The Transcriptional Regulating Protein of 132 kDa (TReP-132) Enhances P450scc Gene Transcription through Interaction with Steroidogenic Factor-1 in Human Adrenal Cells*

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The human P450scc gene is regulated by the tissue-specific orphan nuclear receptor, steroidogenic factor-1 (SF-1), which plays a key role in several physiologic processes including steroid synthesis, adrenal and gonadal development, and sexual differentiation. Several studies have demonstrated the interaction of SF-1 with different proteins. However, it is clear that additional factors not yet identified are involved with SF-1 to regulate different target genes. Recently, it was demonstrated that a novel transcriptional regulating protein of 132 kDa (TReP-132) regulates expression of the human P450scc gene. The overexpression of TReP-132 in adrenal cells increases the production of pregnenolone, which is associated with the activation of P450scc gene expression. Considering the colocalization of TReP-132 and SF-1 in steroidogenic tissues such as the adrenal and testis, and the presence of two putative LXXLL motifs in TReP-132 that can potentially interact with SF-1, the relationship between these two factors was studied. The coexpression of SF-1 and TReP-132 in adrenal NCI-H295 cells cooperates to increase promoter activity. Pull-down experiments demonstrated the interaction between TReP-132 and SF-1, and this was further confirmed in intact cells by coimmunoprecipitation/Western blot and two-hybrid analyses. Deletions and mutations of the TReP-132 cDNA sequence demonstrate that SF-1 interaction requires the LXXLL motif found at the amino-terminal region of the protein. Also, the “proximal activation domain” and the “AF-2 hexamer” motif of SF-1 are involved in interaction with TReP-132. Consistent with previous studies showing interaction between CBP/p300 and SF-1 or TReP-132, the coexpression of these three proteins results in a synergistic effect on P450scc gene promoter activity. Taken together the results in this study identify a novel function of TReP-132 as a partner in a complex with SF-1 and CBP/p300 to regulate gene transcription involved in steroidogenesis.

The conversion of cholesterol to pregnenolone, the first enzymatic step in the biosynthesis of all steroid hormones, is catalyzed by the mitochondrial cytochrome P450 side chain cleavage (P450scc) enzyme (For review see Ref. 1). Pregnenolone is the common precursor in the steroidogenesis pathway, which leads to the synthesis of mineralocorticoids, glucocorticoids, and sex hormones. The enzyme P450scc is encoded by a single human gene, CYP11A1 (2), on chromosome 15q23–24 (3), and its expression is regulated hormonally at the transcriptional level (4). Although expression of the gene has been detected in the central nervous system (5), P450scc transcript and protein are expressed for the most part in steroidogenic tissues such as adrenals, ovaries (6), testis, and the placenta (7–9). The hormonal regulation and developmental pattern of expression of P450scc are species- and tissue-specific (10). ACTH increases steroidogenesis and accumulation of P450scc mRNA in the human adrenal zona fasciculata and reticularis. Luteinizing hormone (LH), follicle-stimulating hormone (FSH), and human chorionic gonadotrophin (hCG) have the same effects in Leydig cells and ovarian granulosa cells (11–13). It is apparent that the cis-acting elements of the 5′-flanking region of the P450scc gene involved in basal and cAMP-dependent regulation are utilized in a developmentally programmed, tissue- and cell-type-dependent manner. For example, in human adrenal NCI-H295 cells, the basal transcriptional activity lies within the first 79 bp upstream from the transcriptional start site of the P450scc gene (14), whereas the region between −89 and −108 was found to significantly increase promoter activity in human placenta JEG-3 cells (15–17).

To identify tissue-specific transcription factors that regulate the expression of cytochrome P450 hydroxylase genes, two separate groups cloned the orphan nuclear receptor steroidogenic factor-1 (SF-1), also known as Ad4BP (18, 19). SF-1 was identified based on its ability to interact with a shared promoter element, PyCAAGGTCA, found in steroid hydroxylase genes and was shown to activate their expression (20–22). It has subsequently become apparent that SF-1 is a central regulator of the endocrine and reproductive system (For review see Refs. 23 and 24). It was shown in mouse and human that SF-1 is expressed at all levels of the hypotalamic-pituitary-adrenal/ gonadal axis, where it is required for the expression of diverse genes that are essential for steroid hormones biosynthesis. For instance, SF-1 is expressed in adrenal and gonadal steroid-
producing cells (5, 25), where it precedes P450scc gene expression (26). In these cells, SF-1 was shown to activate the expression of P450 hydroxylases (22, 27, 28), the ACTH receptor (29), and the gene encoding steroidogenic acute regulatory protein (StAR) (30). In addition, it was demonstrated that SF-1 is expressed in Sertoli cells, where it participates directly in the process of male sex determination, and synergizes with SOX9 and WT1 in activation of Mullerian inhibitory substance (MIS) gene expression (31–33). SF-1 is also expressed in pituitary gonadotropes, where it activates the promoter for MIS and WT1 in activation of Mu MIS process of male sex determination, and synergizes with SOX9 expressed in Sertoli cells, where it participates directly in the regulation of MIS expression (26). In these cells, SF-1 was shown to activate the expression of the testis and adrenal cortex, which is consistent with their functional interaction in steroidogenesis and sexual differentiation, targeted SF-1 gene disruption produced knockout mice which completely lacked the ventromedial hypothalamic nucleus, adrenal glands, and gonads (reviewed in Ref. 23). Consequently, these developmental defects were associated with phenotypic male-to-female sex reversal and adrenocortical insufficiency, resulting in neonatal death (36, 37). However, despite the many studies describing the physiological functions and mechanisms of action of SF-1, it is apparent that additional regulatory mechanisms involving this factor remain to be elucidated.

