellular cAMP, which acts as the second messenger in a cAMP-dependent pathway to increase P450scc gene transcription (6).

The human P450scc cDNA has been cloned and the gene mapped to chromosome 15q23-24 (7). Preliminary studies with the 5'-flanking region of the gene demonstrated the ability of a 2.5-kilobase pair DNA fragment to confer basal and cAMP-responsive activity when transiently transfected into mouse adrenal Y1 tumor cells (8–10). Subsequent studies in mouse Leydig MA-10 (11), I-10 (12), human placenta JEG-3 (13), and adrenal NCI-H295 (14) cells identified regions of the 5'-flanking DNA that conferred basal promoter activity and a response to cAMP. However, it is apparent that the cAMP-responsive element identified in human JEG-3 cells is different from the element identified when studies were performed in mouse Y1, MA-10, and I-10 cells.

The orphan nuclear receptor steroidogenic factor 1 (SF-1)1 also known as adrenal 4-binding protein (Ad4BP) has been demonstrated to promote cell-specific expression of the human P450scc gene promoter in Y1 cells (15–17). The human P450scc gene promoter is inactive in nonsteroidogenic CV-1 cells, but could be activated by expression of exogenous SF-1 (15, 18). In addition to P450scc (15–17, 19), the SF-1 protein is involved in the regulation of several other steroidogenic genes including

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1 The abbreviations used are: SF-1, steroidogenic factor 1; Ad4BP, adrenal 4-binding protein; CRE, cAMP response element; CBP, CREB-binding protein; PCR, polymerase chain reaction; GST, glutathione S-transferase; EMSA, electrophoretic mobility shift assay; HA, hemagglutinin, CMV, cytomegalovirus; PAGE, polyacrylamide gel electrophoresis.

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CBP/p300 Mediates SF-1 Activity

**Experimental Procedures**

**Tissue Culture**—Human adrenal NCI-H295 cells obtained from the American Type Culture Collection were cultured in monolayer as described previously (14) in RPMI 1640 medium (Life Technologies, Inc.) supplemented with penicillin (50 mg/liter) streptomycin (10 μg units/liter), and 10% fetal calf serum (Hyclone, Logan, UT).

**Plasmids**—The P450sc luciferase reporter constructs contain fragments of the human P450scc gene which span from nucleotide −1676, −1620, and −110 to the 5′-end to nucleotide +49 at the 3′-end. The DNA fragments were amplified by PCR from a P450sc genomic clone kindly provided by Dr. Bon-chu Chung (Academia Sinica, Nankang, Taipei) using oligonucleotides which introduced a KpnI site and BglII site at the 5′- and 3′-end, respectively. Following amplification, PCR products were digested with KpnI and BglII, subcloned into the pGt3I reporter plasmid (Promega, Madison, WI), and verified byideoxy-nucleotide sequencing.

The construct used to express the GST-SF-1 fusion protein for electrophoretic mobility shift assays (EMSAs) was made by PCR amplification of the mouse SF-1 cDNA (Dr. Keith L. Parker, University of Texas Southwestern) using the oligonucleotides GCGGATCCCGGATACCGGAGGCCCAGGTAGACTAAGCTTCCGCGGTGCCAGCAC (3′- oligo) and GGCCTCTGAGAACGGTCCTGTTGGCCTGCAGCAT (5′-oligo) which was PCR-amplified by priming with BamHI, subcloned into the BamHI/EcoRI (blunt) site of the pGEXZTK prokaryotic expression plasmid (Pharmacia, Baie d’Urfe, PQ, Canada), and verified by nucleotide sequencing. The construct was used to express an SF-1 fusion protein containing a hemagglutinin (HA) tag at the carboxyl end (SF-1-HA) was made by subcloning a PCR product of SF-1 into the KpnI/XbaI site of the pcDNA3-3HA plasmid (provided by Dr. Claude Labrie, CHUL Research Center) (48). The SF-1 cDNA was amplified by PCR using the oligonucleotides CCGGATCCGGGATACCGGAGGCCCAGGTAGACTAAGCTTCCGCGGTGCCAGCAC (3′-oligo) and GGCCTCTGAGAACGGTCCTGTTGGCCTGCAGCAT (5′-oligo) and GGCCTCTGAGAACGGTCCTGTTGGCCTGCAGCAT (5′-oligo) and GGCCTCTGAGAACGGTCCTGTTGGCCTGCAGCAT (5′-oligo).

