Convergence of Wnt Signaling and Steroidogenic Factor-1 (SF-1) on Transcription of the Rat Inhibin α Gene*

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The action of a variety of peptide hormones is critical for proper growth and differentiation of the urogenital ridge, which ultimately gives rise to the kidney, adrenal cortex, and gonad. One such class of peptides is the Wnt family of secreted glycoproteins that is classically involved in development of cell polarity and cell fate determination. Notably, alterations in Wnt-4 expression in mice and humans result in profound defects in urogenital ridge development, including dysregulation of kidney, gonadal, and adrenal growth. The nuclear receptor steroidogenic factor-1 (SF-1) has been implicated as a downstream effector of peptide hormone signaling during urogenital ridge development as evidenced by both the activation of SF-1-dependent transcription in the adrenal cortex by signaling molecules such as protein kinase A and by the adrenal and gonadal agenesis in mice with null mutations in SF-1. We hypothesized that Wnt-dependent signaling cascades regulate SF-1-dependent transcription of genes required for adrenogonadal development. Specifically, the data demonstrate that β-catenin synergizes with SF-1 to activate the α-inhibin promoter through formation of a transcriptional complex. The activation requires an intact SF-1 RE and is independent of TCF/Lef. These data support the recent observation that β-catenin can participate in nuclear receptor-mediated transcriptional activation and extend the findings to the monomer binding class of orphan nuclear receptors.

Steroidogenic factor-1 (SF-1)† was originally cloned as a transcriptional regulator of steroidogenic enzyme gene expression (1, 2). Consistent with its role in the activation of steroidogenic enzyme gene transcription, SF-1 expression is restricted to a subset of endocrine tissues including the gonads, adrenal cortex, ventromedial hypothalamus, and the pituitary gonadotropes (3–6). The generation of SF-1-deficient mice has defined an additional role for SF-1 in adrenal and gonadal development as evidenced by adrenal and gonadal agenesis in these mice (7, 8). Expression of adrenal-specific SF-1 target genes is regulated by activation of signaling cascades initiated at the cell surface (9) and the subsequent formation of transcription complexes composed of SF-1 and a variety of co-regulatory factors (10–24). Consistent with this mechanism, SF-1 can be post-translationally modified by phosphorylation (17), dephosphorylation (25), and acetylation (26); affecting stability of protein structure (27) and ultimate coregulator interactions (17), dephosphorylation (25), and acetylation (26); affecting stability of protein structure (27) and ultimate coregulator interactions (17). A number of transcriptional coregulators directly interact with SF-1 in the adrenal cortex including dose-sensitive sex reversal adrenal hypoplasia congenital determining region on the X-chromosome-1 (Dax-1) (20, 28) and the p160 family members steroid receptor coactivator-1, -2, -3 whose interactions are modulated by protein kinase A (PKA) (15, 17, 18), lending support to the hypothesis that SF-1 serves as an interface between peptide hormone-initiated signal cascades and transcriptional activation.

