Mutations in the Activation Function-2 Core Domain of Steroidogenic Factor-1 Dominantly Suppresses PKA-dependent Transactivation of the Bovine CYP17 Gene*

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Steroidogenic factor-1 (SF-1) is a nuclear receptor that is essential for the proper development and function of steroid hormone-producing cells. The activation function-2 (AF-2) domain in SF-1 is a short α-helix in the C terminus that is conserved with respect to other nuclear receptors and is important for transactivation of target genes. In order to investigate the possible role of the AF-2 domain of SF-1 in cAMP-dependent transcriptional regulation of the bovine steroid hydroxylase gene CYP17, mutations were introduced and the effects were characterized. The mutant SF-1 proteins were expressed at comparable levels in nonsteroidogenic Cos-1 cells that lack SF-1, and their abilities to bind an SF-1 site from the CYP17 gene were not affected. Transient transfections of wild-type and mutant SF-1 in Cos-1 cells showed that the capacity to transactivate a reporter gene under the control of the SF-1 site from CYP17 was reduced by the mutations in the AF-2 domain of SF-1. A point mutation in the AF-2 region, E454A, resulted in a relative reporter gene activity that was 21% of that observed with wild-type SF-1. Co-transfections of adrenocortical Y-1 cells, which express endogenous SF-1, with the catalytic subunit of cAMP-dependent protein kinase (PKA-C) and the SF-1-dependent reporter gene showed on average a 16-fold increase in activity in the presence of PKA-C. Introduction of the AF-2 mutants of SF-1 into Y-1 cells completely abolished the PKA-C-mediated stimulation of the reporter gene. The transdominant negative effect of the mutant SF-1 proteins suggests that the AF-2 domain is essential for the activation of SF-1 by the cAMP-dependent protein kinase-dependent signaling pathway.

Steroid hormone biosynthesis in the adrenal cortex, testis, and ovary is under the control of the trophic hormones adrenocorticotrophic hormone, luteinizing hormone, and follicle-stimulating hormone from the pituitary (1, 2). Through signaling via G-protein coupled cell surface receptors, these hormones increase intracellular cAMP levels and activate cAMP-dependent protein kinase (PKA). As a consequence, steroidogenesis is stimulated through a rapid mobilization and movement of the substrate cholesterol to the enzymes, which convert it to biologically active hormones. In addition, cAMP and PKA increase the transcription of the genes encoding the biosynthetic enzymes, including the steroid hydroxylases which belong to the cytochrome P-450 superfamily of enzymes (2). Steroidogenic factor-1 (SF-1) or Ad4BP is a transcription factor belonging to the nuclear receptor superfamily, which appears to be crucial for not only the expression of essentially all components of the steroidogenic pathways but also for the development of the steroidogenic tissues per se (3–5). Thus, disruption of the SF-1 gene in mice leads to complete agenesis of the adrenals and gonads (6).

Several lines of evidence have suggested that SF-1 may be directly involved in the cAMP-dependent regulation of steroid hydroxylase gene expression. Often SF-1-binding sites in target genes reside within regions that have been functionally assigned cAMP-responsiveness and require an intact PKA to be active (7, 8). Also, mutations that interfere with SF-1 binding often attenuate cAMP responsiveness (9). Finally, SF-1 contains a putative PKA phosphorylation site (10), can be phosphorylated by PKA in vitro (11) and is immunoprecipitated as a phosphoprotein from adrenocortical cells grown in the presence of [32P]orthophosphate (12). However, the exact role of SF-1 in mediating the cAMP response is not well understood, and results in the literature are in part conflicting. In the bovine CYP17 gene, which encodes the cytochrome P-450 17α-hydroxylase, an SF-1 site is present within cAMP-responsive sequence 2 (CRS2) (13), and mutations that interfere with SF-1 binding correlate with decreases in cAMP-stimulated transcription of a linked reporter gene (9). Furthermore, transfection of SF-1 into a nonsteroidogenic cell lacking this transcription factor activates an otherwise inactive CRS2-dependent reporter construct, and co-transfection of the catalytic subunit of PKA (PKA-C) increases the activity severalfold. In order to determine whether this response relies on structures within SF-1 itself, amino acid substitutions and deletions were introduced. Here we show that mutations within a conserved core region of a putative C-terminal activation function-2 (AF-2) domain of SF-1 compromises its transactivating ability. Furthermore, such mutant SF-1 proteins are able to dominantly suppress the activity of wild-type SF-1 in adrenocortical tumor cells transfected with PKA-C.