As with other nuclear receptors, different mechanisms, including phosphorylation and interaction with co-regulators, have been shown to regulate basal and cAMP-responsive activity of SF-1. For example, a consensus protein kinase A phosphorylation motif has been identified in SF-1 (38) and has been found to mediate cAMP-responsive P450c17 hydroxylase gene expression (39). Furthermore, SF-1 has already been shown to be a component of several multiprotein complexes involved in the tissue- and promoter-specific hormonal responses of target genes. For instance, SF-1 interacts with CBP/p300 (40) to activate the P450scc gene promoter. Moreover, it was recently suggested that the c-Jun protein acts synergistically with SF-1 to activate this promoter (41). Identifying new factors that interact with SF-1 in protein complexes, and understanding how they function with SF-1 to regulate the P450scc gene will provide important insight into understanding how SF-1 modulates transcription.

The transcriptional regulating protein of 132 kDa (TReP-132) was recently cloned and shown to activate the P450scc gene via the 5'-flanking DNA region from nucleotides −155 to −131 (42). Analysis of the predicted primary structure of TReP-132 revealed motifs characteristic of transcription factors and coactivators, which include regions rich in glutamate, proline, and glutamine residues, as well as two LXXLL motifs, suggesting the interaction of the protein with nuclear receptors (43). SF-1 and TReP-132 transcripts colocalize in many tissues, including the testis and adrenal cortex, which is consistent with their functional interaction in steroidogenesis (5, 42).

This present study addresses the role of TReP-132 in the regulation of the human P450scc gene mediated by SF-1 in human adrenal carcinoma NCI-H295 cells. The overexpression of exogenous TReP-132 in these cells led to increased pregnenolone production, which concurs with the ability of this nuclear protein to increase P450scc expression. Reporter gene assays showed that TReP-132 cooperates with SF-1 to induce P450scc promoter activity; and the proximal SF-1 binding site at position −46 to −38 was sufficient to confer responsiveness to both proteins. The physical interaction between SF-1 and TReP-132 was demonstrated and was shown to involve the LXXLL sequence found at the amino-terminal domain of TReP-132 as well as the proximal activation domain and the AF-2 hexamer of SF-1. Considering that p300 was previously shown to interact with SF-1 and TReP-132 to regulate the P450scc gene promoter (40, 42), coexpression of these three factors showed cooperativity among them, leading to synergistic activation of the promoter. The results of this study taken together demonstrate the ability of TReP-132 to form a complex with SF-1 and CBP/p300 to regulate gene transcription involved in steroidogenesis.

EXPERIMENTAL PROCEDURES

Cell Culture—Human NCI-H295 adrenal tumor cells were obtained from the American Type Culture Collection (Manassas, VA). NCI-H295 were cultured in monolayers as described previously (14) in Dulbecco’s modified Eagle’s medium/F12 medium supplemented with penicillin (50 mg/ml), streptomycin (100 units/ml) (Invitrogen), 5% fetal bovine serum (Hyclone, Logan, UT), 1% ITS (containing insulin, transferrin, selenium), Roche 1% red phenol, 10−8 M, and 10−M OH-cortisone.

Plasmids—The following constructs were made as described by Monté et al. (40): P450scc luciferase reporter constructs containing fragments of the human P450scc gene spanning from nucleotides −1676, −155, or −110 at the 5'-end to nucleotide +49 at the 3'-end subcloned in pGL3; the construct used to express the GST-SF-1 fusion protein for electrophoretic mobility shift assays; and the construct used to express the SF-1 fusion protein containing a hemagglutinin (HA) tag at the carboxyl end (SF-1-HA). The P450scc genomic clone was kindly provided by Dr. Bon-Chu Chung (Academia Sinica, Nankang, Taipei). The SF-1 expression construct containing the mouse SF-1 cDNA in the pCMV5 expression plasmid was kindly supplied by Dr. Keith L. Parker (University of Southwestern Texas).

The TReP-132 mutants TReP-132m1, TReP-132m2, and TReP-132m2 were mutated in NR-box 1, NR-box 2, and in both boxes, respectively. Leucines were changed to alanines (see Fig. 4A for the sequence).

The human p300 cDNA in the pCMV-p300-HA and the mouse pCMV-CBP-HA were kindly provided by Dr. Richard Goodman (Oregon Health Sciences University) (44). The constructs expressing GST-CBP fusion proteins were supplied by Dr. Ralph Janneke (Hanover Medical School, Hanover, Germany) (45).

The pFR-LUC plasmid, which comprises five GAL4 elements up-stream of a minimal E1b TATA-box followed by the luciferase gene, was purchased from Stratagene. The pcDNA3–GAL4 vector was generated as described previously (42) from the pBSQ424 pLUCIISM (a gift from Dr. Michael R. Green, Howard Hughes Medical Institute Research Laboratories, University of Massachusetts Medical Center, Worcester, MA). The GAL4-TReP-132 plasmid was created by subcloning a PCR product corresponding to the entire coding region of TReP-132 into the pcDNA3–GAL4 vector.

Transfections and Luciferase Assays—NCI-H295 cells were cultured in 12-well plates at a density of 3 × 104 cells/plate and grown for 24 h before transfection. The medium was then changed, and transient transfections were carried out for 12 h. NCI-H295 cells were transfected with Effectene transfection reagent (Qiagen, Mississauga, Ontario, Canada) at a ratio of 1 μg of DNA to 25 μl of Effectene. Following transfection for 12 h, the cells were washed and incubated in fresh medium for 36 h in NCI-H295 cells. For luciferase assays, cells were harvested, and 20 μl of the cell lysate were assayed for luciferase activities with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Firefly luciferase activities were normalized to Renilla luciferase activity. The efficiency of transfection was verified by green fluorescent protein expression plasmid and efficiencies obtained were 60% or better.