The Wnt genes encode a family of highly conserved glycoproteins that have been implicated in a variety of embryologic programs including gonadal and adrenal development (29–33). As such, they are attractive candidates for modulators of SF-1-dependent transcriptional programs. Wnt ligands bind to cell-surface Frizzled receptors, initiating a cytoplasmic signaling cascade that results in β-catenin stabilization, cytosolic accumulation, and subsequent translocation to the nucleus. Within the nucleus, β-catenin classically complexes with a member of the T-cell factor (TCF) family of transcription factors to activate target-gene transcription (34). However, recent studies indicate that β-catenin can interact directly with a variety of coregulator proteins and transcription factors, including CRE-binding protein (CREB)-binding protein (CBP), the SMAD family of proteins, and the nuclear androgen receptor (AR) to enhance both TCF-dependent (35, 36) and TCF-independent transcription (37–39). In situ hybridization has demonstrated that Wnt-4, Wnt-11, and Wnt-2b are all expressed in the adrenal cortex. Wnt 11 localizes to the definitive zone, while Wnt-2b localizes exclusively to the subcapsular cells beneath the adrenal capsule (32, 33). Wnt-4 knockout mice die within 24 h postpartum due to severe urogenital abnormalities including kidney failure secondary to a failure of nephrogenesis (40). Female Wnt-4 knockout mice demonstrated surprising masculinization of urogenital ridge structures due to the aberrant up-regulation of the SF-1 target genes Müllerian inhibitory substance and P450sc17, and the resultant excess testosterone biosynthesis (29). In addition, ectopic expression of the SF-1 target gene, P450sc21 (critical for corticosterone biosynthesis) was observed in the gonads of Wnt-4-null mice. An increase in plasma corticosterone was also observed in Wnt-4-
null mice. On the contrary, P450-aldosterone synthase and P450c17 (females only) expression were decreased in the embryonic adrenal of Wnt-4 knockout mice, supporting a role of Wnt-4 in the development of the organs arising from the urogenital ridge (31). Recently, a patient with adrenal hypoplasia and male to female sex-reversal was shown to have a duplication of chromosomal locus 1p32–36, which contains the Wnt-4 gene. Up-regulation of the SF-1 target gene Dax-1 in cells from this patient suggests an interaction between SF-1 and Wnt signaling on the Dax-1 promoter (41), as recently confirmed by the demonstration of synergy between SF-1 and β-catenin on activation of Dax-1 transcription in the developing female gonad (42). Because knockout of the SF-1 target gene, α-inhibin, results in the absence of gonadal and adrenal sex steroid excess similar to the Wnt-4-null mice, and transcription of α-inhibin, like Dax-1 (43), is absolutely dependent upon SF-1, we hypothesized that components of the Wnt signaling cascade directly interact with SF-1 to regulate the α-inhibin promoter in the adrenal cortex (44–46). In this article we have specifically explored the activation of SF-1-dependent transcription of the α-inhibin gene by β-catenin in the adrenal cortex.

MATERIALS AND METHODS

Cell Culture—JEG3 placental carcinoma cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Transient transfection of JEG3 cells was performed using calcium phosphate coprecipitation (Speciality Media, Phillipsburg, NJ) according to the manufacturer’s directions. Y1 adrenocortical carcinoma cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 7.5% horse serum and 2.5% fetal calf serum.

Plasmid Constructs—The following plasmids have been previously described: α-inhibin-lucerase (and deletion constructs), pci-neo-HA-SF-1, pcDNA3-S33Y-β-cat, CMV-mTCF-4B-FLAG, Lex-A-Luc, CMV-LexA-SF-1, CMV-LexA-DDD, TOPFLASH, p-65-luc, and myc-LacZ (6, 46–52). Inhibin-Luc-5’SF-1-RE was generated by introducing a point mutation in to the SF-1 binding site using QuikChange (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. The oligonucleotides were designed to generate the ΔSF-1-RE mutant were: 5’-GTTGAGGAGATAGCAGGGC-3’ and 5’-CAGGGCCTGTGCCCTTTCTGT-3’. 5 μl of extracted DNA and 35 rounds of amplification were used. PCR amplification of the distal α-inhibin promoter (−7.5 to −7 kb) was performed using the following primers: 5’-GGACCACCAAGCGCAACTCCAGAG-3’ and 5’-TCAGCCTCCATACGGCCACAG-3’.

Luciferase Assays—JEG3 cells were plated at a density of 1 × 10⁵ cells per well in 12-well plates. Twenty-four hours after plating, cells were transiently transfected using calcium phosphate coprecipitation (Speciality Media, Phillipsburg, NJ). Cells were lysed 48 h post-transfection, and a luciferase assay (Promega, Madison, WI) was performed in triplicate on a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA). Luciferase assays were normalized for transfection efficiency by co-transfecting CMV-myc-LacZ and subsequent determination of β-galactosidase activity (Applied Biosystems, Foster City, CA).