EXPERIMENTAL PROCEDURES

Chemicals—Cell culture media and sera were purchased from Life Technologies, Inc. 32 The ECL (chemiluminescence) kit for Western blotting was from Amersham Pharmacia Biotech, and the luciferase assay kit was from BIO Themes AB (Luciferase Assay kit, production number 484-001, Dalaro, Sweden). All other chemicals were from Sigma or Merck.

Cell Culture and Transient Transfection—Y-1 cells were maintained in Dulbecco’s modified Eagle’s medium (high glucose) supplemented

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1 The abbreviations used are: PKA, cAMP-dependent protein kinase; SF-1, steroidogenic factor-1; hGR, human glucocorticoid receptor; EMSA, electrophoretic mobility shift analysis; PKA-C, catalytic subunit of PKA; CRS2, cAMP-responsive sequence 2; AF-2, activation function-2; Ad4BP, adrenal 4-binding protein.

2 A. L. Jacob and J. Lund, unpublished observations.
with 10% fetal calf serum, 10 units penicillin/ml, and 10 μg streptomycin/ml. The day before transfection, Y-1 cells were plated in 6-well plates at a density of 1 × 10⁶ cells/well. Before transfection, the standard growth medium was replaced by fresh medium, and the cells were incubated for a minimum of 2 h. The calcium phosphate co precipitation method was used to transfect Y-1 cells as described previously (13). Cells and 3 μg of total DNA was added per well. Cos-1 cells were cultured in Dulbecco’s modified Eagle’s medium (low glucose) supplemented with 10% fetal calf serum, 10 units penicillin/ml, and 10 μg streptomycin/ml. The day before transfection, Cos-1 cells were plated in 6-well plates at a density of 1 × 10⁶ cells/well. Cos-1 cells were transfected by the DEAE-dextran method according to standard procedures (13). Cells were incubated with transfection medium containing 3 μg of DNA for 1 h. After removing the transfection medium, 52 mg/liter of chloroquine was added in culture medium, and the cells were incubated for an additional 5 h. The culture medium containing chloroquine was removed, and the cells were incubated for another 18 h with culture medium before they were harvested.

Luciferase Assay—After 24 h of transfection, cells were washed with cold phosphate-buffered saline, and 200 μl of lysis buffer was used to harvest the cells by scraping. 40 μl of the cell extracts were used for luciferase determinations on a LUCY-1 luminometer (Anthos, Austria). Preparation of lysis buffer and the luciferase enzyme assay was according to the procedures of a commercial kit (BIO Themia AB, Luciferase Assay kit, production number 484-001 Dalaro, Sweden).

Plasmid Constructions—Constructions of the reporter plasmid pTS1-4CRS2 and the expression plasmid pCMV5-SF-1 are as described (15). For mutagenesis of the AF-2 domain, the QuickChange® Site-directed Mutagenesis kit (Stratagene) was used. Briefly, the double-stranded plasmid pCMV5-SF-1 and two synthetic oligonucleotide primers with the desired mutations, each complementary to opposite strands, were annealed and extended by means of Pfu DNA polymerase. Following temperature cycling, the products were treated with DPN I in order to digest the parental plasmid. The DNA vectors with desired mutations were transformed into E. coli (Epicurian Coli® XL1-supercompetent cells), and the mutations were confirmed by the dideoxy nucleotide sequencing method (USB, Sequenase, version 2.0).

Western Blot Analysis—To determine expression levels, Cos-1 cells were transfected with plasmids encoding wild-type SF-1 and AF-2 mutants. After 24 h, cells were lysed, and extracts were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose filters. Western blot analysis of SF-1 proteins was carried out with a polyclonal antiserum to Ad4BP or SF-1 (kindly provided by Dr. Ken Morohashi), and immunoreactive proteins were visualized by the enhanced chemiluminescence method (ECL, Amersham Pharmacia Biotech).

Electrophoretic Mobility Shift Analysis (EMSA)—Double-stranded synthetic oligonucleotides containing the SF-1-binding site in CRS2 of the bovine CYP17 gene (15) was labeled with γ-32P and purified by native polyacrylamide gel electrophoresis. 10 μg of nuclear extracts from transfected Cos-1 cells containing wild-type and mutant SF-1 protein were incubated with the 32P-labeled probe. The reaction conditions were as described previously (13). Protein-DNA complexes and unbound probe were separated by nondenaturing electrophoresis on a 4% polyacrylamide gel in 0.5 × TBE (0.045 M Tris borate, 0.001 M EDTA).