Quantification by HPLC of Pregnenolone Levels Secreted by NCI-H295 Cells—NCI-H295 cells were cultured in 60 mm tissue culture plates at a density of 1 × 106/plate, grown for 24 h before transfection, and then transfected with 2 μg of pcDNA3 or TReP-132. At 24 or 48 h after transfection, two extractions of culture media were carried out using ethyl ether, and the organic solution was evaporated under nitrogen. The dried extract was dissolved in 100 μl of isopropanol, and a 10-μl aliquot was diluted with 90 μl of HPLC elution solvent for injection. The HPLC was carried out using Waters Alliance Millennium System (Waters, Milford, MA). Solvent A was 0.2% TFA in H2O, and solvent B was 0.2% TFA in ACN (1:1), reverse phase column, and model 440 absorbance detector at 205 nm) and isocratic elution at 1 ml/min with acetonitrile-water (1:1). The retention time of the standard pregnenolone was 13.8 min. Results are expressed as nmol/mg protein.

Reverse Transcription and Quantitative PCR—NCI-H295 cells were first transfected with pcDNA3 or TReP-132 as described above. Then,
total RNA was isolated using Trizol reagent according to the manufacturer’s protocol, and the levels of P450sc mRNA were assessed by quantitative reverse transcription-PCR. Total RNA was reverse transcribed using random hexamer primers and SuperScript reverse transcriptase (Invitrogen). Next, quantification by Real-Time PCR was performed on a MX 4000 apparatus (Stratagene, La Jolla, CA) using specific pairs of oligonucleotide primers, 5′-gagcaggccacagcttggcc-c3′ and 5′-gagctgctgaagagctgcc-3′, chosen by using the gene sequence XM_007646. Actin transcripts were quantified simultaneously for normalization using the following oligonucleotide primers: 5′-gagcaggccacagctttgga-3′ and 5′-gagctgctgaagagctgcc-3′. PCR amplification was performed in a final volume of 25 μl containing 100 nm each primer, 4 mM MgCl2, the Brilliant Quantitative PCR Core Reagent Kit mix as recommended by the manufacturer (Stratagene), and SYBR Green 0.33X (Sigma). PCR conditions were 95 °C for 10 min, followed by 40 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C. P450sc mRNA levels were subsequently normalized to actin mRNA levels.

In Vitro Protein Binding Assay—SF-1 protein was expressed as a GST fusion protein and was immobilized on glutathione-coupled Sepharose as described (46) prior to incubation with radiolabeled TReP-132 protein and washing, also as described previously (40). The pGEX2TK-containing GST-SF-1 fusion proteins were transformed in Escherichia coli strain BL21 (DE3) pLysS following induction with 0.1 mM isopropyl-1-thio–β-D-galactopyranoside at 28 °C overnight. The [35S]methionine-labeled TReP-132 was synthesized using the rabbit reticulocyte lysate and T7 RNA polymerase system (Promega) according to the manufacturer’s protocol. Bound proteins, released from the Sepharose by boiling in SDS sample buffer, were resolved by 8% SDS-PAGE and visualized by autoradiography.

Co-immunoprecipitation—For co-immunoprecipitation experiments, 1 × 10^7 HeLa cells were plated in a 100-mm culture dish and incubated for 24 h. Cells were transfected using Exgen 500 at 40 μg/μg of DNA of SF-1 tagged with a HA epitope (SF-1-HA) and 5 μg of TReP-132 tagged with a FLAG epitope (TReP-132-FLAG) or pcDNA3 for 24 h. Immunoprecipitation was performed according to the protocol of Santa Cruz Biotechnology, Inc. Cells were collected in radioimmune precipitation assay buffer (1 × PBS, 0.1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors). Cell debris was removed by centrifugation, and the resulting cell extract was preincubated for 30 min with a rabbit non-immune antibody (sc-2027, Santa Cruz Biotechnology) or non-immune rabbit non-immune antibody (sc-2027, Santa Cruz Biotechnology). The quantity of protein in the supernatant was assessed by Bradford assay as instructed by the manufacturer (BioRad, Hercules, CA). 1 mg of protein was incubated overnight at 4 °C with 10 μg of the rabbit anti-SF-1 (06-431, Upstate Biotechnology) or non-immune antibody. Immunoprecipitates were subjected to 8–12% SDS-PAGE and analyzed by Western blot using an anti-FLAG antiserum (05-447, Upstate Biotechnology) at a ratio of 1/10,000 as the first antibody. After three washes with 2% Tween-20 in PBS, membranes were incubated with a horseradish peroxidase-coupled anti-mouse antisera (1/10,000, Jackson Immunoresearch Laboratories, West Grove, PA) at room temperature for 45 min. Membranes were washed three times with 0.2% Tween 20 in PBS then with 2% Tween 20 in 10× TBS and revealed with the Renaissance ECL Plus kit according to the manufacturer’s protocol (PerkinElmer Life Sciences).

RESULTS

TReP-132 Increases Pregnenolone and P450sc Transcript Levels in Human Adrenal Cells—In a previous study it was demonstrated that TReP-132 increases P450sc gene promoter activity (42), which would indicate that this transcriptional regulating protein is potentially able to increase steroid synthesis. To confirm that an increased level of TReP-132 can stimulate the conversion of cholesterol to pregnenolone catalyzed by P450sc, NCI-H295 cells were transfected with a pcDNA3 expression plasmid encoding TReP-132 or with the empty pcDNA3 vector alone. As determined by reverse HPLC, overexpression of exogenous TReP-132 led to an increased level of pregnenolone in the medium of NCI-H295 cell (Fig. 1A). Thus it is apparent that TReP-132 expression increases pregnenolone production in intact human adrenal cells, probably via its positive activity on P450sc gene expression.

