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Regulation of differential COUP-TF-coregulator interactions in adrenal cortical steroidogenesis☆

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Abstract

Hyperfunctioning adrenocortical adenomas produce excessive amounts of various corticosteroids due to dysregulated expression of steroidogenic enzymes. Since no genetic mutations in steroidogenic enzyme genes have been identified as yet, the dysregulated expression at the transcription level may be crucial. Chicken ovalbumin upstream promoter-transcription factors (COUP-TFs) and steroidogenic factor-1 (SF-1) play key roles in the transcriptional regulation of steroidogenic *P450* genes. Transfection studies showed that SF-1 activated and COUP-TFs repressed the transcription of bovine *CYP17* gene promoter from the CRS2 element in a mutually exclusive manner in Y-1 cells. The results indicate that COUP-TFs negatively regulate the transcriptional activity of SF-1, a steroidogenic cell-specific activator of various steroidogenic *P450* genes. Expression of both COUP-TFI and COUP-TFII was significantly decreased in the cortisol-producing adenomas, in which CYP17 was drastically overexpressed, indicating that decreased expression of COUP-TFs play a key role in overexpression of CYP17 in this type of tumors. We then screened for COUP-TFI-interacting proteins from a cortisol-producing adenoma cDNA library using a yeast two-hybrid system and identified a novel RING finger-containing protein which can function as a coregulator for COUP-TFI. Notably, COUP-TFI activated rather than repressed several target genes including the human *CYP11B2* gene promoter, the results of which were opposite to those of the *CYP17* promoter. The bifunctional activities of COUP-TFI may be derived from the promoter context and our newly identified COUP-TFI coregulator.

Keywords: COUP-TFs; Corepressor; Coactivator; N-CoR; SMRT; Steroidogenesis; Adrenal cortex; Cushing's syndrome

1. Introduction

Hyperfunctioning adrenocortical adenomas produce excessive amounts of various corticosteroids, resulting in secondary hypertension and several metabolic disturbances. The excessive steroid production in tumors results from dysregulated expression and activity of specific steroidogenic enzymes; the overexpression of CYP11B2 in aldosterone-producing adenomas and the overexpression of CYP17 and CYP21 in cortisol-producing adenomas [1–5]. Since no genetic mutations in *P450 genes* have been identified in adrenal tumors, dysregulated expression at the transcriptional level may be crucial for the excessive hormone production that is characteristic of this type of tumor. However, the mechanism of such expression of P450s is totally unknown. Nuclear orphan receptors, chicken ovalbumin upstream promoter-transcription factors (COUP-TFs), steroidogenic factor-1 (SF-1) and dosage-sensitive sex reversal, adrenal hypoplasia congenital critical region on the X chromosome-1, encoded by *NR0B1* (DAX-1) are shown to play important roles in the transcriptional regulation of several steroidogenic enzyme genes. To elucidate the mechanism, we have investigated role of COUP-TFs and other nuclear receptors in the regulation of expression of steroidogenic enzymes in the adrenal cortex. We have subsequently identified a novel RING finger-containing COUP-TFI-interacting protein using a yeast two-hybrid system. We also showed the bifunctional properties of COUP-TFI with activator and repressor functions may be derived from the promoter contexts and our newly identified coregulator.

2. Molecular mechanism of COUP-TFs action

COUP-TFs play a key role in the regulation of organogenesis, neurogenesis, and cellular differentiation during

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Table 1
Target genes for COUP-TFs

