Ubc9 and Protein Inhibitor of Activated STAT 1 Activate Chicken Ovalbumin Upstream Promoter-Transcription Factor I-mediated Human CYP11B2 Gene Transcription*

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Isao Kurihara‡, Hirotaka Shibata§§, Sakiko Kobayashi‡, Noriko Suda†, Yayoi Ikeda‡, Kenichi Yokota‡, Ayano Murai‡, Ikuo Saito§§, William E. Rainey**, and Takao Saruta‡

From the ‡Department of Internal Medicine, School of Medicine, Keio University, Tokyo 160-8582, Japan, the §Health Center, Keio University, 33 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan, the ¶Department of Fine Morphology, Yokohama City University Graduate School of Medicine, Yokohama 236-0004, Japan, and the **Division of Reproductive Endocrinology, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9032

Aldosterone synthase (CYP11B2) is involved in the final steps of aldosterone biosynthesis and expressed exclusively in the adrenal zona glomerulosa cells. Using an electrophoretic mobility shift assay, we demonstrate that COUP-TFI binds to the −129/−114 element (Ad5) of human CYP11B2 promoter. Transient transfection in H295R adrenal cells demonstrated that COUP-TFI enhanced CYP11B2 reporter activity. However, the reporter construct with mutated Ad5 sequences showed reduced basal and COUP-TFI-enhanced activity, suggesting that binding of COUP-TFI to Ad5 is important for CYP11B2 transactivation. To elucidate molecular mechanisms of COUP-TFI-mediated activity, we subsequently screened for COUP-TFI-interacting proteins from a human adrenal cDNA library using a yeast two-hybrid system and identified Ubc9 and PIAS1, which have small ubiquitin-related modifier-1 (SUMO-1) conjugase and ligase activities, respectively. The coimmunoprecipitation assays confirmed that COUP-TFI forms a complex with Ubc9 and PIAS1 in mammalian cells. Immunohistochemistry showed that Ubc9 and PIAS1 are markedly expressed in rat adrenal glomerulosa cells. Coexpression of Ubc9 and PIAS1 synergistically enhanced the COUP-TFI-mediated CYP11B2 reporter activity, indicating that both proteins function as coactivators of COUP-TFI. However, sumoylation-defective mutants, Ubc9 (C93S) and PIAS1 (C351S), continued to function as coactivators of COUP-TFI, indicating that sumoylation activity are separable from coactivator ability. In addition, chromatin immunoprecipitation assays demonstrated that ectopically expressed COUP-TFI, Ubc9, and PIAS1 were recruited to an endogenous CYP11B2 promoter. Moreover, reduction of Ubc9 or PIAS1 protein levels by small interfering RNA inhibited the CYP11B2 transactivation by COUP-TFI. Our data support a physiological role of Ubc9 and PIAS1 as transcriptional coactivators in COUP-TFI-mediated CYP11B2 transcription.

1 Aldosterone is exclusively produced in adrenal zona glomerulosa cells due to its unique expression of aldosterone synthase cytochrome P450 (CYP11B2), the enzyme required for the final steps of aldosterone biosynthesis. In aldosterone-producing adrenal cortical adenomas of patients with primary aldosteronism, overexpression of CYP11B2 is demonstrated at the transcriptional level (1, 2). Although the reason for aberrant expression of CYP11B2 in these adenomas is not known, mutations in the CYP11B2 gene do not appear to be the cause (3, 4). We therefore postulated that transcription factors and/or coregulators may play important roles in CYP11B2 overexpression in the tumors.

The trans-acting factors that regulate CYP11B2 expression remain poorly defined. The orphan nuclear receptor, steroidogenic factor-1 (SF-1),1 is shown to play a crucial regulator of most steroid hydroxylase genes, including CYP17 and CYP11B1 (5, 6). However, SF-1 actually represses rather than activates expression of hCYP11B2 (7–9). In addition, other transcription factors that are expressed in the adrenal cortex include the NGFI-B family of orphan nuclear receptors, such as Nur1, NGFI-B, and NOR-1. The NGFI-B family receptors are highly expressed in the adrenal zona glomerulosa cells as well as in aldosterone-producing adenomas (8, 10, 11). These three nuclear receptors are rapidly induced early response genes that enhance transcription by binding to a consensus sequence, named NRE-1, as well as an Ad5 element of the hCYP11B2 promoter. In addition, CREB and ATF-1 enhance transcription of the hCYP11B2 gene by binding to a CRE (9, 12, 13). Our previous data (12) showed that human adrenocortical H295R nuclear proteins containing chicken ovalbumin upstream promoter-transcription factors (COUP-TFs) were bound to the −129/−114 sequence, designated as the Ad5 element of the hCYP11B2 promoter by electrophoretic mobility shift assays. The COUP-TFI was originally identified as an activator of the chicken ovalbumin gene (14, 15); however, COUP-TFs mostly function as transcriptional repressor of many target genes.

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† To whom correspondence should be addressed. Tel.: 81-3-3353-1211 (ext. 62312); Fax: 81-3-5363-3635; E-mail: hiro-406@cb3.so-net.ne.jp.

1 The abbreviations used are: SF-1, steroidogenic factor-1; STAT, signal transducers and activators of transcription; PIAS1, protein inhibitor of activated STAT 1; COUP-TFI, chicken ovalbumin upstream promoter-transcription factor I; SUMO, small ubiquitin-related modifier; NRE-1, NGFI-B response element; CREB, cAMP response element-binding protein; EMSA, electrophoretic mobility shift assay; siRNA, small interfering RNA; ChIP, chromatin immunoprecipitation; E1, ubiquitin-activating enzyme; E2, SUMO carrier protein; E3, SUMO-protein isopeptide ligase; CMV, cytomegalovirus; IP, immunoprecipitation; GFP, green fluorescent protein; EGFP, enhanced GFP; RSV, Rous sarcoma virus; GR, glucocorticoid receptor; Ang II, angiotensin II; DsRed, Discosoma sp. Red.