Immunoprecipitation Assay—Nontoxic sex adenals were injected intraperitoneally with either 150 mM NaCl or 150 mM LiCl with an injection volume of 20 μl/kg (53). At either two or three hours post-injection, adrenals were harvested and either snap-frozen in liquid nitrogen (for subsequent RNA isolation) or stored in ice-cold phosphate-buffered saline. Adrenals from six to nine mice were pooled, homogenized in 1 ml of a buffer containing 25 mM HEPES, 5 mM KCl, 0.5 mM MgCl₂, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 0.5% Nonidet P-40 at pH 7.6. Nuclei were isolated from cytoplasmic protein by spinning at 2500 rpm for 1 min. Nuclear proteins were extracted for 60 min at 4 °C using 500 μl of a buffer containing 25 mM HEPES, 10% sucrose, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.01% Nonidet P-40, and 350 mM NaCl. Insoluble proteins were removed by spinning at maximum speed for 10 min. Protein concentrations were determined by the method of Bradford (Bio-Rad, Hercules, CA). Two hundred micrograms of total nuclear extract for each condition were precleared with 10 μl (bed volume) of protein A/G-agarose for 30 min at 4 °C. Precleared nuclear extract was incubated with either rabbit polyclonal α-HA antibodies (Santa Cruz Biotechnology) or rabbit polyclonal α-SF-1 antibodies (Upstate Biotechnology, Lake Placid, NY) for 16 h at 4 °C with rotation. Subsequently, 10 μl of protein A/G-agarose was added to samples and incubated at 4 °C with rotation for 60 min. The samples were then washed four times with radioimmunoprecipitation assay buffer (Tris-HCl, 50 mM, pH 7.4; Nonidet P-40, 1%; sodium deoxycholate, 0.25%; NaCl, 150 mM; EDTA, 1 mM; phenylmethylsulfonyl fluoride, 1 mM; leupeptin and pepstatin, 1 μg/ml each; NaF, 1 mM). Immunoprecipitates were resolved on 10% denaturing polyacrylamide gels, transferred to polyvinylidene difluoride membrane followed by immunoblot analysis using polyclonal α-β-catenin antibodies (Santa Cruz Biotechnology).

Chromatin Immunoprecipitation—Chromatin immunoprecipitation (ChIP) assays were performed using a chromatin immunoprecipitation assay kit (Upstate Biotechnology) according to the manufacturer’s instructions with the minor modification that isolated nuclei were used as the starting material instead of whole cells. Briefly, cells were cross-linked with 1% formaldehyde at 37 °C for 10 min. Cells were rinsed three times with ice-cold phosphate-buffered saline and collected into phosphate-buffered saline and centrifuged for 5 min at 1000 rpm. Crude nuclei were isolated using the protocol described for co-immunoprecipitation assays. Nuclei were prepared in the SDS lysis buffer provided in the kit. Lysates were sonicated at 30% power, 5 × 10 s using a Sonic Dismembrator model 300 (Fisher Scientific, Pittsburgh, PA) to shear genomic DNA. The following antibodies were used to perform immunoprecipitations, polyclonal α-SF-1 (Upstate Biotechnology), polyclonal α-β-catenin (Santa Cruz Biotechnology), and polyclonal rabbit IgG antibodies (Pierce). PCR amplification of the proximal α-inhibin promoter region spanning the SF-1 response element was performed using the following primers: 5’-GGTGGTGTGATCTGTCCT-3’ and 5’-GCTGGCCCTGTGCCCTTTCTGT-3’. 5 μl of extracted DNA and 35 rounds of amplification were used. PCR amplification of the distal α-inhibin promoter (−7.5 to −7 kb) was performed using the following primers: 5’-GGACCCCAACAGCGCAACTCCAGAG-3’ and 5’-TCAGCCTCCATACGGCCACAG-3’. RNA Isolation and Quantitative PCR—Snap frozen adrenals from NaCl- or LiCl-injected mice (see “Co-Immunoprecipitation Assay” above) were manually disrupted using a mortar and pestle. Total cellular RNA was isolated using an RNAeasy RNA isolation kit (Qiagen, Valencia, CA). One microgram of total cellular RNA was reverse-transcribed using the Superscript First-Strand Synthesis System for cDNA Synthesis (Invitrogen, San Diego, CA). Quantitative PCR for α-inhibin transcript was performed on an Optionix Thermocycler (MJ Research, Waltham, MA) using the QuantiTect SYBR Green PCR Kit (Qiagen). The sequence of primers targeted against the α-inhibin cDNA were 5’-AAGACATGC-GCTTGCGGTTTACA-3’ and 5’-CTATTCGGCCGCTGCTGCTC-3’. 18 S ribosomal RNA was used as an internal standard using commercially available primers (Ambion, Austin, TX).