**Transfactions and Luciferase Assay**—NCI-H295 cells were plated at a density of 3 × 10^5 cells per well in 6-well plates (35 mm per well). Twenty-four hours later, cells were transfected with plasmid constructs using Lipofectin as described by the manufacturer (Life Technologies, Inc.) and incubated for 48 h. Following transfection, the medium was removed and cells were lysed by addition of 500 μl of lysis buffer (1% Triton X-100, 25 mm glycerol, pH 7.4, 50 mm KCl, 2 mm EDTA, pH 8.0) for 15 min. Twenty μl of the cell lysate were assayed for luciferase activity with a luciferase assay system (Promega, Madison, WI) in a Berthold LUMAT LB9501 luminometer. All experiments were normalized by cotransfection of a CMV-β-galactosidase expression vector, and 10 μl of the cell lysate were assayed for β-galactosidase activity with a Galacto-Light Plus (Tropix, Bedford, MA).

**Western Blot Analyses**—NCI-H295 cells were transiently transfected as described above for luciferase assays and were harvested in SDS-sample buffer (0.2% SDS, 12.5 mm Tris-Cl, pH 6.8, 30 mm 2-mercaptoethanol, 2.5% glycerol). 20 μg of total protein were separated by 10% SDS-PAGE and electrophoretically transferred to nitrocellulose (53). The blot was probed with an anti-HA antibody (Babco, Richmond, CA) to detect the SF-1-HA, and the signal was visualized by chemiluminescence using the Renaissance system as described by the manufacturer (DuPont NEN).

**EMSA**—EMSAs were performed using double-stranded oligonucleotides containing the human P450scc gene sequence from nucleotide −52 to −31, which contains the putative wild type SF-1 binding site TCAAGGGCCA in the noncoding strand. The mutant probe contains the identical sequence but has two nucleotide changes in the SF-1 binding site (see Fig. 3A for the sequence). The probes were end-labeled using γ-32P]ATP and T4 polynucleotide kinase (New England Biolabs, Beverly, MA). For all experiments, 2 μg of total protein extract from BL21 Escherichia coli cells expressing GST-SF-1 fusion protein was incubated with 100 fmol (150,000 cpm) of DNA probe in the presence of 2 μg of poly(dI-dC) in 15 mM Heps, pH 7.9, 50 mM KCl, 42 mM NaCl, 0.16 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, 2.5% glycerol, and 4%
to ascertain the presence of SF-1 in the observed DNA-protein complex, the protein extract was preincubated with a polyclonal anti-SF-1 antiserum (Upstate Biotechnology, Lake Placid, NY) for 10 min prior to the addition of the DNA probe. In the competition experiments, reactions contained a 500-fold molar excess of unlabeled double-stranded wild type or mutant SF-1 oligonucleotides. DNA-protein complexes were resolved by native 5% PAGE in 0.5 × Tris borate-EDTA for 2 h at 150 V. Gels were dried and exposed to film for 6 h at −80 °C.