Gonads	LH receptor, FSH receptor		↓
Adrenal cortex	CYP17	17α-hydroxylase/17,20-lyase P450	↓
	CYP11B2	Aldosterone synthase P450	↑
	CYP19	Aromatase P450	↓
	DAX-1		↓
Pituitary gland	Oxytocin		↓
Cerebellum	PCP-2	Purkinje cell protein-2, cerebellar Purkinje cell-specific gene	↓
Liver	Angiotensinogen	Renin substrate	↓
	HNF-1	Hepatocyte nuclear factor-1	↑
Heart	ANF	Atrial natriuretic factor	↑
	Calreticulin	Ca^{2+} binding chaperone of the endoplasmic reticulum	↑
	NHE-1	Na^{+}/H^{+} exchanger-1	↑
Adipose tissues	PEPCK	Phosphoenolpyruvate carboxykinase	↑
	LPL	Lipoprotein lipase	↑
Prostate	NGFI-A	An early response gene expressed in prostate cancer	↑
Others	CaMKIV	Ca^{2+}/ calmodulin-dependent protein kinase IV	↓
	Vitronectin		↑

The arrows (↑) and (↓) indicate that COUP-TFs upregulate and downregulate (a target gene), respectively.

embryogenic development [6–14]. COUP-TFs are also involved in the regulation of several genes that encode metabolic enzymes, including phosphoenolpyruvate carboxykinase [15] and several steroidogenic P450s [16–18] (Table 1).

Members of the COUP-TF subfamily of nuclear receptors are generally considered to be repressors of transcription [8,19,20]. However, it is important to note that COUP-TF proteins may also activate transcription in some promoter and/or cellular contexts. Indeed, COUP-TFI, purified from HeLa cells, was originally characterized as a positive factor in the regulation of the ovalbumin promoter [21]. Other groups have subsequently demonstrated that COUP-TF can also function as a transcriptional activator in transient transfection experiments and in vitro. These findings, when considered together with the possibility that an activating ligand(s) for COUP-TFs may exist, raise the possibility that homodimeric or heterodimeric complexes composed of COUP-TF family members may positively regulate transcription of target genes in a cell-specific manner. Moreover, COUP-TFI has recently been shown to serve as an accessory factor for some ligand-bound nuclear receptors, suggesting that it may modulate, both negatively and positively, a wide range of hormonal responses (Table 1).

COUP-TF can form strong homodimers and bind to a wide spectrum of response elements with various arrays of the AGGTCA core motif [22], allowing COUP-TF to bind to a variety of hormone response elements recognized by other members of the subfamily, including receptors for retinoic acid (RAR), 9-*cis* retinoic acid (RXR), thyroid hormone (TR), and vitamin D_3, peroxisome proliferators-activated receptor (PPARs); and hepatocyte nuclear factor 4 (HNF-4). An important consequence of this promiscuous DNA binding is the inhibition of transcriptional activities of TR, RAR, RXR the vitamin D_3 receptor, PPARs, and HNF-4 on both artificial and native response elements. In this manner, the function of COUP-TFs has some similarities to that of RXR; both receptors can form heterodimers with a variety of nuclear receptors. Remarkably, the function of RXR is essentially silent on that of partner receptors; however, COUP-TFs repress the function of the partner receptors by several possible mechanisms. COUP-TFs compete for the response elements of the receptors, thus acting as passive repressors of the transcriptional activation induced by them. Another mechanism of passive repression by COUP-TFs involves their ability to heterodimerize with the RXR, reducing its availability for other nuclear receptors that uses it as a partner. COUP-TFs also function as a repressor by quenching of transactivator-dependent transcription and transrepression of activated transcription [8,19]. In addition, COUP-TF has been found to be capable of actively repressing the basal promoter activity of several target genes through interaction with corepressors nuclear receptor corepressor (N-CoR) and silencing mediator of retinoid and thyroid hormone receptor (SMRT) [18,20,23–25].