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COP1-TFs inhibit the transcription of other nuclear receptor such as retinoic acid receptor and thyroid hormone receptor (14). Furthermore, COP1-TFI represses basal transcriptional activity by active repression utilizing transcriptional corepressors, such as N-CoR and SMRT (16). We and other investigators have previously demonstrated that COP1-TFI and SF-1 regulate the bovine CYP17 expression in a mutually exclusive manner (17, 18). We have previously reported that COP1-TFI is expressed in the normal adrenal cortex and that expression levels of COP1-TFI is inversely correlated with those of CYP17, but correlated with those of N-CoR in adrenal cortical adenomas (18–23).

We, therefore, have screened COP1-TFI-interacting proteins from a human adrenocortical adenoma cDNA library using a yeast two-hybrid system and identified Ubc9 (24) and PIAS1, which are small ubiquitin-related modifier-1 (SUMO-1)-conjugating enzyme and SUMO-1 ligase, respectively. The SUMO post-translational modification modifies many proteins with roles in diverse processes, including regulation of transcription, chromatin structure, and DNA repair (25–30). The SUMO modification has not been generally associated with increased protein degradation. Rather, similar to non-proteolytic roles of ubiquitin, SUMO modification regulates protein localization and activity. The SUMO E1-activating, E2-conjugating enzymes, and E3-ligase are involved in the sumoylation machinery. In contrast to the ubiquitin system where dozens of E2 enzymes have been identified, Ubc9 is the only known SUMO-E2-conjugating enzyme. Several SUMO-E3 ligases have been identified that promote transfer of SUMO from E2 to specific targets. Such E3-ligases have been identified, Ubc9 is the only known SUMO-E2-conjugating enzyme. Several SUMO-E3 ligases have been identified that promote transfer of SUMO from E2 to specific substrates. To date, three unrelated proteins have been suggested to have SUMO-E3 ligase activity; the protein inhibitors of activated STAT1 (PIAS1) proteins (31–33), RanBP2 (34, 35), and polycystin protein P2 (36). The present study described that both Ubc9 and PIAS1 can function as transcriptional coactivators of COP1-TFI for the hCYP11B2 gene transcription in a sumoylation-independent manner. These proteins are shown to form a complex in the nucleus and exhibit a very unique localization in the adrenal zona glomerulosa cells. We demonstrated here that COP1-TFI, Ubc9, and PIAS1 are recruited to an endogenous CYP11B2 promoter, thus contributing to aldosterone biosynthesis in adrenal zona glomerulosa cells.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—Several COP1-TF constructs, such as pGBK7-COP1-TFI, pGBK7-COP1-TFI-(55–315), pGBK7-COP1-TFI-(86–183), pGBK7-COP1-TFI-(150–183), pGBK7-COP1-TFI-(315–423), pGBK7-COP1-TFI-(55–423), pRSV-COP1-TFI, pRSV-COP1-TFI-(1–85), pRSV-COP1-TFI-(86–183), pRSV-COP1-TFI-(150–183), pRSV-COP1-TFI-(315–423), pRSV-COP1-TFI-(55–423), were subcloned into pGADT7 vector using a PCR amplification procedures. Based on other nuclear hormone receptors as a reference, the sequence from amino acids 1–85, 86–149, 150–183 and 184–423 have been designated domains A/B, C, D and E/F, respectively. (B) The PIAS1 fragments shown were cloned into the pGADT7, pEGFP, and pDNA3.1His expression vectors as described under “Experimental Procedures.” The N terminus of PIAS1 contains NR box, a coactivator motif that interacts with nuclear receptors, whereas the C terminus of PIAS1 contains two CoRNR motifs, the corepressor of which interacts with nuclear receptors. In the middle of the PIAS1 proteins, the RING domain, which is necessary for SUMO-1 ligase activity, is localized.

FIG. 1. Diagram of various deletion mutants of COP1-TFI (A) and PIAS1 (B), A, the COP1-TFI fragments shown were cloned into the pGBK7 expression vector as described under “Experimental Procedures.” Based on other nuclear hormone receptors as a reference, the sequence from amino acids 1–85, 86–149, 150–183 and 184–423 have been designated domains A/B, C, D and E/F, respectively. (B) The PIAS1 fragments shown were cloned into the pGADT7, pEGFP, and pDNA3.1His expression vectors as described under “Experimental Procedures.” The N terminus of PIAS1 contains NR box, a coactivator motif that interacts with nuclear receptors, whereas the C terminus of PIAS1 contains two CoRNR motifs, the corepressor of which interacts with nuclear receptors. In the middle of the PIAS1 proteins, the RING domain, which is necessary for SUMO-1 ligase activity, is localized.

