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Switching of NR5A Proteins Associated with the Inhibin α-Subunit Gene Promoter after Activation of the Gene in Granulosa Cells

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The inhibin α-subunit gene is transcriptionally activated by FSH in ovarian granulosa cells during follicular growth. We have investigated the roles of the NR5A family nuclear receptors steroidogenic factor 1 (SF-1) and liver receptor homolog 1 (LRH-1) in transcriptional activation of the inhibin α-subunit gene. Transfection assays using an inhibin α-subunit promoter reporter in GRMO2 granulosa cells show that LRH-1 and SF-1 act similarly to increase promoter activity, and that the activity of both transcription factors is augmented by the coactivators cAMP response element-binding protein-binding protein and steroid receptor coactivator 1. However, chromatin immunoprecipitation experiments illustrate differential dynamic association of LRH-1 and SF-1 with the α-subunit inhibin promoter in both primary cells and the GRMO2 granulosa cell line such that hormonal stimulation of transcription results in an apparent replacement of SF-1 with LRH-1. Transcriptional stimulation of the inhibin α-subunit gene is dependent on MAPK kinase activity, as is the dynamic association/dissociation of SF-1 and LRH-1 with the promoter. Inhibition of the phosphatidylinositol 3-kinase signaling pathway influences promoter occupancy and transcriptional activation by SF-1 but not LRH-1, suggesting a possible mechanistic basis for the distinct functions of these NR5A proteins in inhibin α-subunit gene regulation. (*Molecular Endocrinology* 20: 1090–1103, 2006)

THE PITUITARY gonadotropins FSH and LH regulate the expression of numerous gonadal genes. For example, expression of the inhibin α- and β-subunit (1), aromatase, the LH receptor, and protein kinase A (PKA) RIIβ (2) genes is stimulated by FSH in granulosa cells. After the LH surge, the expression of these genes rapidly declines (1–3). FSH binds specific G protein-coupled receptors on ovarian granulosa cells of small follicles, inducing proliferation and subsequent differentiation of these cells (4). Receptor activation promotes the formation of cellular second messengers, including cAMP, which activates multiple signaling pathways resulting in myriad cellular changes. For example, cAMP activates PKA, thus leading to the phosphorylation of protein substrates (4–6) including the transcription factor CREB [cAMP response element (CRE)-binding protein] (7–9). FSH and cAMP also activate the MAPKs, ERK 1 and ERK 2 (10), phosphatidylinositol 3-kinase (PI3K), protein kinase B (PKB/Akt), and serum- and glucocorticoid-induced kinase (Sgk) (11) in preantral granulosa cells.

Inhibin was isolated from ovarian follicular fluid based on its ability to suppress secretion of FSH (12–15). Inhibin is a heterodimer of two subunits; α, which is unique to inhibin, and β_A or β_B (14, 15), which are shared by the functional antagonist activin (16–18). In female rats, inhibin levels fluctuate depending on reproductive cycle stage, with both inhibin A and inhibin B increasing throughout metestrus and diestrus (19). The increase in inhibin levels is due, in large part, to the influence of FSH (1). Phosphorylated CREB binds an atypical CRE in the inhibin α-subunit gene proximal promoter, thus activating the promoter. Increasing phosphorylated CREB by stimulating granulosa cells with FSH (9) or by using forskolin, which activates adenylyl cyclase, results in inhibin α-subunit gene activation (20, 21). Mutation of the nonconsensus CRE within the promoter eliminates CREB binding and inhibin α-subunit gene activation by FSH and forskolin (21).

The inhibin α-subunit gene proximal promoter also contains a nonconsensus binding site for steroidogenic factor-1 (SF-1) (22), a member of the NR5A nuclear receptor family (23, 24). SF-1 is necessary for normal development and function of endocrine tissues including the adrenals, gonads, anterior pituitary gonadotropes, and the ventromedial hypothalamus (25). Granulosa cell-specific SF-1 knockout mice are infer-

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Abbreviations: CBP, CREB-binding protein; ChIP, chromatin immunoprecipitation; CMV, cytomegalovirus; CRE, cAMP response element; CREB, CRE-binding protein; LRH, liver receptor homolog; LY, LY294002; MEK, MAPK kinase; PD, PD098059; PI3K, phosphatidylinositol 3-kinase; PIP, phosphatidyl inositol lipid; PKA, protein kinase A; PKB, protein kinase B; PMSG, pregnant mare serum gonadotropin; SBS, SF-1 binding site; SDS, sodium dodecyl sulfate; SF-1, steroidogenic factor 1; SRC, steroid receptor coactivator; WT, wild type.

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tile and have no corpora lutea, demonstrating that SF-1 also plays an essential role in follicular development (26). Transfection and mutation experiments illustrate that the inhibin α-subunit proximal promoter SF-1 binding site (SBS) contributes to inhibin α-subunit gene activation and acts in concert with the CRE to regulate gene expression (22).