3. Transcriptional regulation of bovine CYP17 by COUP-TFs, SF-1, and DAX-1

Bovine CYP17, encoding one of the key enzymes in glucocorticoid and adrenal androgen production, has a cyclic AMP-response sequence 2 (CRS2) within the promoter region of the gene [16,17,26]. Steroidogenic factor-1/adrenal 4 binding protein (SF-1/Ad4BP, encoded by the gene *NR5A1*) [27–31] and COUP-TFI (encoded by *NR2F1*) can bind to the CRS2 region in a mutually exclusive manner to activate and repress, respectively, the transcription of the *CYP17* gene. COUP-TF, SF-1/Ad4BP and DAX-1 belong to the superfamily of nuclear hormone receptors and are classified as nuclear orphan receptors because their ligands have yet to be identified.

To determine what region of the COUP-TFI molecule is essential for repression, we carried out cotransfection experiments with various deletion mutants of COUP-TFI. Increasing concentrations of SF-1 cDNA transfected with the catalytic subunit of protein kinase A (PKA-C) cDNA into Y-1 cells increased the *CYP17* promoter activity from the CRS2 element (3CRS2-Luc) in a dose-dependent manner (lanes 1–4 in Fig. 1). As expected, co-expression of full-length COUP-TFI (1–423) repressed SF-1-mediated transactivation of the 3CRS2-Luc (lanes 5–7 in Fig. 1).