bait. A human adrenocortical adenoma cDNA library was prepared as shown previously (24). Yeast strain AH109 containing pGBK7-COP1-TFI-(55–423) was transformed with a human adrenocortical adenoma cDNA library in pGADT7 (Clontech) and plated on synthetic complete medium lacking tryptophan, adenine, leucine, and histidine. His+ and Ade+ colonies exhibiting β-galactosidase activity by filter lift assay were further characterized according to the manufacturer’s protocol (Clontech). β-Galactosidase activity was determined with chlorophenol red β-galactopyranoside as described previously (16, 24). To recover the library plasmids, total DNA from the yeast was isolated with a Zymoprep™ yeast plasmid Miniprep kit (Zymo Research, Orange, CA) and used to transform Escherichia coli (HB101) in the presence of ampicillin. To ensure that the correct cDNAs were identified, the library plasmids isolated were transformed into Y187 containing pGBK7-COP1-TFI-(55–423), and β-galactosidase activity was determined. The specificity of the interaction of #2–3 (PIAS1-(5–651)) and #2–4 (Ubc9-(1–158)), both of the 20 positive clones, with COP1-TFI was determined by mating with Y187, which contains pGBK7-COP1-TFI-(55–423), and the β-galactosidase activities of these diploids were examined by the filter lift and chlorophenol red β-galactopyranoside methods. The sequence of the #2–3 and #2–4 clones was identical to the GenBank™-submitted sequence of PIAS1 and Ubc9, respectively. The yeast two-hybrid system was also used to determine protein-protein interaction between COP1-TFI/COP1-TFIAd/GFP/DAX-1/R3 and these clones.

Western Blot Analysis and Coimmunoprecipitation—The cells were lysed with lysis buffer (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride), and Western blots were performed before the immunoprecipitation (IP) steps to confirm protein expression by corresponding antibodies as described previously (24). The primary antibodies used for immunoprecipitation were rabbit polyclonal anti-CP71-TFI antibody (generous gift by Dr. Ming-Jer Tsai) (15), and the antibodies used for the Western blots were anti-COP1-TFI, anti-Xpress (Invitrogen), anti-FLAG (Sigma), anti-Ubc9 (BD Biosciences Pharmingen), anti-PIAS1 (Santa Cruz Biotechnology), and anti-α-ubulin (OncoGene Research Product) antibodies.

Fluorescence Imaging—The images of EGFP-tagged Ubc9 and
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DeRed-COUPTFI were described previously (24). COS-1 cells were transiently transfected with expression vectors of pEGFP-PIAS1, and pDsRed-COUPTFI. Live cell microscopy of GFP fusion and DeRed fusion proteins was performed on a confocal microscope (Axiovert 100M, Carl Zeiss Co., Ltd.). Imaging for GFP and DeRed was performed by excitation with 488 and 543 nm, respectively, from an argon laser, and the emissions were viewed through band passes ranging from 500 to 550 nm, and 550 to 600 nm, respectively, by band pass regulation with LSM510 (Carl Zeiss Co., Ltd.). All images were processed as TIFF (tagged image file format) files on Photoshop 7.0 using standard image-processing techniques.

Northern Blot Analysis—The human tissue Northern blots (Clontech) were hybridized at 42 °C overnight with 32P-labeled cDNA probes of the full-length 1.1-kb hUbc9, 1.9-kb hPIAS-1, full-length 1.3-kb hCOUP-TFI, or 1.1-kb glyceraldehyde-3-phosphate dehydrogenase (Clontech) cDNAs according to the manufacturer’s protocol. The membranes were washed at a final stringency of 0.1 M NaCl/0.01% SDS at 50 °C and analyzed with a BAS 2000 image scanner (Fuji Film Co.). The mRNA levels were determined by comparison with glyceraldehyde-3-phosphate dehydrogenase mRNA levels.

Immunohistochemistry—Formalin-fixed tissues were embedded in paraffin, sectioned at 6 μm, and mounted on silane-coated slides. For immunohistochemistry, sections were dewaxed, rehydrated, followed by blocking endogenous peroxidase using 3% (v/v) hydrogen peroxidase in phosphate-buffered saline, which were then subjected to microwave antigen retrieval in 0.1% citrate buffer. Thereafter, they were washed in phosphate-buffered saline and blocked with a blocking solution containing 5% bovine serum albumin in phosphate-buffered saline for 30 min. They were subsequently incubated overnight at 4 °C with primary antibodies diluted appropriately with the blocking solution. Primary antibody immunohistochemistry was performed using rabbit anti-human antibodies against anti-Ubc9 (BD Biosciences, Pharmingen), anti-PIAS1 (Santa Cruz Biotechnology). After two washes in phosphate-buffered saline, immunoreactivities were detected using a Vectastain ABC Elite kit (Vector Laboratories, CA) and a Vecta DAB substrate kit (Vector Laboratories). As negative controls, sections were incubated with the preimmune or control serum in place of the primary antibody.

Electrophoretic Mobility Shift Analysis—Nuclear extracts were prepared as described previously (8, 11, 12). For in vitro transcription/translation, 0.5 μg of pGBT9-Ad4BP/SF-1 and pFL-COUPTFI was used in conjunction with the TnT-coupled reticulocyte lysate system (Promega), as directed by the manufacturer. EMSA conditions were as described previously (8, 11, 12) using 5 μg of H295R nuclear extract or 5–50 μl of reticulocyte extract. Protein-DNA complexes were separated in a native gel (10% polyacrylamide, 0.5× TBE) at 2500 V for 3 h at 4 °C. Protein-DNA complexes were detected by autoradiography. Gel images were scanned with an imaging analysis system (Storm 840, Molecular Dynamics), and the results were visualized by using the program Quantity One 4.0.3. The identity of the nuclear protein-DNA complexes was confirmed by competition assays and supershift assays (25). The specificity of the DNA-protein complexes was confirmed by the addition of 100-fold excess of unlabeled probe.

Luciferase Assays— Twenty-four hours after transfection, cells were harvested, and luciferase activities were determined by using the Dual-Luciferase Reporter Assay System (Promega). Relative luciferase activity was determined as the ratio of luciferase to Renilla luciferase activities for each sample.