In Vitro Protein Binding Assay—To ascertain the interaction between CBP/p300 and SF-1 in vitro, fragments of CBP polypeptides from different regions of the protein were expressed as GST fusion proteins and were immobilized on glutathione-coupled Sepharose as described by Frangioni and Neel (54) prior to incubation with radiolabeled SF-1 protein.35S-Labeled SF-1 protein was produced from an SF-1 expression construct provided by Dr. Keith Parker (University of Texas Southwestern) using rabbit reticulocyte lysate and T7 RNA polymerase (Promega). GST-CBP fusion proteins were produced from cDNA constructs in E. coli BL21 following induction with 0.1 mM isopropylthiogalactopyranoside. Equal quantities of GST-CBP fusion proteins were incubated with glutathione-coupled Sepharose for 30 min at 4 °C in binding buffer (100 mM NaCl, 1 mM EDTA, pH 8, 20 mM Tris, pH 8.0, 0.5% Nonidet P-40). The beads were then washed three times with phosphate-buffered saline, once with binding buffer, and then incubated with radiolabeled SF-1 for 2 h at 4 °C in the same buffer. After incubation, the beads were washed five times with washing buffer (20 mM Tris-Cl, pH 8.0, 500 mM NaCl, 5 mM EDTA, pH 8.0, and 0.1% Triton X-100). Bound proteins were released from the Sepharose by boiling in SDS sample buffer and were analyzed by SDS-PAGE (55). The gels were stained with Coomassie Blue to ascertain that equal amounts of GST proteins were loaded, after which the gels were incubated for 30 min with Amplify (Amersham Corp., Oakville, Ontario, Canada), dried, and exposed to film.

RESULTS

Effect of SF-1 and CBP/p300 on the Human P450scc Promoter—Previous gene transfer experiments have demonstrated the ability of the 5′-flanking region of the human P450scc gene to confer significant promoter activity in NCI-H295 cells (14). To determine the ability of CBP/p300 to mediate SF-1 regulation of the human P450scc gene, luciferase reporter constructs (−1676Luc, −1620Luc, and −110Luc) which contain 5′ deletions of the 5′-flanking region of the gene, were cotransfected with SF-1 and p300 or CBP in human adrenal NCI-H295 cells (Fig. 1). In transient transfection experiments, expression of exogenous SF-1 protein increased luciferase activity of the three constructs by 3-fold, indicating that the putative SF-1 binding site at position −46 is sufficient to confer a response. Transfection of p300 alone activated transcription above basal levels; however, cotransfection of SF-1 and p300 further increased expression of the three reporter constructs. Similar results were obtained with the CBP protein transfected in place of p300 (Fig. 1). To determine if the putative SF-1 binding site TCAAGGCCA, which is found in the inverted position at nucleotides −38 to −46, can confer a response to SF-1, p300, and CBP, the site in the −110 construct was mutated to TCAATTCCA. Mutation of the putative SF-1 binding site abolished the response to SF-1, SF-1 cotransfected with p300, and SF-1 cotransfected with CBP (Fig. 1). Since it is possible that the response of promoter activity to SF-1 is concentration-dependent, Western blot analysis was performed to measure the level of exogenous SF-1 (Fig. 2). Coexpression of p300 and the HA-tagged SF-1 fusion protein (SF-1-HA) led to a 3-fold increase in the level of exogenous SF-1 protein over the transfection of SF-1-HA alone. However, transfection of an 8-fold increase of the SF-1-HA expression plasmid alone, which led to a 8-fold increase in the level of expressed protein, conferred a significantly lower level of P450scc promoter activity than the combined effect of p300 and SF-1. Thus, activation of the P450scc promoter is at least partially dependent on p300 and indicates a functional interaction between CBP/p300 and SF-1.

To determine if SF-1 can interact with the putative binding site at nucleotides −38 to −46 of the human P450scc gene, EMSAs were performed, and a prominent protein-DNA complex was observed when the wild type DNA probe (Fig. 3A) was incubated with GST-SF-1 expressed in E. coli (Fig. 3B). The protein-DNA complex was effectively competed in the presence of a 500-fold molar excess of unlabeled wild type SF-1 DNA probe; however, the complex was not affected by an excess of the mutant probe. To ascertain whether SF-1 was implicated in the protein-DNA complex, incubation of the protein extract with a polyclonal anti-SF-1 antiserum was found to abolish complex formation. As well, a protein-DNA complex was not observed when the radiolabeled mutant probe was incubated with the SF-1-containing extract.