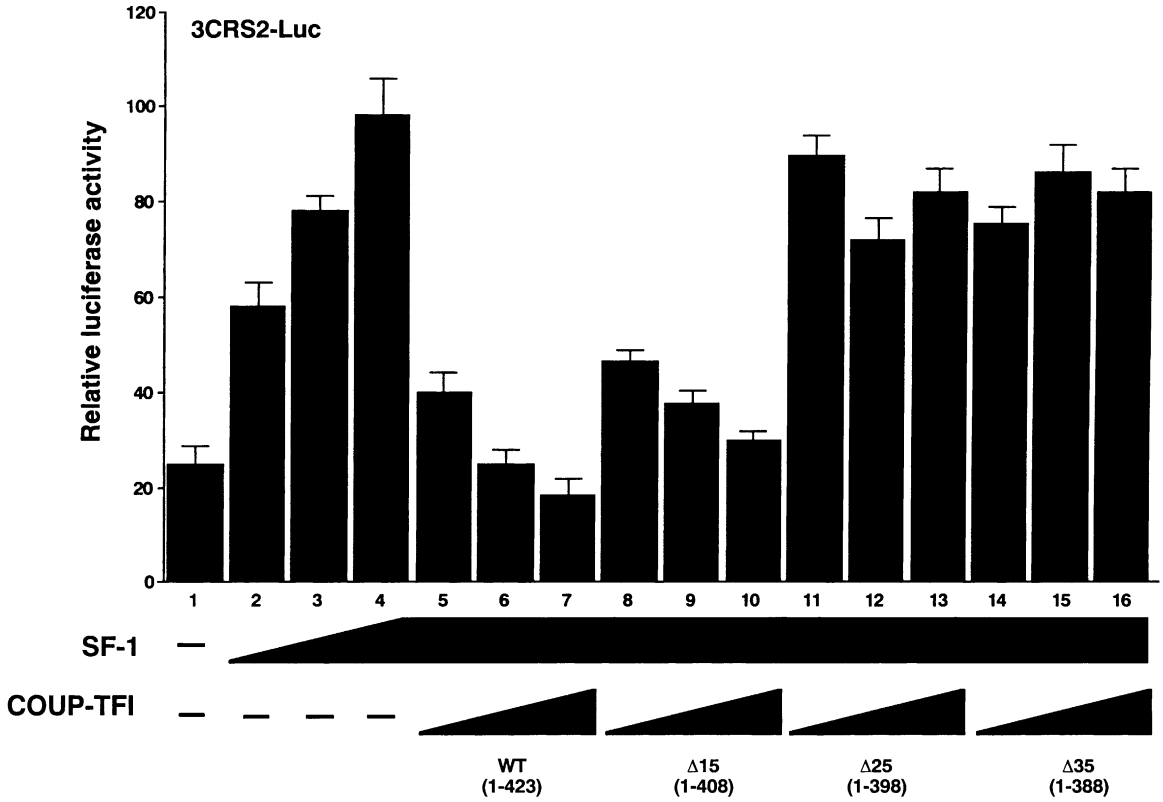

Fig. 1. The silencing function of the COUP-TFI was significantly diminished when 25 or 35 amino acids but not 15 amino acids were deleted from the C-terminal end in Y-1 cells. Y-1 cells were transfected with increasing amounts of SF-1 cDNA (0.1 μg (lane 2), 0.2 μg (lane 3) and 0.3 μg (lanes 4–16)), wild type or deletion mutant COUP-TFI cDNA (0.1 μg (lanes 5, 8, 11 and 14), 0.2 μg (lanes 6, 9, 12 and 15) or 0.3 μg (lanes 7, 10, 13 and 16)). All wells were transfected with 3CRS2-SV40-Luc reporter DNA (0.5 μg) and PKA-C (0.1 μg) cDNA.

Deletion of the last 15 amino acids from the C-terminal had no effect on its ability to repress the SF-1-dependent transcription (lanes 8–10 in Fig. 1). However, deletion of additional 10 or 20 amino acids, to give the COUP-TFIΔ25 (1–398) or COUP-TFIΔ35 (1–388) constructs, abolished its ability to repress transcription (lanes 11–16 in Fig. 1). The expression of these wild type and mutant COUP-TFI protein was confirmed at almost comparable levels with Western blot analysis (data not shown). These data suggest that the region of COUP-TFI encoding amino acids 398–408 is required for transcriptional repression of the bovine *CYP17* promoter.

Next, we examined the involvement of corepressors in COUP-TFI repression of SF-1-dependent activation of 3CRS2 promoter activity. As shown in Fig. 2A, SF-1-dependent transcription was repressed by overexpression of COUP-TFI in Y-1 cells (lanes 1–3 in Fig. 2A). Overexpression of N-CoR or SMRT further potentiated this repression activity in a dose-dependent manner (lanes 4–9 in Fig. 2A). However, neither N-CoR nor SMRT have a