RESULTS

Identification of Ubc9 and PIAS1 as COUP-TFI-interacting Proteins by Yeast Two-hybrid System—To search for proteins that might regulate the activity of the COUP-TFI, we performed a yeast two-hybrid screen with COUP-TFI encoding amino acids 55–423 as bait and a cDNA library prepared from a human adrenocortical adenoma as described previously (24). In this manner, we identified a full-length clone of Ubc9 and a partial clone of PIAS1, which are SUMO E2-conjugating enzyme and E3-ligase, respectively. This report further describes function and expression of COUP-TFI, Ubc9, and PIAS1 in the transcriptional regulation of the human aldosterone synthase gene (CYP11B2).

We have recently shown that Ubc9 specifically interacted with COUP-TFI and mapped interaction domains that C terminus of Ubc9 encoding amino acids 59–158 interacted with the C terminus of COUP-TFI encoding amino acids 383–403 (24). In the present study we performed yeast two-hybrid assays to demonstrate that PIAS1 interacts specifically with COUP-TFI. Both Ubc9 and PIAS1 interacted with COUP-TFI, and the interactions were specific, as no interaction between Gal4 DBD-COUP-TFI (amino acids 55–423) fusion and Gal4 activation domain (Gal4 AD; empty vector) was observed (Fig. 2A). In contrast, as a positive control, we showed strong interaction between Gal4 DBD-COUP-TFI and Gal4 AD-COUP-TFI, because COUP-TFI readily forms homodimers (data not shown). In addition, both Ubc9 and PIAS1 did not interact with unrelated bait corresponding to lamin (data not shown). Besides interacting with COUP-TFI, both Ubc9 and PIAS1 also interacted with COUP-TFI and SF-1, but not with DAX-1 or unliganded TRα 168–456 (Fig. 2A).

To identify interaction domains more precisely, various fragments of COUP-TFI and PIAS1 were cotransformed in yeast, and β-galactosidase liquid assays were performed to quantitate the protein-protein interaction (Fig. 2, B and C). Both Gal4 DBD-COUP-TFI encoding amino acids 86–183 and amino acids 150–183 strongly interacted with the full-length PIAS1, suggesting that the DNA-binding domain and hinge regions of COUP-TFI were necessary to interact with Ubc9. In contrast, the C-terminal fragments of PIAS1 encoding amino acids 301–651 and amino acids 406–651 strongly interacted with COUP-TFI, whereas PIAS1 fragments encoding amino acids 1–150, 1–300, 1–405, and 5–73/664–651 showed minimal interaction with COUP-TFI. Although the N and C termini of PIAS1 contain nuclear receptor-interacting domains, named nuclear receptor box (NR box), and CoRNR motifs, respectively, these domains are not involved in the interaction with COUP-TFI.
Taken together with the interaction data described above, the most C-terminal fragments of PIAS1 encoding amino acids 406–563 interacted with DNA binding and hinge regions of COUP-TFI in yeast.

Interaction and Subcellular Localization of Ubc9, PIAS1, and COUP-TFI in Mammalian Cells—We previously showed that Ubc9 interacts specifically and directly with COUP-TFI (24). The association between COUP-TFI, Ubc9, and PIAS1 was further investigated by coimmunoprecipitation assays (Fig. 3). COS-1 cells were transfected with Xpress-tagged Ubc9 (pcDNA3.1/His-Ubc9), FLAG-tagged PIAS1 (p3xFLAG-CMV-10-PIAS1), and RSV-promoter-driven COUP-TFI expression vectors (pRSV-COUP-TFI). Polyclonal anti-COUP-TFI antibody was first used to precipitate the protein complexes containing COUP-TFI, and the amount of DNA was kept constant by the addition of empty expression vectors. Whole cell extracts were subjected to immunoprecipitation (IP) with anti-COUP-TFI antibody, and immunoprecipitates were subsequently analyzed by immunoblotting (blots) with anti-COUP-TFI (upper), anti-FLAG (middle), and anti-Xpress antibodies (lower). B–G, subcellular localization of COUP-TFI and PIAS1. EGFP-PIAS1 (B–D) or EGFP-PIAS1(C351S) (E–G) was cotransfected with DsRed-COUP-TFI in COS-1 cells. These proteins are colocalized in the nuclei of the transfected COS-1 cells.

Tissue Distribution of mRNA of COUP-TFI, Ubc9, and PIAS1 by Northern Blot Analysis—We next examined the expression of COUP-TFI, Ubc9, and PIAS1 mRNA in human endocrine tissues (Fig. 4). Because Ubc9 and PIAS1 were cloned from a human adrenocortical adenoma cDNA library, we confirmed that these mRNAs were expressed in normal human adrenal cortex (Fig. 4). Ubc9 mRNA is widely distributed in many endocrine tissues, and the expression profile of Ubc9 is similar to that of COUP-TFI as shown previously (24). PIAS1 mRNA is also relatively highly expressed in steroidogenic tissues, such as testis, ovary, and adrenal cortex (Fig. 4).

Immunohistochemistry of COUP-TFI, Ubc9, and PIAS1 in Rat Adrenal Glands—We next examined immunohistochemistry of COUP-TFI, Ubc9, and PIAS1 in rat adrenal glands (Fig. 5). Strikingly, both Ubc9 and PIAS1 are exclusively expressed in the nuclei of zona glomerulosa cells of adult rat adrenal
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binding activity of in vitro translated proteins was assessed. In vitro translated SF-1 bound to the Ad5 probe, producing a complex with mobility similar to that of complex 3 (lane 1 in Fig. 6B). In the presence of either in vitro translated COUP-TFI or COUP-TFII, a complex with mobility similar to that of complex 1 was formed (lanes 3 and 6 in Fig. 6B). All three in vitro synthesized proteins were recognized by their respective antibodies (lanes 2, 4, and 5 in Fig. 6B).