RESULTS AND DISCUSSION

Several lines of evidence suggest that the nuclear receptor SF-1 may be directly involved in cAMP-dependent transcriptional regulation of steroid hydroxylase genes (see the Introduction). However, direct evidence of functionally important structures in SF-1 for the cAMP effect is lacking. The ligand-binding domain of nuclear receptors is located in the C-terminal region and consists of approximately 225 amino acids involved not only in ligand binding but also in hormone heterodimerization, hormone-dependent transcriptional activation as well as hormone-reversal function (transcriptional repression) (16, 17). The C-terminal activation function domain (AF-2) is common to all ligand-activated members of the nuclear receptor superfamily, and SF-1 has been shown to be activated by 25-hydroxy cholesterol in an AF-2-dependent manner (18). The AF-2 region in SF-1 and the alignment with various nuclear receptors are shown in Fig. 1A. The AF-2 sequence is LLIEML (Fig. 1B), and the domain structure of SF-1 is shown. The DNA-binding domain (DBD), the ligand-binding domain (LBD), and the C-terminal AF-2 core domain are indicated. B, alignment of the C-terminal amino acid sequence of SF-1 with various nuclear receptors. Ad4BP or SF-1 (ADHβ), thyroid receptor α and β (THA1 and THB1), and retinoic acid receptor α, β, γ (RAR1, RAR2, and RRG1) were aligned. The AF-2 sequence LLIEMI is represented as ΦΦΦΦΦΦΦ, where Φ represents hydrophobic residues and E (glutamic acid) is common to most of the nuclear receptors. C, the amino acids substitutions and deletions in the putative AF-2 core domain of SF-1 are shown. In the L and LL mutants, the amino acid leucine is substituted for alanine. The amino acid glutamic acid is changed to alanine in the E mutant. LL deletion, AF-2 core deletion, and 5’-extended AF-2 core deletion are deletion mutants.

In mutants 1–3, the nonpolar Leu 451 and Leu 452 were changed to the small nonpolar residue alanine or leucine. The conserved glutamic acid at position 454 was substituted for alanine in mutant 4. The entire AF-2 core was deleted in mutant 5, whereas in mutant 6, the arginine and the two asparagine residues N-terminal to the AF-2 core were also deleted (Fig. 1C). Wild-type and mutant plasmids were transfected into Cos-1 cells together with the reporter plasmid pTS1-4CRS2Luc containing the SF-1-binding site from the proximal promoter region of the bovine CYP17 gene.

Proper expression from wild-type and mutant plasmids was analyzed by Western blot analysis and EMSA. Using a polyclonal antiserum to SF-1, whole cell extracts from transfected Cos-1 cells show the expression of both wild-type and mutant SF-1 proteins at comparable levels (Fig. 2A). 10 μg of nuclear extracts of transfected Cos-1 cells were also used in EMSA, and no significant differences in DNA binding were observed when comparing wild-type and mutant SF-1 proteins (Fig. 2B). It was therefore concluded that wild-type and mutant SF-1 proteins...
are expressed at comparable levels and that the proteins carrying mutations in their AF-2 core domain have retained their ability to bind DNA. To positively identify the mobility of SF-1, steroidogenic H295 cell extracts incubated in the presence or absence of antibodies against SF-1 before loading on the gel are also shown (Fig. 2B, right-hand two lanes).

Transactivation by Wild-type and Mutant SF-1 in Nonsteroidogenic Cos-1 Cells—To determine the effect of mutations in the AF-2 core domain of SF-1 with respect to its ability to stimulate the expression of pT81-4CRS2LUC, wild-type and mutant plasmids were transfected into Cos-1 cells. After 24 h of transfection, cells were lysed, and the luciferase activity was determined. No activity was observed in extracts from cells transfected with the empty expression vector and the reporter plasmid (data not shown). Significant differences in transactivation were obtained when comparing wild-type and mutant SF-1 (Fig. 3). All the six mutants showed decreased ability to transactivate pT81-4CRS2LUC. In mutants 4, 5, and 6, the relative luciferase activity obtained was 21, 32, and 14% of that obtained with wild-type SF-1, respectively. The α-helical region of AF-2 is strongly hydrophobic and direct contact with ligand has been shown in the thyroid receptor (T3Rα) and the retinoic acid receptor (RARγ) (19, 20). According to the crystal structures of T3Rα and RARγ, the leucine residues Leu451 and Leu452 as well as Glu454 in SF-1 are predicted to be surface exposed in the helical core and form part of an interaction surface recognized by cofactors or ligand. Leu451 and Glu454 are among the most conserved residues in other nuclear receptors, and Glu454 is not only surface exposed but also extends into the solvent. The dramatic effect of mutating Glu454 in SF-1 supports the notion that this is an essential residue for transactivation by nuclear receptors.