significant effect on transcription activity of the reporter construct in the absence of cotransfected SF-1 and COUP-TFI (data not shown). These data suggest that both N-CoR and SMRT function as a corepressor of COUP-TFI in SF-1-mediated 3CRS2-Luc transcription in Y-1 cells (lanes 4–9 in Fig. 2A). Similar results were also observed in COS-1 cells (data not shown). To determine the domains of SMRT critical for repression of the CYP17 CRS2, cotransfection experiments were performed with several SMRT deletion mutants. In the absence of exogenous SMRT, the COUP-TFI expression construct decreased SF-1-mediated repression by 67–75% (lanes 1–3 in Fig. 2B). Overexpression of exogenous SMRT encoding amino acids 1023–2517, 1051–1586 and 1587–2311 drastically potentiated COUP-TFI-mediated repression of the reporter activity (lanes 4–6 in Fig. 2B). In contrast, constructs containing the C-terminus of the SMRT protein (amino acids 2214–2517) had no effect on the repression activity of COUP-TFI (lane 7 in Fig. 2B). These results suggest that the domains of SMRT encoding both amino acids 1051–1586 and 1587–2311 can interact with CRS2-bound COUP-TFI in Y-1 and COS-1 cells to induce transcriptional repression; however, the C-terminal domain of SMRT encoding amino acids 2214–2517 does not interact with COUP-TFI.

In addition, the presence of trichostatin A, a potent inhibitor of histone deacetylases (HDACs), at a concentration between 10^{-8} and 10^{-6} M reversed COUP-TFI-mediated repression of *CYP17* promoter activity (data not shown), indicating that HDAC activity is involved in the COUP-TFI-mediated repression of the 3CRS2-Luc reporter activity.