To determine whether the overexpression of exogenous TReP-132 can indeed increase the level of P450sc transcript, quantitative reverse transcription-PCR analyses was performed on RNA isolated from NCI-H295 cells transfected with the pcDNA3 expression plasmid encoding TReP-132. The over-
expression of TReP-132 led to an increased level of P450scc transcript 2.9-fold over that obtained when cells were transfected with the empty pcDNA3 vector alone, thus suggesting that TReP-132 increases expression of the human P450scc gene.

**TReP-132 Increases SF-1 Activation of the Human P450scc Gene Promoter**—During characterization of TReP-132, it was demonstrated that this protein can increase the expression of reporter plasmids via the 5′-flanking region of the human P450scc gene. Considering that TReP-132 was isolated based on its ability to interact with the −155/−131 element by screening a human placenta cDNA expression library, this protein was demonstrated to interact with the −155/−131 element in electrophoretic mobility shift assays and to activate promoter activity via this element (42). TReP-132 was shown to activate expression of the P450scc gene promoter via a fragment of the gene from −1676 to +49 (42). However, our subsequent studies in NCI-H295 cells showed the ability of TReP-132 to activate the −110/+49 sccLuc reporter plasmid which does not have the −155/−131 element but contains a single putative SF-1 binding site at position −46. The primary structure of TReP-132 contains two copies of the LXXLL motif, which is found in transcriptional coregulators and is involved in the interaction with nuclear receptors. Considering that the SF-1 nuclear receptor is an important regulator of P450scc gene expression, the present study has ascertained whether TReP-132 interaction with SF-1 is one mechanism by which TReP-132 can influence promoter activity.

Prior to determining the ability of TReP-132 to regulate P450scc gene promoter activity via SF-1 and its binding site at position −46, the relevance of this site was assessed. Previous experiments have demonstrated the ability of SF-1 to confer significant promoter activity via the 5′-flanking region of the human P450scc gene in NCI-H295 cells. It was shown that a fragment of the promoter region from nucleotides −110 to +49, which contains a single SF-1 binding site at position −46, is sufficient to confer response to SF-1 (14, 40). However, the 5′-flanking region of the gene from nucleotides −1676 to −110 contains four additional putative SF-1 binding sites; thus the implication of the site at position −46 in the context of the other upstream putative SF-1 cis-acting elements remained to be determined. In agreement with previous results, SF-1 is able to activate the −1676/+49 sccLuc construct, which contains the five putative SF-1 sites, as well as the −155/−49 sccLuc and −110/−49 sccLuc constructs, which contain the single site (Fig. 2, A and B). In addition, mutation of the SF-1 binding site at position −46 in the −1676/+49 sccLuc, −155/+49 sccLuc, and −110/+49 sccLuc constructs diminished the basal promoter activities and abolished the responses to SF-1. Together these results indicate that SF-1 activates the human P450scc gene promoter via the site at position −46, which was previously shown to form a protein-DNA complex with the nuclear receptor (40).

To determine the ability of TReP-132 to influence SF-1 regulation of the human P450scc gene, the −1676/+49 sccLuc, −155/−49 sccLuc, and −110/−49 sccLuc reporter constructs were cotransfected with the expression plasmids encoding SF-1 and TReP-132 in NCI-H295 cells (Fig. 2C). The expression of exogenous SF-1 or TReP-132 alone increased the expression of all the wild-type reporter constructs tested, and the coexpression of both proteins led to the greatest increase in promoter activity. However, the constructs bearing a mutated SF-1 binding site at position −46 exhibited no response to SF-1 or TReP-132 when expressed either alone or in combination. These results suggest a functional interaction between SF-1 and TReP-132, which confers increased promoter activity via the SF-1 binding site at position −46.

**The TReP-132 and SF-1 Proteins Interact Directly**—To assess whether TReP-132 and SF-1 interact directly, a pull-down assay was performed. The immobilized glutathione S-transferase (GST)-SF-1 fusion protein was shown to bind specifically to 35S-labeled TReP-132 (Fig. 3A). To determine whether TReP-132 interacts directly with SF-1 in intact cells, immunoprecipitation analyses were performed on cell extracts in which TReP-132 tagged with the FLAG epitope (TReP-Flag) was coexpressed with SF-1 in HeLa cells, which do not express endogenous SF-1. Potential protein complexes were immunoprecipitated using the anti-SF-1 antibody and subjected to Western blot analysis using a monoclonal anti-Flag antibody (Fig. 3B). The results show that the anti-SF-1 antibody precipitated a complex that contained the TReP-Flag protein. The exogenous TReP-132 was precipitated neither in the absence of transfected SF-1 nor when the immunoprecipitation was performed with non-immune serum. Thus, these results are indicative of a specific interaction between SF-1 and TReP-132 in intact cells.

To further confirm that SF-1 interacts with TReP-132 in intact cells, two-hybrid assays were performed in HeLa cells. Two chimeric proteins consisting of TReP-132 fused to the GAL4 DNA-binding domain (GAL4-TReP) and SF-1 fused to the transactivation domain of VP16 (VP16-SF-1) were coexpressed with the pFRLuc reporter plasmid containing five GAL4 binding sites (Fig. 3C). The coexpression of both fusion proteins yielded an induction of promoter activity over the levels obtained by coexpression of VP16-SF-1 with GAL4 or GAL4-TReP with VP16, which is consistent with an interaction between TReP-132 and SF-1.