Inhibition of SF-1 and p300 Activity by E1A—Based on previous studies it is apparent that gene regulation mediated by CBP and p300 is repressed by the adenovirus 12 S E1A oncoprotein (56). Cotransfection of an E1A expression construct had an inhibitory effect on the activity of SF-1, and it also significantly inhibited the stimulatory activity mediated by p300 (Fig. 4). It has been shown that the positive arginine residue at position 2 of E1A is important for stable p300 interaction where
the RG2 mutation abrogates p300 binding selectively (49). To ascertain the specificity of the inhibitory effect of E1A on p300 and SF-1, co-transfection of RG2 in place of E1A was found to abolish the inhibitory effect and did not decrease the stimulatory activity and had no effect on SF-1- and p300-mediated promoter activity. Whereas deletion of residues 120–140 was able to abolish the inhibitory effect and did not decrease the stimulatory activity and had no effect on SF-1- and p300-mediated promoter activity.

Interaction between CBP/p300 and SF-1—To determine if SF-1 interacts with CBP/p300, GST fusion proteins that contain different regions of CBP were bound to glutathione-Sepharose and incubated with 35S-labeled SF-1 protein produced from rabbit reticulocyte lysate. SF-1 interacted with the region between residues 1–451 and 1460–1891 of CBP, but no interaction was observed with the regions between residues 451–721, 721–1100, 1100–1460, and 1892–2441 (Fig. 6). Similar experiments performed with fragments of the p300 protein demonstrate interaction of SF-1 with both the carboxy- and amino-terminal region of the protein (data not shown). To ascertain if SF-1 can interact with p300 in vivo, two-hybrid assays were carried out in NCI-H295 cells. The amino region of p300 (Np300) from residues 1 to 1275 and the carboxy region from residues 1257 to 2378 (Cp300) were fused to VP16 and cotransfected with SF-1 and the −110Luc reporter plasmid (Fig. 7). The coexpression of SF-1 with Np300/VP16 or Cp300/VP16 yielded a higher activity than cotransfection of SF-1 with VP16, which indicates the interaction of both the amino- and carboxy-terminal region of p300 with SF-1. These in vivo results also indicate a stronger interaction between the carboxy region of p300 and SF-1, which concurs with the interaction experiments performed in vitro.

**DISCUSSION**

Regulation of the human P450scc gene has been examined in several studies in which the promoter region was transfected into different cell lines. However, depending on the cells used, different putative cis-acting elements have been implicated to confer regulation. The first studies in mouse adrenal Y1 cells (8–10) identified an upstream region in the 5′-flanking DNA, which confers responsiveness to cAMP and implicates a putative element TGATGTCA homologous to the consensus CRE. These results were later confirmed in mouse Leydig MA-10 (11) and I-10 cells (12); however, experiments in human JEG-3 cells localized a cAMP response element to a different region further
downstream and implicate an Sp1 site (13, 58). Interestingly, recent studies in human adrenal NCI-H295 cells were unable to localize a cAMP-responsive element that correlates with the elements found in the other cells (14). The substantial differences seen between the cell lines were generally thought to reflect tissue-specific differences, since trans-acting nuclear factors are believed not to vary much among mammalian species. However, the recent results in human adrenal NCI-H295 cells when compared with results in mouse adrenal Y1 cells further raise the possibility of species-specific differences. The putative CRE at nucleotide −1633 of the human P450scc gene 5′-flanking region, which confers cAMP responsiveness in Y1, MA-10, and I-10 cells, is found 11 nucleotides upstream of an SF-1 binding site TCAAGGTCA, and it has been proposed that both elements are required to confer a full cAMP response (17). However, neither of these elements can confer a response to cAMP in NCI-H295 cells as demonstrated in transient gene transfer experiments (14). As seen in this study, the addition of 5′-flanking sequences from nucleotides −2110 to −21676, which introduced several putative SF-1 binding sites, conferred only a slight increase of SF-1 response beyond the activity obtained with the −2110 construct. These results suggest that the SF-1 site TCAAGGCCA found between nucleotides −38 and −46 confers a response to SF-1. The relevance of this site to regulate the P450scc gene in NCI-H295 cells is further demonstrated by the mutation of two nucleotides which abrogates the SF-1/DNA complex and abolishes any functional response to SF-1.