RESULTS

SF-1 and β-Catenin Synergistically Activate the α-Inhibin Promoter—Based upon previous data suggesting a relationship between Wnt-dependent signaling pathways and SF-1-mediated transcription in adrenocortical and gonadal development, we examined the potential for Wnt-dependent signaling to participate in the regulation of SF-1-mediated transcription of the α-inhibin gene. It has previously been shown that SF-1 is capable of transcriptionally activating the rat α-inhibin promoter (46). Co-transfection assays were performed to determine the ability of β-catenin, the downstream effector of Wnt signaling, to synergize with SF-1 in transcriptional activation of the α-inhibin gene. An α-inhibin-luciferase (Inh-luc) construct containing 201 bp of the rat α-inhibin promoter driving luciferase was used in co-transfection assays. JEG3 placental carcinoma cells were co-transfected with the reporter plasmid and increasing doses of a constitutively active β-catenin mutant (S33Y) that cannot be phosphorylated by GSK-3β (54) in the presence or absence of SF-1. In the presence of SF-1, a dose-dependent increase in the activation of the reporter construct was observed (Fig. 1A). At maximal concentrations of S33Y tested, a 9-fold increase in activation was observed when compared with SF-1 alone. However, in the absence of SF-1, S33Y was unable to activate transcription of the Inh-luc reporter construct, demonstrating an absolute requirement of SF-1 for the transcriptional activation of the α-inhibin promoter by β-catenin.
**FIG. 1.** β-catenin transcriptionally activates the α-inhibin promoter using a cis-acting sequence within the proximal 165 bp to synergize with SF-1. A, β-catenin functionally synergizes with SF-1 to transactivate the α-inhibin promoter. A constitutively active β-catenin mutant (S33Y) synergizes with SF-1 in a dose-dependent manner on a 2021-bp fragment of the α-inhibin promoter in JEG3 choriocarcinoma cells. β-catenin’s ability to transactivate the α-inhibin promoter requires the presence of SF-1 and results in a 9-fold activation over SF-1 alone. 300 ng of Inh-Luc, 25 ng of SF-1, serial dilutions (41–333 ng) of S33Y β-catenin, and 33 ng of myc-LacZ were used per condition in triplicate (n = 5). Raw data was normalized for transfection efficiency (LacZ), and values represent the mean fold activation above SF-1 levels ± S.E. B, a 165-bp cis-acting region is sufficient for β-catenin activation of the α-inhibin promoter. Serial deletions of the α-inhibin promoter, generated by restriction digest, were co-transfected with SF-1 and S33Y β-catenin into JEG3 cells. Each deletion construct maintained the synergy between SF-1 and β-catenin, including −165 inhibin-luc, which contains the proximal 165 bp of the α-inhibin promoter. S33Y was able to activate −165 inhibin-luc 6.5-fold over SF-1, which is similar to its ability to activate the 1641-bp promoter fragment, indicating that the cis element required for β-catenin transactivation of α-inhibin is contained within the proximal 165 bp. 300 ng of each reporter construct, 25 ng of SF-1, 333 ng of S33Y β-catenin, and 33 ng of myc-LacZ were used per condition in triplicate (n = 3). Raw data was normalized for transfection efficiency (LacZ) and values represent the mean fold activation above SF-1 levels ± S.E. C, β-catenin results in a dose-dependent increase in SF-1-mediated transcriptional activation of the proximal 165 bp of the α-inhibin promoter. The maximal concentration of β-catenin activated this promoter fragment 4.5-fold over SF-1. As with the 2-kb promoter fragment, β-catenin was barely able to transactivate the promoter in the absence of SF-1. 300 ng of −165 Inh-Luc, 25 ng of SF-1, serial dilutions (41–333 ng) of S33Y β-catenin, and 33 ng of myc-LacZ were used per condition in triplicate (n = 5). Raw data was normalized for transfection efficiency, and values represent the mean fold activation above SF-1 ± S.E.
A Proximal 165-bp Cis-acting Element Is Sufficient for β-Catenin Transactivation of α-Inhibin—In order to determine the mechanism of activation of the α-inhibin promoter by β-catenin, we systematically analyzed this promoter in an attempt to identify putative TCF binding sites. A panel of serial deletions of the α-inhibin promoter fused to a luciferase reporter was utilized to locate the region of the promoter required for functional synergy between SF-1 and β-catenin. Co-transfection of each reporter construct in the presence or absence of SF-1 expression plasmid was performed with or without a maximal dose of β-catenin. These experiments demonstrated that β-catenin was capable of synergizing with SF-1 on all deletion constructs tested (Fig. 1B). In addition, β-catenin activated the full-length reporter construct in a dose-dependent manner, similar to both the −1641 bp construct and the full-length construct described in Fig. 1A. To further characterize this synergy, JEG3 cells were co-transfected with increasing amounts of S33Y and −165 Inh-luc in the presence or absence of SF-1. A dose-dependent increase in SF-1-mediated transcriptional activation, up to −4.5-fold, was observed in response to increasing quantities of S33Y (Fig. 1C). Similar to results obtained with full-length α-inhibin-luc, S33Y was barely able to activate transcription of the reporter plasmid in the absence of SF-1, confirming that the cis-acting element required for β-catenin synergy with SF-1 is located within the proximal 165 bp of the α-inhibin promoter and that SF-1 is required for β-catenin transcriptional activation of the α-inhibin promoter.