These data suggest that complexes 1 and 3 represent binding of COUP-TF and SF-1, respectively. The nature of the protein(s) forming complexes 2 is unknown at present. Because formation of this complex was abolished in the presence of anti-COUP-TF antibody, but not reproduced by recombinant COUP-TFI or II, complex 2 may represent binding either of a heterodimer between COUP-TF and another unidentified protein or binding of a protein related to, but distinct from COUP-TF.

COUP-TFI-mediated Human CYP11B2 Gene Transactivation Is Potentiated by Ubc9 and PIAS1 Independently of the sumoylation Activities—To explore how COUP-TF regulates human CYP11B2 gene transcription, we transiently transfected exogenous COUP-TFI in H295R cells and measured the luciferase activity of the CYP11B2 reporter gene. Overexpression of COUP-TFI activated the CYP11B2 gene transcription in a dose-dependent manner (lanes 2–4 in Fig. 7A). The mutation of Ad5 sequences, to which COUP-TF binding was disrupted, abrogated basal and COUP-TFI-mediated transactivation of the gene, thus suggesting that Ad5 sequences are crucial for COUP-TFI-mediated CYP11B2 transactivation (Fig. 7B). In addition, overexpression of COUP-TFIΔ35, in which 35 amino acids are deleted from the C terminus, effectively removing repressor domain, did not activate this reporter activity (lanes 5–7 in Fig. 7A), indicating that the deleted region of the COUP-TFI C terminus is indispensable for this activation of CYP11B2 gene.

Furthermore, cotransfection of Ubc9 or PIAS1 with COUP-TFI transactivated COUP-TFI-mediated activation of the CYP11B2 gene transcription (lanes 5 and 8 in Fig. 8A), whereas Ubc9 or PIAS1 alone did not influence the reporter gene activity (lanes 2 and 3 in Fig. 8A). Cotransfection of both Ubc9 and PIAS1 with COUP-TFI showed that their transactivating effect on the COUP-TFI-mediated CYP11B2 transcription was synergistic (Fig. 8B). In addition, coexpression of Ubc9(1–58) or PIAS1(1–150), which does not contain COUP-TFI-interacting domain, had no effects on the COUP-TFI-mediated transactivation (lanes 7 and 10 in Fig. 8A), indicating that interaction between COUP-TFI and Ubc9 or PIAS1 is crucial for COUP-TFI-mediated transactivation of the CYP11B2 promoter. These findings indicate that both Ubc9 and PIAS1 can function as transcriptional coactivators of COUP-TFI for the CYP11B2 gene transcription. Although Ubc9 and PIAS1 are enzymes that are responsible for SUMO modification, the sumoylation-defective mutants Ubc9 (C93S) or PIAS1 (C351S) continued to function as activators of COUP-TFI (lanes 6 and 9 in Fig. 8A). These findings suggest that both Ubc9 and PIAS1 function as coactivators for the COUP-TFI-mediated CYP11B2 gene transcription in a sumoylation-independent manner.

Binding of Ubc9 and PIAS1 to COUP-TFI, but Not to SF-1, Are Crucial for COUP-TFI-mediated CYP11B2 Gene Transactivation.—The Ad5 sequence contains a direct repeat of a consensus (AGGGTA) and variant (AGGCTG) nuclear receptor half-site (DR0) on the non-coding strand. To define the precise nucleotides required for binding of SF-1 and COUP-TFI, a series of oligonucleotides containing progressive stepwise 2-bp mutations across these putative half-sites (m1–m9) were radiolabeled and used in EMSA (Fig. 9A). When H295R nuclear
extracts were used as the source of protein (top panel of Fig. 9A), m1 through m4 formed specific complexes similar to those formed using the wild-type probe. The m5 through m8 failed to form complexes 1 or 2, although complex 3 was still observed using m7 and m8 as probe. Similar results were observed using in vitro translated proteins (Fig. 9A). SF-1 was bound to all mutant oligonucleotides except m5 and m6, whereas COUP-TFI and COUP-TFII failed to bind to m5 through m8. These data further support the hypothesis that complexes 1 and 3 represent binding of COUP-TF and SF-1, respectively, because the abilities of in vitro synthesized proteins and H295R nuclear extracts to bind to the various mutant oligonucleotides were

FIG. 6. Binding of COUP-TF and SF-1 to the CYP11B2 –129/–114 (Ad5) cis-element. A, electrophoretic mobility shift analysis (EMSA) was performed using H295R nuclear extract (NE, 5.0 μg) and a 32P-labeled oligonucleotide probe corresponding to human CYP11B2–129/–114. Protein-DNA complexes (shown by arrows) were separated from free probe (shown by arrowheads) by electrophoresis on a 4% native polyacrylamide gel. Nonradioabeled self competitor DNA was added to a 200-fold molar excess (lane 3) to identify nonspecific protein-DNA interactions (NS). Lanes 4–6 show binding activity in the presence of antisera directed against either SF-1 (S) or COUP-TF (C). The position of the resulting supershifted complexes is indicated by a bracket. B, EMSA was performed as described above using in vitro translated SF-1, COUP-TFI, or COUP-TFII as the source of protein.