SF-1 Mutated in the AF-2 Domain Dominantly Suppresses cAMP-stimulated Transcription—In Y-1 cells (mouse adreno-
cortical tumor cells), which express endogenous SF-1, transfection with the reporter plasmid pT81-4CRS2LUC resulted in clearly detectable levels of luciferase activity (Fig. 4). When the mutant SF-1 proteins were expressed, a significant reduction in the level of basal activity was observed. Transfection of the mutants 5 and 6, which carry deletions of the AF-2 domain, gave rise to relative luciferase activities that were 44 and 32%, respectively, of the activities obtained when the empty expression vector was used for transfection. These results show that the basal activity of the pT81-4CRS2LUC is attenuated through transfection with plasmids encoding SF-1 proteins deleted in the AF-2 core region.

SF-1 is an activator of CRS2-mediated transcription, and this activity has previously been shown to be stimulated in the presence of forskolin and PKA-C (9). In agreement with these results, co-transfection of Y-1 cells with pT81-4CRS2LUC and 100 ng of plasmid encoding PKA-C plasmid led to a highly significant increase in luciferase activity (Fig. 4). However, the transcriptional activation by endogenous SF-1 was drastically reduced (up to 90% inhibition) when the AF-2 mutants were expressed in the presence of PKA-C. These results suggest that the AF-2 mutants of SF-1 compete with the endogenous SF-1 for transactivation in a dominant negative fashion. In contrast, transfection of Y-1 cells with a plasmid encoding the fusion protein between green fluorescent protein and the human glucocorticoid receptor (hGR) did not affect the activity of pT81-4CRS2LUC either in the presence or absence of PKA-C (Fig. 5).

The mechanism(s) underlying the transdominant negative effect by these AF-2 mutants of SF-1 could theoretically involve dimerization of the mutant SF-1 proteins and endogenous SF-1. However, heterodimer formation is not supported by evidence from the literature, which indicates that SF-1 binds DNA as a monomer (21). Other possible explanations might be the competition for binding of DNA and co-activators such as steroid receptor co-activator-1 and CREB-binding protein. Interaction of steroid receptor co-activator-1 in an AF-2-depend-ent manner with SF-1 has been recently demonstrated (22).

We have noticed up to a 40-fold increase in luciferase activity by co-transfection of Y-1 cells with pT81-4CRS2LUC and PKA-C. The results from the AF-2 mutations suggest that the increased activity relies on this domain in SF-1. What could therefore be the possible site(s) of cAMP regulation? Previous studies show that the activation of the PKA signaling pathway does not increase the mRNA levels for SF-1 in Y-1 and MA-10 cells (11) nor does it affect SF-1 protein levels in Y-1 cells. So the enhanced transactivation observed in PKA-stimulated Y-1 cells should not be the consequence of a change in the abundance of SF-1. It is possible that PKA-C expression in Y-1 cells increases the levels or the availability of a possible ligand for SF-1. 25-Hydroxy cholesterol has been shown to activate SF-1 (18), but it is not known whether the enzyme responsible for the synthesis of this ligand is induced by PKA in adrenocortical cells.

Studies on other nuclear receptors have shown that phosphorylation can lead to conformational changes in the proteins and can effect DNA binding and protein-protein interactions as well as nuclear translocation (24). In vitro phosphorylation of SF-1 by PKA-C has been reported (11). This could suggest that PKA-dependent phosphorylation would be a possible mechanism for activation of SF-1. However, increased phosphorylation led to a decreased DNA binding affinity of SF-1 (11). This is analogous to NGFI-B (Nur77), another nuclear receptor, where increased levels of in vivo phosphorylation of NGFI-B protein correlated with decreased DNA binding affinity (25). However, it is difficult to envision how PKA-dependent inhibition of DNA binding by SF-1 could lead to increased transactivation of target genes and why an intact AF-2 domain would be required. Whether SF-1 protein can be phosphorylated in vivo by PKA and whether such phosphorylation achieves receptor activation via the AF-2 domain should therefore be experimentally tested. It is also possible that activated PKA does not directly phosphorylate SF-1 to regulate its transactivation capacity but that it instead elicits a change in the phosphorylation status by other mechanisms. There is precedence in the literature for phosphorylation-dependent activation of nuclear receptors that appears to be direct and not dependent on the presence of ligand (23, 26), e.g. the estrogen receptor can be activated in the absence of ligand by cAMP treatment of cells (23).

In conclusion, our results clearly show that even a single point mutation in the AF-2 core domain of SF-1 had a significant effect on transactivation and transformed SF-1 into a dominant negative mutant with respect to PKA-C-dependent activation of a SF-1-regulated reporter gene. Co-activators or ligands involved in transactivation by SF-1 in an AF-2-depend-ent manner in the presence of PKA-C remain to be determined.

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