The SF-1 RE Is Necessary and Sufficient for the Synergistic Activation of the α-Inhibin Promoter by β-Catenin and SF-1—Since the SF-1 response element (RE) resides within the proximal 165 bp of the α-inhibin promoter, we tested whether the SF-1 RE was an absolute requirement for the synergistic effects of β-catenin on the α-inhibin promoter. Previously, mutation of the SF-1 RE has been shown to result in loss of binding of SF-1 (46). We therefore introduced identical point mutations in this binding site in the context of the full-length reporter. As expected, disruption of the SF-1 RE resulted in the complete loss of SF-1-dependent activation of the reporter construct (Fig. 2A). In addition, S33Y was unable to transactivate the mutated promoter in either the absence or presence of SF-1, indicating that sequences between −165 and +1 are sufficient, and the SF-1 RE within this promoter region is necessary for the synergistic activation of the α-inhibin promoter by β-catenin and SF-1.

In order to test whether the SF-1 RE was sufficient for synergy between SF-1 and β-catenin, we utilized a reporter construct containing 5 tandem copies of a consensus SF-1 RE driving the luciferase gene reporter (p-65-luc). β-catenin was able to transcriptionally synergize with SF-1 on this reporter construct in a dose-dependent manner, showing a 9-fold activation over SF-1 levels (Fig. 2B). As expected, β-catenin was unable to activate this reporter in the absence of SF-1. These data demonstrate that the SF-1 RE is a necessary and sufficient DNA promoter element for transcription synergy between SF-1 and β-catenin.

TCF/LEF Is Not Required for the Synergy between SF-1 and β-Catenin on the α-Inhibin Promoter—Since β-catenin transactivation is classically propagated through TCF/LEF (lymphoid enhancer factor), we evaluated the potential role of TCF/LEF in the synergy between SF-1 and β-catenin. However, sequence analysis of the proximal 165 bases of the α-inhibin promoter did not reveal a strong consensus TCF/LEF binding site, and the sufficiency of the SF-1 RE for the synergy between SF-1 and β-catenin predicts a potential TCF-independent mechanism of β-catenin synergy. Using TOPFLASH, an artificial promoter containing three tandem consensus TCF binding sites as a positive control for TCF/LEF-mediated transcriptional response, co-transfection assays were performed. JEG3 cells were co-transfected with either (a) TOPFLASH, S33Y, and increasing quantities of TCF-4 or (b) Inh-luc, SF-1, S33Y, and increasing
quantities of TCF-4. As expected, S33Y strongly activated the TOPFLASH artificial promoter, and addition of TCF-4 led to further dose-dependent transcriptional activation (Fig. 3A), consistent with a TCF-dependent β-catenin effect. However, when TCF-4 was titrated with Inh-luc, a dose-dependent repression of α-inhibin transactivation was observed, indicating that TCF is not required for the synergy between SF-1 and β-catenin. In addition, the repression predicts that TCF sequestration of β-catenin might prevent transcriptional synergy with SF-1 on the α-inhibin promoter. These data suggest that the interaction between SF-1 and β-catenin is independent of the action of the TCF family of transcription factors predicting a novel mechanism of action for β-catenin-mediated transcriptional activation by orphan receptors.