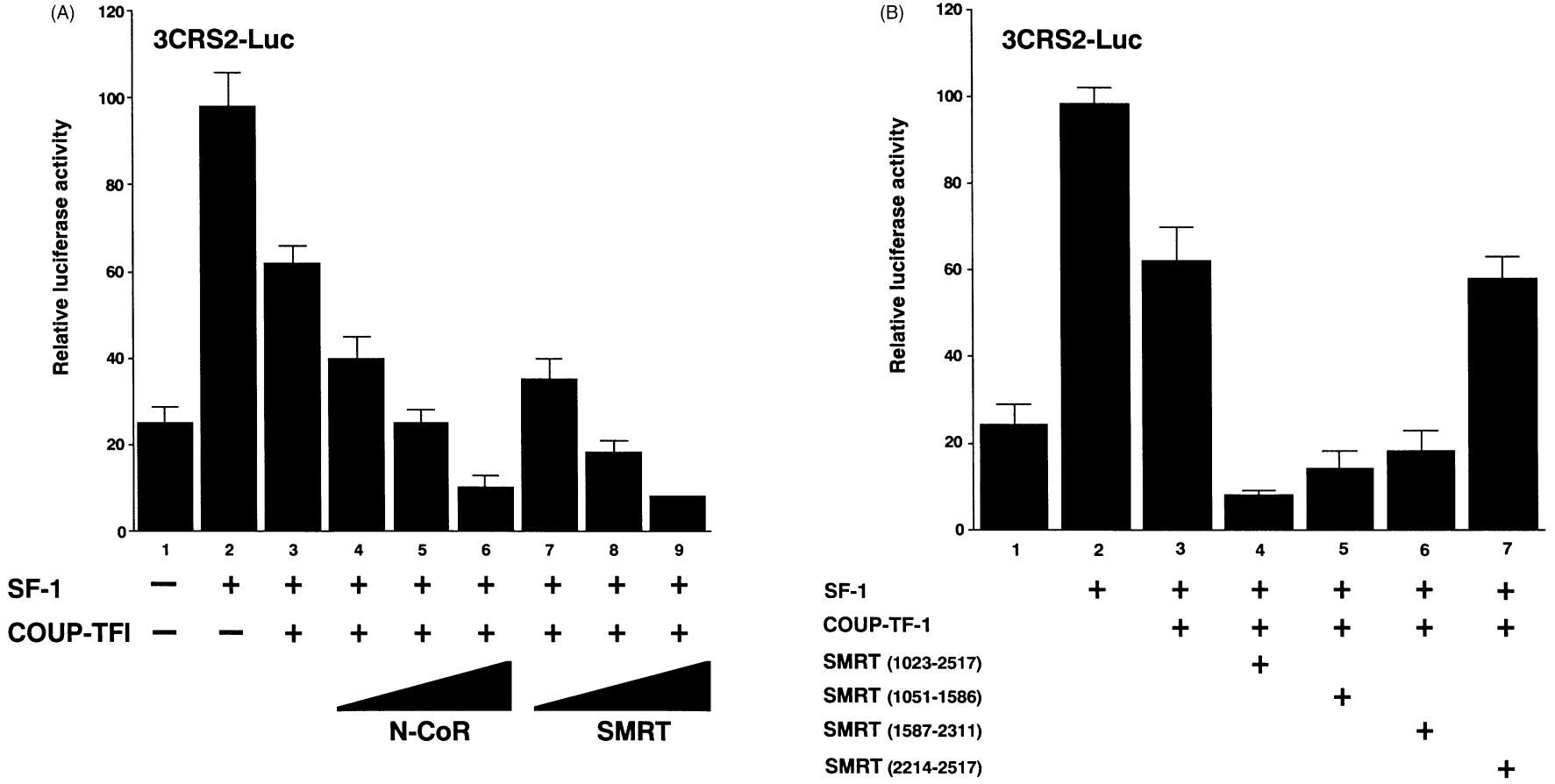

Fig. 2. (A) Both N-CoR and SMRT potentiate COUP-TFI-mediated repression of 3CRS2-Luc activity in Y-1 cells. Y-1 cells were also cotransfected with increasing amounts of N-CoR or SMRT expression plasmid (0.1 μg (lanes 4 and 7), 0.2 μg (lanes 5 and 8) or 0.3 μg (lanes 6 and 9)). (B) Domains of SMRT critical for COUP-TFI-mediated repression activity of 3CRS2-Luc in Y-1 cells. Y-1 cells were transfected with 0.2 μg SF-1, 0.2 μg COUP-TFI cDNAs, 0.5 μg 3CRS2-SV40-Luc reporter DNA and PKA-C (0.1 μg) cDNA. Y-1 cells were also cotransfected with expression plasmid carrying various deletion mutants of SMRT encoding amino acids 1023–2517, 1051–1586, 1587–2311 or 2214–2517 (0.3 μg).

4. Expression profiles of CYP17 and its relevant nuclear receptors in normal adrenals and adrenocortical adenomas

Since COUP-TFs, SF-1 and DAX-1 are shown to be crucial regulators for *CYP17* gene transcription in mammalian cells, we therefore examined the expression levels and immunolocalization of COUP-TF, DAX-1 and SF-1 in human adrenal glands and adrenocortical adenomas and compared the results with data on CYP17 expression and enzyme activity levels to study their potential correlation with adrenocortical steroidogenesis. In the normal adrenal cortex, nuclear immunoreactivities for COUP-TF, DAX-1 and SF-1 are readily detected in the zonae glomerulosa, fasciculata and reticularis of the adrenocortical cells (data not shown) [25,32,33]. The colocalization of the three orphan receptors suggests that COUP-TF1, DAX-1 and SF-1 play an important role in adrenocortical steroidogenesis. Interestingly, immunoreactivity for COUP-TF was also observed in the nuclei of adrenocortical stromal cells, suggesting that COUP-TF has some other role in addition to its involvement in steroidogenesis [25,32,33]. In