It has been demonstrated that steroidogenic gene promoters that are inactive when transfected into nonsteroidogenic cell lines such as CV-1 (15, 18) and Hela (59) can be induced by expression of exogenous SF-1. Considering the essential role of this factor to regulate steroidogenic gene expression including P450scc, relatively little is known about the mechanism of SF-1...
It is becoming clear that CBP and p300 are general coactivator proteins, which can interact specifically with a wide array of transcription factors and may serve as integrators of multiple signal transduction pathways (62). CBP/p300 have been demonstrated to mediate the activity of several factors such as MyoD (63), AP1 (64), SRC-1 (65, 66), p65 (67) including the nuclear receptors ER (68), RAR, RXR, and TR (62, 69). It has been postulated that the negative cross talk between nuclear hormones receptors and AP1, which all interact with CBP/p300, may be the result of competition for interaction with limiting amounts of coactivator proteins (64). Recently, Cheng et al. (70) reported the positive cross-talk between nuclear hormone receptors and p45/NF-E2 mediated by CBP. Similarly, the interaction of SF-1 with CBP/p300 may be a mechanism by which SF-1 can exert its effect on P450sc gene expression by influencing the interaction of p300 with other factors to trigger RNA polymerase II-dependent transcription. In addition to the results shown in this study with the human P450sc gene promoter, activation of the human P450c17 gene promoter by SF-1 is also mediated by CBP/p300 in NCI-H295 cells.2 Similar to other nuclear receptors, SF-1 interacts with the amino-terminal region of p300; however, SF-1 also binds p300 at the carboxyl-terminal region, which have been shown to interact with several transcription factors including SRC-1 (50). The association of SF-1 with both the amino- and carboxy-terminal regions of CBP/p300 is similar to their interaction with p65 of the NF-xB family of transcriptional activators (67).

One possible explanation for association with CBP/p300 at two independent sites is the simultaneous interaction of two or more molecules of SF-1. Although it was demonstrated that the SF-1 site at position −46 of the P450sc gene is functional, the addition of putative sites further upstream led to a higher response to SF-1 and p300. Binding of SF-1 to one site of the coactivator may facilitate occupancy of the other SF-1 site, leading to recruitment of other factors required for transcriptional activity. Interaction with nuclear hormone receptors and receptor-mediated transactivation by CBP/p300 have been shown to be ligand-dependent. However, the ligand for the orphan nuclear receptor SF-1 has not been determined, and our results demonstrate interaction between SF-1 and p300 without the addition of exogenous ligand. Recently, it has been demonstrated that oxysterols can enhance SF-1-dependent transcriptional activity, however, it is unclear if these compounds serve as a ligand (71).

Human p300 was identified initially by its ability to bind the

2 F. DeWitte and D. W. Hum, unpublished data.
adenoviral E1A oncprotein (72), which can transform primary cells, block cellular differentiation, and inhibit certain transcriptional enhancer elements. Binding of E1A to CBP/p300 has been shown to abolish or down-regulate the stimulatory effects of c-Fos (45), cJun, JunB (52), MyoD (63), and CREB (39, 56). In this study, the positive effect of SF-1 on P450c21 gene promoter activity was also inhibited by E1A, which most likely is mediated by binding to endogenous p300. In agreement with previous studies, which show that mutation of E1A at position 2 can abolish interaction with p300 and alleviate its inhibitory effect, we show that RG2 is unable to inhibit the stimulatory effect of SF-1 and p300 on the P450c21 gene promoter. As well, the deletion of residues 2–36 and 37–68 in E1A, which are regions postulated to interact with p300, also abolished its effect on SF-1 and p300.

Analogous to the action of E1A that can alter cellular differentiation, SF-1 plays an important role in endocrine differentiation as well as regulating sex determination and P450 steroid hydroxylase gene expression. In addition to the many identified genes including P450c21, P450c27, P450c17, P450 17α-HSD, and Müllerian inhibiting substance, which are regulated by SF-1, it is likely that other novel targets of SF-1 will be identified. Regulation of the numerous steroidogenic genes by SF-1 most likely involves CBP/p300 to integrate the effects of such factors as Sp1 and CREB. The interaction of SF-1 with p300 is potentially a key step in the mechanism of SF-1 action; however, further studies will be required to identify the other factors involved in the SF-1/p300 protein complex.

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