A modified mammalian two-hybrid assay was performed to confirm whether the functional synergy between SF-1 and β-catenin was independent of TCF/Lef DNA binding. In this assay, increasing amounts of S33Y β-catenin were tested for the ability to increase expression of a LexA-RE-Luc reporter plasmid in the presence and absence of the LexA-SF-1 fusion construct in which the SF-1 DNA binding domain (DBD) was replaced with the LexA DBD. A dose-dependent increase of LexA-RE-Luc transcription was observed in the presence of increasing amounts of S33Y when co-transfected with LexA-SF-1. No effect of S33Y was observed with LexA DBD alone, demonstrating that the activation of LexA-Luc by S33Y was propagated through interactions with SF-1 (Fig. 3B). The ability of β-catenin to activate LexA-RE-Luc transcription in the presence of LexA-SF-1 confirms that the synergy between SF-1 and β-catenin is independent of TCF/Lef DNA binding and suggests that the synergy is mediated by a physical interaction between SF-1 and β-catenin.

**β-Catenin Is a Component of the SF-1 Transcription Complex Assembled on the Endogenous α-Inhibin Promoter in the Adrenal**—Direct evidence of a transcriptionally active protein complex including SF-1 and β-catenin was explored through a combination of co-immunoprecipitation and ChIP assays. To evaluate the significance of protein complex formation in vivo, we utilized standard co-immunoprecipitation assays on nuclear lysates from adrenals of mice injected with either NaCl or LiCl, which is a potent inhibitor of GSK-3β and hence should result in a stabilization of β-catenin. Immunoblot analysis revealed that endogenous β-catenin was elevated in the nuclear fraction of adrenals from LiCl-injected mice when compared with adrenals from mice injected with NaCl, while SF-1 levels were unaffected (Fig. 4). Immunoprecipitation was performed on nuclear lysates using α-SF-1 antibodies or α-HA antibodies (negative control) followed by immunoblot analysis for β-catenin using polyclonal α-β-catenin antibodies. As expected, no β-catenin was detected in α-HA immunoprecipitates. While only minimal β-catenin was detected in α-SF-1 immunoprecipitates from nuclear extracts of adrenals from NaCl-injected mice, a strong β-catenin signal was observed in α-SF-1 immunoprecipitates following LiCl treatment, indicative of the dynamic assembly of a protein complex that includes SF-1 and β-catenin following LiCl treatment. Such a result is consistent with the activation of the canonical Wnt signaling cascade in the adrenal cortex, inhibition of GSK-3β, stabilization of β-catenin and subsequent association with the SF-1 transcription complex (Fig. 4).

In order to test whether β-catenin is a component of the SF-1 transcription complex on the endogenous α-inhibin promoter within the adrenal, chromatin immunoprecipitation assays were performed. Y1 nuclear lysates were immunoprecipitated with rabbit polyclonal IgG, α-β-catenin or α-SF-1 antibodies. PCR amplification was performed using primers designed to
Since we observed an increase in adrenocortical cell lines, we examined whether LiCl treatment would activate transcription of the endogenous SF-1 promoter when primers targeted against the distal SF-1 CRE within the Wnt-4 promoter. Rabbit polyclonal IgG served as a negative control. No PCR amplification was observed from samples immunoprecipitated with IgG (Fig. 5). These data demonstrate that chromatinized template.

**Injection of LiCl into Mice Increases α-inhibin Expression within the Adrenal Cortex**—Since we observed an increase in β-catenin stabilization together with an increase in the association between SF-1 and β-catenin in response to LiCl injection, we examined whether LiCl treatment would activate transcription of the endogenous α-inhibin gene within the adrenal cortex of mice. Using an identical paradigm for LiCl treatment as utilized in co-immunoprecipitation studies, quantitative RT-PCR reveals a relative 3.50 ± 0.95-fold increase in α-inhibin transcript levels after 3 h of LiCl treatment when compared with NaCl-treated controls, with a relative expression level of 1.0 ± 0.26 (Fig. 6). These data are consistent with a Wnt-mediated stabilization of nuclear β-catenin and synergistic activation of SF-1-dependent transcription of the α-inhibin gene in the mouse adrenal cortex.