FIG. 7. COUP-TFI functions as a transcriptional activator for the human CYP11B2 gene transcription through Ad5 element in H295R cells. A, H295R cells were transiently transfected with 0.6 μg of total DNA, including COUP-TFI (lane 2, 30 ng; lane 3, 100 ng; lane 4, 300 ng) or COUP-TFIΔ35 cDNA (lane 5, 30 ng; lane 6, 100 ng; lane 7, 300 ng), and 0.3 μg of CYP11B2 (–1521/+2)-luciferase reporter DNA for each well of the 24-well dish as indicated. Forty-eight hours post-transfection, cells were harvested, and the extracts were assayed for luciferase activity. B, H295R cells were transiently transfected with 0.6 μg of total DNA, including COUP-TFI (lane 2, 30 ng; lane 3, 100 ng; lane 4, 300 ng) or COUP-TFIΔ35 cDNA (lane 5, 30 ng; lane 6, 100 ng; lane 7, 300 ng), and 0.3 μg of CYP11B2 reporter with mutated Ad5 sequences was used (mutated Ad5 (–129/–114)-luciferase) instead of the wild-type CYP11B2 reporter. Forty-eight hours post-transfection, cells were harvested and the extracts were assayed for luciferase activity. Assays were performed in three separate experiments, each with triplicate samples.
identical. Further, because only mutations within the consen-
sus nuclear receptor half-site (AGGTCA, m5-m8) disrupt bind-
ing of COUP-TF, it is likely that this sequence represents the
key site for the COUP-TF binding site. Utilizing Ad5 luciferase
reporters containing the wild-type, m5 or m7 sequences, we
investigated how Ubc9 and PIAS1 affect the COUP-TFI-regu-
lated CYP11B2 promoter activities. In compared with the find-
ning that Ubc9 and PIAS1 potentiated the COUP-TFI-mediated
transactivation of the wild-type Ad5 promoter (lanes 4–7 in
Fig. 9B), both proteins activated neither m5 nor m7 Ad5 re-
porter activities (lanes 8–21 in Fig. 9B). These findings indicate
that both Ubc9 and PIAS1 enhance the Ad5 reporter activity
mediated by COUP-TFI, but not SF-1.

Endogenous Ubc9 and PIAS1 Are Required for the CYP11B2
Transcriptional Activation by COUP-TFI—If Ubc9 and PIAS1
are coactivators of COUP-TFI, reducing the endogenous level
of Ubc9 or PIAS1 should decrease the transcriptional activity by
COUP-TFI in transient transfection assays. As described pre-
viously (Figs. 7 and 8), overexpression of COUP-TFI activated
human CYP11B2 reporter activities by 6-fold (lane 3 in Fig.
10A). Cotransfection of siRNA of two sets of Ubc9 (Ubc9a (lane
5) and Ubc9b (lane 6) in Fig. 10A) or two sets of PIAS1 (PIAS1a
(lane 7) and PIAS1b (lane 8) in Fig. 10A), but not negative
control (lane 4 in Fig. 10A), effectively reduced the endogenous
levels of Ubc9 or PIAS1 proteins but had no effect on the α-
tubulin protein level seen in Western blot (Fig. 10B). Reduc-
tion of endogenous Ubc9 or PIAS1 protein level decreased the
COUP-TFI-mediated transactivation by ~30–50%. These find-
ings indicate that endogenous Ubc9 and PIAS1 normally func-
tion as transcriptional coactivators for the COUP-TFI-medi-
atmed transactivation.

COUP-TFI, Ubc9, and PIAS1 Are Specifically Recruited to
the Ad5 Element of Human CYP11B2 Gene Promoter—
As mentioned above, COUP-TFI-Ubc9-PIAS1 complex activated the
human CYP11B2 gene transcription. Chromatin immunoprecipitation (ChIP) assays were used to test whether Ubc9 and PIAS1 are recruited to the endogenous CYP11B2 gene promoter in H295R cells. The cross-linked, sheared chromatin preparations were subjected to immunoprecipitation with various antibodies, and the precipitated DNA was analyzed by PCR amplification of the Ad5 element of the CYP11B2 promoter. We have confirmed the size of the sonicated DNA is ~300–600 bp and these bands looked uniform (data not shown). When COUP-TFI, Xpress-Ubc9, and FLAG-PIAS1 were aberrantly overexpressed in H295R cells, antibodies against COUP-TFI, Xpress, or FLAG efficiently immunoprecipitated the Ad5 element of the CYP11B2 promoter. Normal IgG and no antibody failed to precipitate the CYP11B2 promoter (Fig. 11). In contrast to the Ad5 element of the CYP11B2 promoter, the 3′-untranslated control region of the CYP11B2 gene was not detected in association with COUP-TFI, Ubc9, or PIAS1 (Fig. 11). Thus Ubc9, PIAS1, and COUP-TFI were recruited to a native COUP-TFI-regulated CYP11B2 promoter, demonstrating a functional interaction between COUP-TFI and Ubc9-PIAS1 occurring in an in vivo setting. The results from ChIP and RNA interference experiments strongly support a physiological role of Ubc9 and PIAS1 in COUP-TFI-dependent transcription of the human CYP11B2 gene.

**DISCUSSION**

In this report, we have identified and described Ubc9 and PIAS1, which interact with the COUP-TFI and can function as coactivators for the COUP-TFI-mediated transcription of the human CYP11B2 gene. We detected that COUP-TFI, Ubc9, and PIAS1 form a complex in the nuclei of mammalian cells. Expression of Ubc9 and PIAS1 was markedly detected in rat adrenal zona glomerulosa cells, in which CYP11B2 is exclusively expressed. Transient transfection assays together with small interfering RNA and ChIP assays indicated a physiological role of Ubc9 and PIAS1 in COUP-TFI-dependent transactivation of the human CYP11B2 gene in normal adrenal glomerulosa cells. Both Ubc9 and PIAS1 have SUMO-1-conjugating enzyme (E2) and SUMO-1 ligase (E3) activity, respectively (25–30). The C93S substitution of Ubc9 and the C351S substitution of PIAS1, which abrogate sumoylation activity, continued to interact with COUP-TFI and to potentiate transcriptional activation mediated by COUP-TFI, indicating that their sumoylation enzyme activities are not required for function as coactivators of COUP-TFI.