**Interaction of TReP-132 with SF-1 Involves the Putative NR-box LRQLL of TReP-132**—To further characterize the interaction between TReP-132 and SF-1, which leads to increased promoter activity of the P450scc gene, the interacting domains involved in each of the proteins were further defined. To identify the domain(s) implicated in TReP-132, the full-length GAL4-TReP protein as well as fusion proteins containing 5′ or 3′ deletions of TReP-132 were coexpressed with VP16-SF-1 in HeLa cells. The deletions were designed to remove specific regions corresponding to domains that are potentially important in protein-protein interaction and transcriptional activity, such as the glutamine-, proline-, and glutamic acid-rich regions and the zinc-finger domains (Fig. 4A). In the constructs containing progressive deletion from the 3′-end of TReP-132, removal of putative functional domains in GAL4-TRePdel1 to del3 led to slight variations on basal promoter activity and SF-1 interaction (Fig. 4B). However, it is clear that the shortest fusion protein (GAL4-TRePdel4) containing a single LXXLL motif (LRQLL) at residue 181 retained the ability to interact with SF-1. All of the GAL4-TReP constructs that were deleted from the amino-terminal end, which do not contain the LRQLL motif, also did not interact with SF-1. To further determine the functional relevance of the LRQLL sequence, the leucine residues were changed to alanines, thereby abolishing the LXXLL motif in the full-length GAL4-TRePm protein, which resulted in ablation of the ability of the fusion protein to interact with SF-1. Similar results were obtained when the same mutations were introduced in the GAL4-TRePdel4 construct (Fig. 4B).

To further determine the functional importance of the two LXXLL motifs in TReP-132, the sequences were mutated individually and in combination in the full-length protein, and the effects on the ability of TReP-132 to activate the P450scc gene promoter was assessed with the −110/−49 sccLuc reporter construct transfected in the presence and absence of exogenous SF-1 in NCI-H295 cells. Mutation of the LRQLL sequence either alone or in combination with the mutation of the LEMLL...
sequence abolished the ability of TReP-132 to increase promoter activity (Fig. 4C). The increased promoter activity obtained when wild-type TReP-132 is coexpressed with SF-1 was also abolished with these mutations. Although the individual mutations of the LEMLL sequence also led to decreased transcriptional activation, the effect was consistently less than that seen when TReP-132 is mutated at the LRQLL motif.

TReP-132 Interacts with SF-1 via the AF-2 Domain and the Proximal Activation Domain—As reported by Crawford et al. (48), the carboxyl-terminal end of SF-1 contains an AF-2 hexamer, shown to be included in the essential conserved motif for the transcriptional activation function 2 (AF-2) domain of many nuclear receptors (49–56). The AF-2 hexamer was shown to be required for transcriptional activation, for functional interaction with SRC-1 (57, 58), and for the ability of SF-1 to confer the steroidogenic phenotype to embryonic stem cells by stable transfection (57). Another functional domain found in SF-1, described by Crawford et al. (48), is the proximal activation domain, located between residues 187 and 245 and involved in interaction with SRC-1. Additionally, the FTZ-F1-box and the proline cluster of SF-1 were previously proposed to interact with TFIIB and c-Jun (59). To test which domain of SF-1 is required for interaction with TReP-132, a mammalian two-hybrid analysis was performed in HeLa cells by expressing

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**FIG. 2.** TReP-132 potentiates the transactivation of P450scc mediated by SF-1. A, schematic representation of the luciferase reporter constructs used in transcriptional activity assays in NCI-H295 cells, containing progressive deletions of the human P450scc gene 5'-flanking region from nucleotides −1676, −155, and −110 to +49. The constructs denoted as −1676/+49mscluc, −155/+49mscluc, and −110/+49mscluc contain a mutated SF-1 binding site found between nucleotides −38 and −46. B, the promoter-reporter constructs (0.2 µg) were cotransfected with the plasmid expressing SF-1 (0.1 µg). The results are expressed as relative light units of luciferase activity. Transfections were normalized to Renilla luciferase activity expressed from a cotransfected plasmid. SF-1 increases expression of the reporter constructs containing wild-type P450scc promoter regions, but the transcriptional activation is abolished by mutation of the SF-1 binding site. C, the promoter-reporter constructs (0.1 µg) were cotransfected with plasmids expressing SF-1 (0.1 µg) and TReP-132 (0.4 µg). The results are expressed as fold increase of luciferase activity over the value obtained from the empty pGL3 reporter vector alone. Coexpression of TReP-132 yielded significantly higher P450scc promoter activities than obtained with expression of SF-1 alone. TReP-132-mediated transactivation was abolished by mutation of the SF-1 binding site. Results represent the mean of four independent experiments ± S.D.


a series of VP16-SF-1 chimeric proteins coexpressed with GAL4-TReP or with GAL4-TRePdel4 (Fig. 5). The progressive deletion of the amino-terminal end of SF-1 showed that removal of the DNA-binding domain did not diminish interaction with TReP-132. However, removal of the proximal activation domain and the upstream amino-end region between amino acids 119 and 187 led to diminished interaction. Deletions of the carboxyl-terminal region of SF-1 showed that removal of the regions from nucleotides 119–462 (VP16-SF-1del5), 245–462 (VP16-SF-1del4), or of just the AF-2 hexamer motif (VP16-SF-1ΔAF-2) abolished the interaction between SF-1 and TReP-132. Thus, it is apparent that the proximal activation domain and the AF-2 hexamer both have essential roles in the interaction with TReP-132.

CBP/p300 Potentiates Activation of the P450scc Promoter by SF-1 and TReP-132—CBP/p300 was previously shown to interact with SF-1 (40) to increase the activity of the P450scc gene promoter, and it was subsequently demonstrated in a separate study that CBP/p300 can also interact with TReP-132 to increase expression of the same gene promoter (42). Pull-down analyses performed in the present study demonstrate that full-length TReP-132 can interact with CBP at three independent regions (Fig. 6A). Also having shown in this study that TReP-132 can interact with SF-1, it was next ascertained if these three factors can interact synergistically to regulate P450scc gene promoter activity. The greatest increase of reporter gene expression (−1676/+49sccLuc and −110/+49sccLuc) was obtained when expression plasmids encoding SF-1, TReP-132, and p300 were cotransfected into NCI-H295 cells (Fig. 6B). The synergistic effect was observed only during coexpression of the three factors and was not seen when they were expressed alone or in any combination of only two of the proteins. Therefore these results demonstrate the cooperativity between SF-1, TReP-132, and p300, which interact synergistically to activate P450scc gene expression.