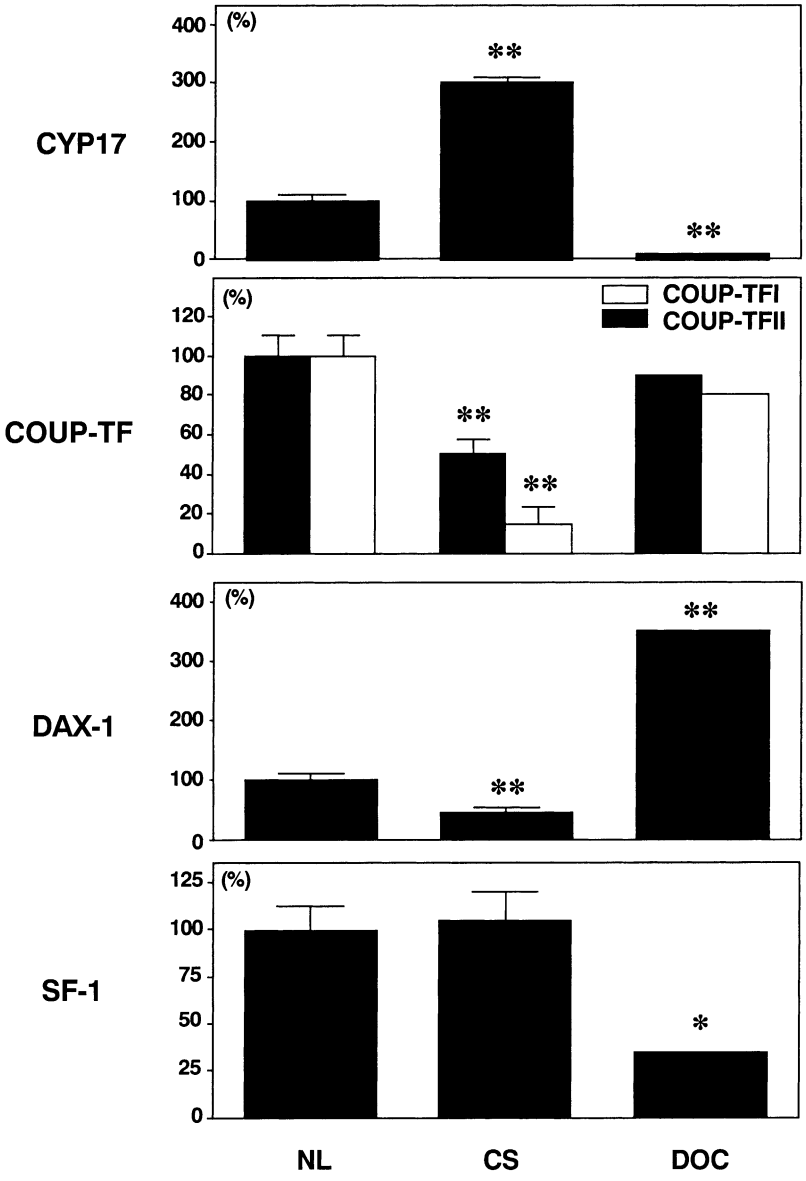

Fig. 3. Expression levels of CYP17 and of its relevant nuclear receptors in normal adrenals and adrenocortical adenomas. The graphs represent the immunointensities of CYP17, COUP-TFI, COUP-TFII, DAX-1 and SF-1 according to Western blot analysis. Western blots of CYP17, COUP-TFI, COUP-TFII, DAX-1 and SF-1 proteins in normal adrenals resected in conjunction with renal cell carcinoma (NL, $n = 5$), Cushing's syndrome (CS, $n = 5$) and deoxycorticosterone-producing adenomas (DOC, $n = 2$) were detected as bands of 53, 46, 46, 53, and 53 kDa, respectively. Immunointensities of specific bands were quantified using NIH Image Version 1.61 software on a Macintosh computer. All values are expressed as the mean ± S.E.M. The statistical significance of differences between groups was determined using an unpaired Student's *t*-test and StatView 5.0 software on a Macintosh computer. $*P < 0.05$ vs. NL, $**P < 0.01$ vs. NL.

contrast, CYP17 expression was significantly upregulated in Cushing's syndrome adenomas, where the expression levels of COUP-TFI, COUP-TFII and DAX-1 were reduced (Fig. 3). In deoxycorticosterone-producing adenomas, on the other hand, CYP17 expression was extremely reduced, whereas DAX-1 expression was markedly elevated. The expression of SF-1 did not differ between normal adrenals and Cushing's syndrome but appeared to be reduced in deoxycorticosterone-producing adenomas (Fig. 3). The results of Western blot analysis were consistent with those of immunohistochemistry [32,34] (data not shown). In summary, the expression profiles of COUP-TF, DAX-1 and SF-1 are totally different in normal adrenals and adrenocortical adenomas, such as in Cushing's syndrome and deoxycorticosterone-producing adenomas [25,32,33,35,36]. These altered COUP-TF and DAX-1 expression profiles appear to play an important role in the production of excessive corticosteroid secretion in adrenocortical tumors by acting as transcriptional repressors of the *CYP17* gene.