**DISCUSSION**

SF-1 regulates critical genes involved in the growth and differentiation of the adrenal cortex. The regulation of SF-1 transcriptional complexes involves both coregulator recruitment and activation by signaling cascades initiated by peptide hormones binding to cognate cell surface receptors. Since the altered expression of Wnt genes results in urogenital ridge defects mediated in part by dysregulated activation of SF-1 target genes, we have explored whether classic components of the Wnt signaling cascade activate SF-1-mediated transcription in the adrenal cortex. Wnt-4 knockout mice display profound kidney, gonadal, and adrenal malformations (29, 31, 55) including female to male sex reversal secondary to dysregulation of the SF-1 target genes, Müllerian inhibitory substance, P450c21, and P450c17, and resultant excessive glucocorticoid and androgen biosynthesis. In addition, the duplication of chromosomal locus 1p32-36, which contains the Wnt-4 gene, results in glucocorticoid and androgen deficiency with male to female sex-reversal in a single patient (41), similar to patients and mice with SF-1 loss of function mutations. Two SF-1 target genes implicated in gonadal and adrenal growth are Dax-1 (28, 56) and α-inhibin (44, 45). Dax-1 is uniquely up-regulated in cells from the one patient with a Wnt-4 duplication (41), an effect recently shown to be mediated by transcriptional synergy between SF-1 and β-catenin in the activation of the Dax-1 promoter (42). Because α-inhibin-null mice exhibit dysregula-
tion of gonadal and adrenal growth together with sex-steroid excess (45, 57), we chose to examine the interaction between SF-1 and the downstream effector of Wnt signaling, β-catenin, on the promoter of the SF-1 target gene α-inhibin.

We demonstrate that β-catenin is able to synergize with SF-1 in a dose-dependent manner to activate transcription of the α-inhibin promoter. The cis-acting element required for synergy between SF-1 and β-catenin maps to the proximal 165 bp of the α-inhibin promoter. While Mizusaki et al. (42) demonstrate a role for TCF in the regulation of Dax-1, the lack of a TCF consensus binding site, coupled with the absence of trans-activation of the α-inhibin promoter by β-catenin alone (without SF-1), suggests a novel mechanism of action for the regulation of α-inhibin by β-catenin. This is supported by the loss of both SF-1-mediated transactivation of the β-catenin promoter and the synergy with β-catenin following introduction of a point mutant in the SF-1 RE within the 165-bp region along with the efficiency of a consensus SF-1 RE to support the synergy between SF-1 and β-catenin. In addition, titration of TCF-4B, while activating the synthetic TCF/LEF RE construct TOPFLASH, disrupts the functional synergy between SF-1 and β-catenin on the α-inhibin promoter, suggesting a sequestera-

The presence of a protein complex containing SF-1 and β-catenin is independent of TCF binding to DNA, which is a novel mechanism of action for β-catenin with nuclear receptors (37–39). In prostate cancer cell lines, nuclear trans-

β-catenin in nuclear receptor-mediated transcriptional activation and extend the finding to the mono-

The presence of a protein complex containing SF-1 and β-catenin was evaluated by modified mammalian two-hybrid, co-immunoprecipitation, and ChIP assays. While the synergistic activation by SF-1 and β-catenin in the mammalian two-hybrid suggests a physical interaction between the two proteins, the ability of SF-1 to co-immunoprecipitate with β-catenin out of adrenal cortical nuclear lysates serves as further evidence that these two factors form a protein complex within the adrenal cortex. ChIP assays reveal that SF-1 and β-catenin antibodies are both able to immunoprecipitate the proximal α-inhibin promoter, indicating that the transcriptional complex seen in the co-immunoprecipitation is active on the α-inhibin promoter in the adrenal cortical cell. The ability of LiCl, which mimics canonical Wnt signaling by inhibiting GSK-3β, to increase both the interaction between SF-1 and β-catenin and α-inhibin expression within the adrenal cortex further supports a model by which Wnt-activated signaling cascades regulate α-inhibin expression.

Not surprisingly, additional factors participate in the regulation of α-inhibin gene transcription. CBP, CREB (46), and GATA-1 (58) stimulate while WT-1 (59) and ICER (60) inhibit α-inhibin transcription. In addition, the binding of CREB to the α-inhibin CRE (61–63) is dependent upon SF-1 binding to the SF-1 RE (46). As such, future studies that examine the interplay between these factors and the regulation of transcription complex formation by membrane signals will be essential. Our preliminary studies indicate that adrenocorticotrophic hormone results in a significant attenuation of the co-immunoprecipitation of SF-1 and β-catenin in mouse Y1 adrenocortical cells.

How Wnt ligands shown or predicted to be involved in adrenal growth and differentiation (Wnt2b, Ref. 33; Wnt-4, Ref. 31; Wnt-11, Ref. 32) influence the SF-1/β-catenin complex will provide important mechanistic insight into the regulation of monomer binding orphan nuclear receptors by membrane-induced signaling cascades.

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