**DISCUSSION**

Cytochrome P450scc, which catalyzes the first step in the synthesis of steroid hormones from cholesterol, is a key determinant of steroid synthesis in steroidogenic tissues (for review see Ref. 60). It is clear that the temporal and spatial specific expression of this gene is required for steroid synthesis, which is necessary for many physiological processes (6, 10, 61–63). However, relatively little is known about the mechanism(s) of its regulation and the factors involved therein. Recently, a novel zinc-finger protein, TReP-132, was isolated and shown to increase P450scc gene promoter activity in human placental JEG-3 and adrenal NCI-H295 cells (42). Although the TReP-132 transcript is expressed in many of the tissues examined, the highest levels were found in the adrenal cortex and the testis, which is consistent with a role for TReP-132 in the regulation of P450scc gene expression in steroidogenic tissues. In the present study, the overexpression of exogenous TReP-132 in adrenal NCI-H295 cells led to increased pregnenolone production, which concurs with the ability of this nuclear protein to increase P450scc expression.

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To determine the mechanism(s) by which TReP-132 increases P450scc expression in adrenal cells, it was found that exogenous TReP-132 can increase the expression of a reporter plasmid under the control of the 5′-flanking region of the P450scc gene from nucleotides −110 to +49. Although it has been shown that TReP-132 can increase P450scc gene promoter activity via a cis-acting element between nucleotides −155 and −131, the present study addressed the ability of TReP-132 to regulate promoter activity via interaction with SF-1.

In previous studies, the region of the P450scc gene from −79 to +49, which contains an inverted SF-1 binding site (TCAAGGCCA) between nucleotides −38 and −46, was demonstrated to

FIG. 4. SF-1 and TReP-132 interaction is dependent on the putative NR-box LRQLL of TReP-132. A, a schematic diagram depicting GAL4-TReP-132 constructs containing progressive deletions of the carboxyl-terminal end (GAL4-TRePdel1 to -del4) and the amino-terminal end (GAL4-TRePdel5 to -del7) of TReP-132 fused to the DNA-binding domain of GAL4. GAL4-TReP constructs mutated in the putative NR-box, LRQLL, are labeled GAL4-TRePm and GAL4-TRePdel4m. B, the GAL4 reporter construct, pFR-Luc (0.55 μg), was cotransfected in SF-1-deficient HeLa cells with the various GAL4-TReP fusion constructs (190 ng) in the presence or absence of VP16-SF-1 (340 ng). Transfections were normalized to Renilla luciferase activity. The values are expressed as relative luciferase activity (mean ± S.D.) and represent three independent experiments, each performed in triplicate. The cotransfection of VP16-SF-1 with each GAL4-TReP construct yielded significant activation of promoter activity, which was abolished by deletion or mutation of the putative LRQLL NR-box. C, transfection studies were performed in human NCI-H295 cells with the 110/49sccLuc reporter construct (0.2 μg) to determine the function of the two putative NR-boxes (LRQLL and LEMLL) in full-length TReP-132 in the presence or absence of exogenous SF-1. Cells were transfected with wild-type TReP-132, mutated at either the LRQLL (TReP-132m1) or LEMLL (TReP-132m2) site, or mutated at both sites (TReP-132m1–2). The results are expressed as fold increase of luciferase activity over the values obtained from the empty pGL3 vector. Transfections were normalized to Renilla luciferase activity. The proteins SF-1 and TReP-132 interact to activate the P450scc gene promoter, but the cotransfection of SF-1 with TReP-132 mutated in the LRQLL NR-box did not confer luciferase activity above the values obtained with transfection of the SF-1 vector alone. Results represent the mean of three independent experiments ± S.D.
confer basal and cAMP-responsive promoter activity in NCI-H295 cells (14). Because the primary structure of TReP-132 contains putative NR-box sequences, which suggests that it may interact with nuclear receptors to confer increased promoter activity, the function of the SF-1 binding site at position +46 was ascertained in the −1676/−49secLuc reporter construct that contains four additional upstream putative SF-1 binding sites. In agreement with previous results (40), the presence of the additional upstream sites did not confer significant additional basal activity nor did it increase responsiveness to SF-1 in NCI-H295 cells. However, mutation of the site at −46 decreased basal promoter activity and abolished the response to SF-1, which concurs with the recent in vivo results of Hu et al. (64) demonstrating in transgenic mice that mutation of the same site greatly reduces human CYP11A1 gene promoter activity in adrenals. Despite the loss of responsiveness to SF-1 in all the reporter constructs that contain the mutated SF-1 site at position −46, the 5′-flanking region retained the ability to express a minimal amount of promoter activity. This is consistent with the results of Guo and Chung (65) showing that the first 34 bp upstream from the transcriptional start site, containing a TATA-box as the only known

![Fig. 5](image-url)
As an initial approach to determining whether TReP-132 is able to interact directly with SF-1, pull-down analysis demonstrated that the GST-SF-1 fusion protein is capable of binding full-length TReP-132. To confirm this interaction in vivo it was demonstrated in cotransfection experiments in HeLa cells that SF-1 protein coprecipitates with TReP-132, which is indicative of the two proteins interacting in intact cells. A third approach, consisting of two-hybrid analyses, showed an interaction between the GAL4-TReP-132 and VP16-SF-1 fusion proteins. The results obtained using these three approaches are thus consistent with the possibility that these two proteins interact without the necessity of TReP-132 to bind DNA.