Ubc9 and PIAS1 Function as Transcriptional Coactivators of COUP-TFI in CYP11B2 Transcription—Both Ubc9 and PIAS1 meet all the criteria for transcriptional coactivator proteins in the modulation of COUP-TFI transcriptional properties. First, as shown in Figs. 2 and 3, Ubc9 and PIAS1 specifically interacted with COUP-TFI in yeast and mammalian cells. The Ubc9 was previously shown to interact with the C-terminal putative ligand-binding domain of COUP-TFI (388–403) that is described as a transcriptional repressor domain (24). The present study showed that PIAS1 interacted with the DNA-binding domain and hinge region of COUP-TFI (86–183). Coimmunoprecipitation and subcellular localization experiments showed that Ubc9 and PIAS1 are colocalized with COUP-TFI in transfected COS-1 cells (Fig. 3). These findings suggest that Ubc9 and PIAS1 specifically interact with COUP-TFI and form a complex in mammalian cells. Second, overexpression of Ubc9 or
PIAS1 had no effect on the reporter activities in the absence of transfected COUP-TFI (Fig. 8A). However, Ubc9 or PIAS1 poten
tiated the transactivation mediated by COUP-TFI. Subse-
quently, coexpression of the Ubc9 or PIAS1 deletion muta
tion, Ubc9-(1–58) or PIAS1-(1–150), which impairs COUP-TFI bind-
ing, did not affect the transactivation mediated by COUP-TFI, in
dicating that interaction of COUP-TFI with Ubc9 or PIAS1 is
required for COUP-TFI-mediated transactivation. In addition,
coexpression of Ubc9 and PIAS1 synergistically enhanced the
COUP-TFI-mediated transactivation. Third, reduction of en-
dogenous Ubc9 or PIAS1 by small interfering RNA decreased
COUP-TFI-mediated transactivation. In vivo, did not affect the transactivation mediated by COUP-TFI, in
dicating that interaction of COUP-TFI with Ubc9 or PIAS1 is
required for COUP-TFI-mediated transactivation. In addition,
coexpression of Ubc9 and PIAS1 normally contributes to COUP-TFI-mediated transactivation. Fourth, ChIP assays clearly showed that COUP-TFI, Ubc9, and
PIAS1 were recruited to a native COUP-TFI-regulated CYP11B2 promoter, demonstrating a functional coupling be-
tween COUP-TFI and Ubc9-PIAS1 in vivo. Therefore, Ubc9
and PIAS1 possess all the characteristics expected for tran-
scriptional coactivator proteins of COUP-TFI in vivo.

To confirm further that Ubc9 and PIAS1 are coactivators of
COUP-TFI, we ruled out several other possible ways in which
these proteins might enhance COUP-TFI-mediated transacti-
vation. First, as SUMO-1 conjugation plays an important role
in protein modification, the effects of Ubc9 and PIAS1 on
COUP-TFI transactivation might be the result of effects of
Ubc9 and PIAS1 on COUP-TFI protein concentrations. Our
preliminary experiments showed that overexpression of Ubc9
or PIAS1 did not alter COUP-TFI protein concentration in
H295R cells (data not shown). Second, it was also possible that
overexpression of Ubc9 or PIAS1 increases the concentrations of
some coactivators or decreases the concentrations of some
corepressors, which have been shown to interact with COUP-
TFI, but the results showed that overexpression of these pro-
tein did not alter the protein concentrations of SRC-1, GRIP-1,
or SMRT in the cells (data not shown). Because these experi-
ments were performed by transient transfection, we are not able
to conclude unequivocally that Ubc9 and PIAS1 have no effects on these coregulator concentrations, and further inves-
tigation is required. Third, another possibility is that overex-
pression of Ubc9 or PIAS1 increased the DNA-binding affinity
of COUP-TFI. To exclude the possibility, we performed electro-
phoretic mobility shift assays to determine whether bacterially
or in vitro transcription-translated Ubc9 or PIAS1 proteins
affect the binding of COUP-TFI to its response element DNA
(Ad5) of the human CYP11B2 promoter region. The results
showed that Ubc9 or PIAS1 have no effects on COUP-TFI
binding to the Ad5 element (data not shown). Fourth, it has
been proposed that SUMO-1 conjugation targets proteins to
different cellular localizations. SF-1 can be directed into nu-
clear speckles and sequestered from the nucleolus in the
presence of SUMO-1, thus resulting in transcriptional re-
pression (39). It is therefore possible that coexpression of
Ubc9 and PIAS1 alters subcellular localization of COUP-TFI.
However, based on the subcellular localization data (Fig. 3),
localization of COUP-TFI continues to be in the nucleus
without re-localization. Taken together with the above-
mentioned findings, Ubc9 and PIAS1 clearly function as
novel coactivators of COUP-TFI in vivo. However, detailed
molecular mechanisms are largely unknown and further in-
vestigation is required.

Role of Ubc9 and PIAS1 in Sumoylation—SUMO-1 conjuga-
tion (sumoylation) has been reported to play an important role
in many cellular processes (25–30). Sumoylation resembles
ubiquitination, but the enzymes involved in these three pro-
cesses are distinct. SUMO-1 is conjugated to target proteins at
the consensus sequence ϕKXE (ϕ is any hydrophobic amino
acid, and X is any amino acid). COUP-TFI has no such SUMO
consensus sequence; however, several proteins, such as Mdm2
and CREB, are also modified at sites other than ϕKXE (26).
Further investigation is required to elucidate whether COUP-
TFI is sumoylated in vivo.