5. Function of COUP-TF-interacting proteins

A number of COUP-TF-interacting proteins have recently been identified using a yeast two-hybrid system (Table 2). We previously showed that gene silencing by COUP-TFI is mediated by N-CoR and SMRT [18,20]. Both N-CoR and SMRT appear to be involved in the recruitment of trichostatin A-sensitive HDACs to the template DNA. Indeed, the level of COUP-TFI expression was highly correlated with those of N-CoR expression, but not of SMRT expression, indicating that COUP-TFI and N-CoR may play a role in steroidogenesis by human adrenocortical adenomas [36]. In addition to these corepressors, the putative ligand binding domain of the COUP-TFI also binds Alien [37], the N-CoR variant RIP13Δ1 corepressor [24] and Friend of GATA-2 (FOG-2) [38]. In the brain, COUP-TFI is co-expressed with the zinc finger proteins CTIP1 and CTIP2 [39], which bind COUP-TFI and act as corepressor proteins. Expression levels of CTIP1 mRNA were highly correlated with those of COUP-TFI mRNA in human adrenocortical adenomas (unpublished observation). Thus, the selective use of tissue-restricted corepressor proteins may be one mechanism by which widely expressed nuclear receptors can induce tissue-specific transcription.

Table 2
A variety of COUP-TF-interacting proteins with versatile functions

Name	Structure and function
Nuclear receptors (TR, RAR, RXR, etc.)	Ligand-mediated transcription factors COUP-TFs inhibit the receptor's function by heterodimer formation
TFIIB	A component of basal transcriptional machinery
N-CoR, SMRT	Corepressor for several nuclear receptors, including TR, RAR, and COUP-TF
Ear2	Orphan nuclear receptor
CTIP1, CTIP2	C_2H_2 zinc finger proteins, corepressor for COUP-TFs
FOG-2	Multi-zinc finger proteins, corepressor for COUP-TFs
$p56^{lck}$	Coactivator for COUP-TFs
Novel COUP-TF-interacting protein	RING finger protein, coactivator and/or corepressor for COUP-TFs

6. Identification of novel COUP-TFI-interacting proteins

The results of our recent studies have suggested that COUP-TFs may play an important role in the regulation of adrenocortical steroidogenesis. To elucidate the mechanisms of COUP-TF-mediated repression, we screened for COUP-TFI-interacting proteins using a yeast two-hybrid system with a COUP-TFI as a bait from a human adrenocortical adenoma cDNA library. Together with known and well characterized COUP-TFI-interacting proteins, we identified a novel RING finger-containing protein [25,40] which interacted with COUP-TFI, COUP-TFII, SF-1, and DAX-1, but not with TRβ. This novel COUP-TFI-interacting protein contains a RING finger in the intermediate portion of the protein and interacts with the DNA binding domain and the hinge region of COUP-TFI through the C-terminal of the protein. The analysis of Gal4 DNA-binding domain-fusion protein demonstrated that this protein has autonomous repression domain at the C-terminus as well as activation domain at the N-terminus. The presence of trichostatin A did not affect transcriptional activity mediated by the Gal4 fusion protein of the coregulator, indicating that trichostatin A-sensitive HDACs may not be directly involved in the coregulator complexes. Transfection studies indicated that overexpression of COUP-TFI repressed the bovine *CYP17* reporter activity from the CRS2 element (Figs. 1 and 2), whereas increased rather than decreased reporter activities of the *Na^+/H^+ exchanger-1*, *NGFI-A* and the human *CYP11B2* genes (data not shown). In addition, co-expression of the novel COUP-TFI-interacting protein significantly enhanced these COUP-TFI mediated reporter activities, indicating that this protein can function as a bifunctional coregulator with both coactivator and corepressor functions. Taken together, these data indicate that COUP-TFI can function as a bifunctional transcription factor with both activator and repressor functions depending on the promoter contexts.

7. Conclusion

The results obtained to date imply that the nuclear orphan receptors, SF-1 and COUP-TFs are positive and negative transcription factors for several steroidogenic enzyme genes. Interestingly, even COUP-TFs can function as an activator as well as a repressor for several steroidogenic enzymes depending on the promoter contexts and allosteric effects

[41]. The bifunctional properties of COUP-TFs may also be derived from the COUP-TF coregulators including our recently identified RING finger-containing protein. Much remains to be explored to clarify the pathophysiological role of COUP-TFs and their coregulators in the adrenal cortex and adrenocortical tumors.

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