TReP-132 contains two nuclear receptor box (NR-box) LXXLL motifs at amino acids 181 and 863, which are frequently found in the nuclear receptor interaction domains of transcriptional coregulatory proteins such as p/CIP, NcoA-1, NcoA-2, RAC3, TIF-2/GRIP-1, SRC-1/p160, CBP/p300, RIP-10, TIF-1, and TRIP-1/SUG-1 (43, 66–70). To determine whether these two putative NR-boxes are involved in the interaction with SF-1, two-hybrid experiments were performed with TReP-132 amino- and carboxy-terminal deletion mutants fused to the DNA-binding domain of GAL4. The GAL4–TRePdel4 protein, which comprises the amino-terminal region of TReP-132 from residues 1 to 248 and contains the LRQLL motif at residue 181, is sufficient to retain interaction with SF-1. In contrast, the GAL4–TRePdel15 to del17 proteins, which contain the LEMLL motif at position 862 but not the LRQLLL sequence at residue 181, were not able to interact with SF-1. Thus, only the TReP-132 constructs containing the LRQLLL motif are able to interact with VP16-SF-1. Moreover, the interactions of GAL4–TRePdel4 and GAL4–TRePdel4 with VP16-SF-1 were abolished when the leucines of the LRQLLL sequence were changed to alanines, further implicating the LRQLLL motif in the interaction with SF-1. To confirm the importance of the LRQLLL motif in the function of TReP-132 as a coactivator of SF-1, mutation of this sequence in full-length TReP-132 abolished the stimulatory effect on P450scc gene promoter activity in NCI-H295 cells cotransfected with SF-1.

The two-hybrid experiments also implicated the two carboxy-terminal zinc-fingers of TReP-132 in transcriptional regulation. Deletion of the region containing the zinc-finger motifs in the GAL4–TRePdel1 construct decreased the ability of TReP-132 to activate basal promoter activity and decreased the functional interaction between TReP-132 and SF-1. The C$_2$H$_2$ zinc-finger domains of transcription factors have previously been implicated in protein–protein interactions. For example, both the aryl hydrocarbon receptor (AhR) and the AhR nuclear translocator (Arnt), which form a trans-acting heterodimer, interact with the zinc-finger domain of Sp1 to confer drug-inducible expression of CYP1A1 (71). Similarly, the carboxy-terminal zinc-fingers of GATA1, which interact and synergize with Sp1 and EKLF (72), are sufficient to induce megakaryocytic differentiation (73). As other examples, p53 and par-4 were shown to interact with the C$_2$H$_2$-type zinc-fingers located at the carboxyl terminus of WT1 (74, 75) and to repress the activity of WT1. Because the transcriptional activation and repression domains of WT1 have been mapped to regions outside of the zinc-finger domains, it was suggested that par-4 does not play a role as a classical cofactor for WT1-mediated transcription but is nonetheless a critical modulator that contributes to the determination of WT1 function as either a transcriptional activator or repressor. Although it is apparent that the zinc-fingers at the carboxyl region of TReP-132 are involved in its transcriptional activity, the mechanism(s) of action and factors implicated remain to be elucidated.

Unlike the classical nuclear receptors, which have been found to contain an active promoter domain, and are able to direct cAMP-dependent transcription in Y1 and NCI-H295 cells. With all of the reporter constructs used in the present study, mutation of the SF-1 binding site at position –46 also abolished the response to TReP-132, which suggests an interaction between the two proteins where the ability of TReP-132 to increase P450scc promoter activity is dependent on SF-1 binding to its cis-acting element.

FIG. 6. TReP-132, SF-1, and CBP/p300 interaction confers synergistic activation of the P450scc promoter. A, TReP-132 interacts with three distinct regions of CBP by pull-down assay. Different regions of CBP between the amino acid residues as indicated at the top of the figure were expressed as GST fusion proteins, and approximately equal amounts were immobilized on glutathione-coupled Sepharose prior to incubation with TReP-132 labeled with [35S]methionine. The regions of the GAL4-TReP-132 input

Transfected vectors

- TReP-132
- +TReP-132

Fold over pGL3

SF-1 p300

-1676/+49secLuc, −1676/+49msecLuc, and −110/+49secLuc (0.1 µg) were each transiently cotransfected with plasmids expressing SF-1 (0.1 µg), TReP-132 (0.2 µg), and p300 (0.4 µg) or a combination of these proteins, as indicated at the bottom of the graph. The results are expressed as fold increase of luciferase activity over the value obtained from pGL3 alone (±S.D.) and were normalized to Renilla luciferase activities. The coexpression of p300, SF-1, and TReP-132 leads to a synergistic increase of promoter activity. This synergistic effect is abolished by mutation of the SF-1 binding site found between nucleotides −38 and −46.

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high affinity ligands that are essential for AF-2-mediated activation (50, 52, 53, 76, 77), a high affinity ligand for SF-1 remains to be identified (78, 79). Mutations in the AF-2 domain of SF-1 suppresses protein kinase A-dependent transactivation of the bovine CYP17 gene (80). Moreover, the AF-2 domain was shown to interact with SRC-1 and to be required for GAL4-SF-1 transactivation (58). In the present study, two-hybrid experiments show that the AF-2 domain of SF-1 is required for interaction with TReP-132, because deletions of the AF-2 hexamer motif in the VP16-SF-1 chimeric proteins alleviated interaction with GAL4-TReP and GAL4-TRePdel4.

In addition to the AF-2 domain, other regions of SF-1 have been shown to be involved in protein-protein interactions. Although SF-1 does not harbor a transcriptionally active AF-1 domain, this nuclear receptor has been shown to contain other active domains in the amino-terminal region. Crawford et al. (48) have shown that full interaction of the SRC-1 coregulator with SF-1 requires an intact AF-2 hexamer motif and the proximal activation domain located between amino acids 187 and 245. The present study shows that the same domains are involved in the interaction between SF-1 and TReP-132, because the deletion of either the proximal activation domain or the AF-2 hexamer motif in SF-1 alleviated interaction with TReP-132. It is also shown here that the addition of an amino region to the proximal activation domain of SF-1 between amino acids 119 and 187 increased interaction with TReP-132, which concurs with the results of Crawford et al. (48) showing that this region also increases the interaction between SF-1 and SRC-1. It can be speculated that SF-1 interacts differentially with coregulator proteins to regulate gene transcription in a promoter- and cell type-specific manner. However, with the recent reports demonstrating the implication of other factors such as Dax-1 (32, 81–84) and N-CoR (85) in SF-1 function, it is clear that other studies will be required to fully understand the mechanisms by which SF-1 regulates the expression of its many target genes.