Our data showed that both wild type and sumoylation-defec-
tive mutants Ubc9 (C93S) and PIAS1 (C351S) similarly en-
hanced COUP-TFI-regulated CYP11B2 promoter activities, in-
dicating that these proteins possess dual distinct functions,
SUMO-dependent and SUMO-independent pathways, such as
coactivator function. However, it is possible that ectopically
produced Ubc9 and PIAS1 regulate COUP-TFI-mediated transactivation through not only sumoylation of COUP-TFI but
also conjugation of SUMO-1 to one or more other cellular
factors involved in transcriptional regulation. Recent data have
raised the intriguing possibility that SUMO modification may
have a specific impact on the ability of some transcription
factors to function synergistically (27, 40, 41). Previous studies
of the glucocorticoid receptor (GR) had identified a region re-
ferred to as a synergy control motif, mutation of which led to a
selective increase in the activity of the GR from promoters
bearing multiple, but not single sites. The synergy control motif
contains a consensus SUMO acceptor site, and recent data
have shown that this is, in fact, the major site of addition of
SUMO in the GR. Considerable numbers of transcription fac-
tors, including GR (41–44), androgen receptor (32, 33, 45–51),
progesterone receptor (47, 52), mineralocorticoid receptor (53,
54), pteroxisome proliferator-activated receptor γ (55), and
steroidogenic factor-1 (SF-1) (39, 56), are modulated by SUMO-1
attachment, and SUMO-modified transcription factors mostly
resulted in attenuation of transcription. Recent studies dem-
onstrated that one of the molecular mechanisms of sumoyla-
tion-mediated repression is protein modified by SUMO-1 re-
cruited histone deacetylases and transcriptional corepressors,
thus repressing transcription (29, 38, 57–59). However, there
are also opposite examples of SUMO-modified proteins, which
lead to transcriptional activation rather than repression. Tran-
scriptional coactivators, such as SRC-1 and GRIP1, are shown
to be modified by SUMO-1, thus resulting in enhanced tran-
scriptional coactivator capacities (60, 61). Therefore, sumoy-
lation does not necessarily induce transcriptional repression de-
pending on the substrates. The molecular mechanisms that
could explain how SUMO modification affects transcriptional
regulation are largely unknown.

COUP-TFI-Ubc9-PIAS1 Complexes Are Crucial for Aldoster-
one Synthesis—We have identified three important cis-ele-
ments in the hCYP11B2 promoter: a CRE at −71/−64, a cis-
element termed Ad5 at −129/−114, and a third cis-element
termed NBRE-1 (−766/−759) (8, 9, 12). The CRE is common to
both hCYP11B1 and hCYP11B2 and is regulated by both pro-
in hormone kinase A- and calmodulin-dependent kinase-dependent
mechanisms. Neither the Ad5 nor NBRE-1 cis-elements found
in the 5′-flanking sequence of hCYP11B2 are present in
hCYP11B1 (8, 9, 12). The present results confirmed Ad5 ele-
ment is crucial for CYP11B2 transactivation, because deletion of
the element dramatically reduces basal and COUP-TFI-
mediated transactivation (Fig. 7B). Based on the present
and previous reports (8, 9, 12), COUP-TFI and/or Nurr1/NGFI-B
play important roles in transactivation of human CYP11B2
gene through binding to the Ad5 element. The physiological
importance of these particular transcription factors should be
investigated in vivo. Our preliminary data demonstrated that
levels of expression of Ubc9 and PIAS1 are not altered in
aldosterone-producing adenomas of patients with primary al-

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dosteronism compared with those in normal adrenals. However, the significance of Ubc9 and PIAS1 in aldosterone-producing adrenomas remains to be further investigated.

We have recently shown that the effects of K+ and angioten-sin II (Ang II) on hCYP11B2 transcription occur through two pathways; increased expression of Nur1/NGFI-B and phos- phorylation of ATF-1/CREB (13). Ang II treatment rapidly induces levels of mRNA and protein of Nur1/NGFI-B, thereby transactivating hCYP11B2 gene (8, 9). Both Nur1 and NGFI-B markedly increased transcription of hCYP11B2 through binding to the NBRE-1 and Ad5 sites, which are unique to hCYP11B2. Buholzer et al. (62) have very recently reported that COUP-TF is a negative regulator of steroidogenesis in bovine adrenal zona glomerulosa cells. They also showed that Ang II treatment dramatically decreased levels of COUP-TF in the cells, thus activating StAR gene expression. Based on these findings, it is tempting to speculate that levels of expression of COUP-TFI, Ubc9, or PIAS1 in aldosterone biosynthesis. Therefore, COUP-TFI, Ubc9, or PIAS1 in real-time reverse transcription-PCR and Western blot analysis (data not shown). The reason why Ang II treatment showed different effects on COUP-TFI levels between other report and ours is not known; however, different cellular context, such as bovine adrenal zona glomerulosa cells and human H295R cells, may be one reason for that. We do not know a physiological role of COUP-TFI and Ubc9-PIAS1 in Ang II and K+ regulation of hCYP11B2 expression in this study, and this should be investigated.

In conclusion, we identified novel COUP-TFI-interacting proteins, Ubc9 and PIAS1, and these proteins function as co-activators of COUP-TFI for human CYP11B2 transactivation. The unique localization profiles of these proteins in adrenal zona glomerulosa cells are consistent with a crucial physiological role in aldosterone biosynthesis. Therefore, the COUP-TFI-Ubc9-PIAS1 complexes shed new light in controlling the long term capacity of the adrenal gland to produce aldosterone. In addition, these studies provide new mechanisms through which COUP-TFI can act as a transcriptional activator through the novel interaction with Ubc9 and PIAS1.

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