The coregulators CBP/p300 have been demonstrated to interact with SF-1 and to increase the promoter activity of the human P450scc gene via the SF-1 binding site located between nucleotides −38 and −46 (40). Subsequently, it has been shown that CBP/p300 also interact with TReP-132, leading to increased promoter activity of the human P450scc gene (42). With the finding of the present study that TReP-132 interacts with SF-1, another question we addressed was whether the three factors CBP/p300, TReP-132, and SF-1 can function in association to increase transcriptional activity. The transfection experiments clearly demonstrate that combined coexpression of all three proteins leads to a synergistic activation of the P450scc gene promoter that is much higher than when coexpressing either of the two proteins alone. This cooperativity is similar to the situation described for the carboxyl-terminal transactivation domains of HIF-1α, which was shown to function in synergy with SRC-1 and CBP to increase transcription (86).

In the study of Monté et al. (40) SF-1 was shown to interact with both the amino- and carboxyl-terminal regions of CBP between amino acids 1–451 and 1460–1891. In a subsequent study, a partial TReP-132 protein that was truncated at the amino-terminal end and thus contained residues 439 to 1200 was shown to interact with CBP at the region from amino acids 1460 to 1891 (42). In the present study, the full-length TReP-132 protein was shown to interact with the regions of CBP between residues 1–451, 1460–1891, and 1892–2441. These three regions of CBP involved in interaction with SF-1 and TReP-132 are also involved in the interaction with other distinct nuclear receptors and transcriptional coregulators (for review see Refs. 87 and 88). The region 1–460 was previously shown to interact with RAR, RXR, TR (89, 90), ER (91), CREB, c-Jun (44, 92), and PPARγ2 (93). The region between residues 1460 and 1891 contains the histone acetyltransferase domain and is involved in interaction with transcriptional activators such as MyoD (94), TFIIB (95), and E2F-1 (96). The most carboxy terminal region of CBP from residues 1892 to 2441, which interacts with TReP-132, contains the domain shown to interact with the corepressor SRC-1 (97, 98). The interaction of TReP-132 and SF-1, with more than one domain of CBP, is not too surprising considering that this has been observed with other transcription factors such as p/CAF (99), STAT1 (100), p65 (101), and AF-1 (102).

TReP-132 was isolated based on its ability to bind to the region between nucleotides −155 and −131 of the P450scc gene promoter. However, the interaction of TReP-132 with SF-1 and the requirement of an intact SF-1 binding site for TReP-132 activity in human adrenal NCI-H295 cells indicate that this protein also functions as a coactivator of the nuclear receptor. Moreover, TReP-132 was shown to activate the reporter construct containing three copies of the SF-1 binding site upstream of the minimal thymidine kinase promoter in NCI-H295 cells; and TReP-132 does not interact with the SF-1 binding site, as determined by electrophoretic mobility shift analyses (data not shown). The function of TReP-132 as a coregulator of SF-1 resembles other factors that have been demonstrated to interact with SF-1 and to influence its activity on gene promoter regulation. The Ptx1 homeobox transcription factor was initially demonstrated to bind to and activate expression of the pituitary pro-opiomelanocortin (POMC) gene (103, 104). Subsequently, Ptx1 was also shown to interact and synergize with SF-1 to activate the pituitary LHβ gene promoter, where Ptx1 apparently does not bind DNA (105). It has also been reported that the orphan nuclear receptor Dax-1 (dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome, gene 1) suppresses SF-1 activity through direct physical interaction (81, 85) and inhibits the expression of several SF-1 target genes including those encoding MIS (32), the high density lipoprotein receptor (82), and hP450c17 (84). In the study of Crawford et al. (85), Dax-1 was shown to recruit the corepressor N-CoR to SF-1 to repress promoter activity. In the study of Nachtigal et al. (32), Dax-1 was demonstrated to interact with SF-1 and to antagonize the synergy between SF-1 and WT1 involved in regulation of MIS gene expression. It is interesting that Dax-1 and WT1 are two transcription factors that, under certain promoter contexts, interact directly with cis-acting elements (106, 107), but in regulation of the MIS gene their mode of action apparently does not involve direct DNA binding when interacting with SF-1 (82, 83).

Results taken from the present and previous studies (40, 42) indicate that TReP-132, SF-1, and CBP/p300 interact together to regulate gene transcription. As described for other transcription factors and coregulators, it is tempting to speculate on a mechanism by which SF-1 and TReP-132 are bridged by CBP/p300 and influence the interaction of p300 with other factors to influence RNA polymerase II transcription. An example of this is the role of CBP/p300 as a “bridging molecule” in the positive cross-talk between STAT3 and Smad1 (108) and the role of CBP/p300 between Myb and C/EBPβ to activate Mim-2 in myelomonocytic differentiation (109). Considering the different coregulators that interact with SF-1 (such as Dax-1, N-CoR, and WT-1), it is possible that TReP-132 also interacts with these proteins to recruit or displace factors in a SF-1-dependent complex required to regulate gene expression. The coexpression of TReP-132 with these SF-1-interacting proteins in the same tissues, which include the adrenal and testis, is consistent with
their interaction. It is clear that additional studies will be required to further decipher and understand the mechanism(s) by which TReP-132 and SF-1 interact to regulate gene expression, which may play a major role in steroidogenesis, organogenesis, and reproductive function.

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