More recently, another NR5A family protein was found in the ovary, liver receptor homolog-1 (LRH-1) (27). LRH-1 was originally characterized as a liver-specific receptor that regulates genes involved in bile acid synthesis (28, 29), but it has been shown to be expressed in pancreas, intestine, ovary, and at low levels in the adrenal gland (29, 30). Although these proteins are derived from two different genes, SF-1 and LRH-1 share a high degree of identity, particularly in their DNA binding domains. Thus, LRH-1 and SF-1 can recognize the same DNA sequence (31, 32), and several reports demonstrate that genes expressed in the adrenal, in adipocytes, and in the ovary previously shown to be regulated by SF-1 can also be transactivated by LRH-1 (30–33). Recent reports also indicate that LRH-1 expression is modulated during the reproductive cycle (34–36).

Like other transcription factors, CREB and SF-1 interact with coactivators to exert their effects. Indeed, we have previously shown that the coactivator, CREB binding protein (CBP), activates the inhibin α-subunit proximal promoter (22). CBP is a member of the p300 family of proteins and was first identified based on its interaction with the phosphorylated form of CREB (33). CBP acts by recruiting RNA polymerase II to its target genes (37, 38), by locally remodeling chromatin through intrinsic histone acetylation activity (39, 40), and by recruiting additional histone modifiers (41, 42). CBP has also been shown to interact with SF-1 (22, 43) and to increase SF-1-regulated transcription (22). Another coactivator, steroid receptor coactivator-1 (SRC-1) (44) has also been shown to interact with SF-1 (45), and LRH-1 can interact with the related p160 family cofactor SRC-3 (46). CBP and SRC-1 can also interact with each other (47, 48). Together, these results suggest that CREB and NR5A proteins recruit a complex of transcriptional coactivators to target gene promoters.

In this report we define a mechanism for the synergistic response of the inhibin α-subunit gene observed with the combination of forskolin and NR5A protein overexpression and discover distinct roles for SF-1 and LRH-1 in inhibin α-subunit gene regulation. We use a combination of cell transfection and chromatin immunoprecipitation (ChIP) assays to demonstrate regulated association of complexes including CREB, SF-1, LRH-1, and the coactivators CBP and SRC-1 with the inhibin α-subunit promoter. We demonstrate that stimulation of granulosa cells results in a dynamic switch of NR5A proteins on the proximal promoter such that LRH-1 replaces SF-1. We provide evidence that this switch and NR5A-mediated activation of the inhibin promoter are mediated, in part, by both the MAPK and PI3K signaling pathways.

RESULTS

Synergistic Activation of the Inhibin α-Subunit Gene Is Due to Formation of a Complex Including Transcription Factors and Coactivators

FSH treatment increases cAMP levels in granulosa cells, and this stimulation can be mimicked in transformed mouse granulosa GRMO2 cells by the adenylyl cyclase activator forskolin. We defined previously a proximal promoter element containing an atypical cAMP response element (CRE) that is necessary for the inhibin α-subunit gene to respond to FSH stimulation and increased cAMP levels (21). This region of the promoter also contains an atypical SBS (22). As recent reports indicate that LRH-1 and SF-1 can bind the same DNA element (30, 31), we tested whether LRH-1 could act at the inhibin α-subunit proximal promoter. GRMO2 cells were transfected with the inhibin α-subunit promoter/reporter construct [wild-type (WT)LUC)] as well as with an empty vector or expression plasmids for mouse SF-1 or mouse LRH-1. Overexpression of SF-1 in this transfection assay increases luciferase activity 4-fold, and this increase is augmented by forskolin treatment (Fig. 1B) as reported previously (22). Transfection of LRH-1 yields a similar result: the transcription factor increases inhibin α-subunit reporter levels 4-fold, and LRH-1 also synergizes with forskolin (Fig. 1B). Cotransfection of SF-1 and LRH-1 together did not further increase luciferase reporter gene expression (data not shown).

The SBS and CRE within the proximal promoter are known to act together to regulate inhibin α-subunit expression (22). To test whether LRH-1 can contribute to this cooperative regulation, transient transfection assays using the CREMUT promoter, in which a 2-bp mutation was made in the CRE, were performed (Fig. 1B). Neither SF-1 nor LRH-1 is able to transactivate this CREMUT promoter. Transfections in mouse GRMO2 cells using a promoter containing a 2-bp mutation in the SBS (SBSMUT), which eliminates SF-1 binding and transactivation (22), illustrate that LRH-1 acts through the same site as SF-1 (Fig. 1B). Although this SBSMUT inhibin α-subunit promoter is not activated by SF-1 or by LRH-1, it does respond to forskolin stimulation, albeit at a much reduced level as compared with WT.

To test whether the synergistic response of the inhibin α-subunit gene was due to an interdependence of CREB and NR5A proteins in binding to the DNA, we performed EMSAs using GRMO2 nuclear proteins and probes corresponding to a sequence including the CRE and SBS in the inhibin α-subunit gene (WT) or probes containing mutations in either the CRE (CREMUT) or the SBS (SBSMUT). Both CREB and NR5A proteins bind the WT DNA probe (Fig. 1C). Mutation of

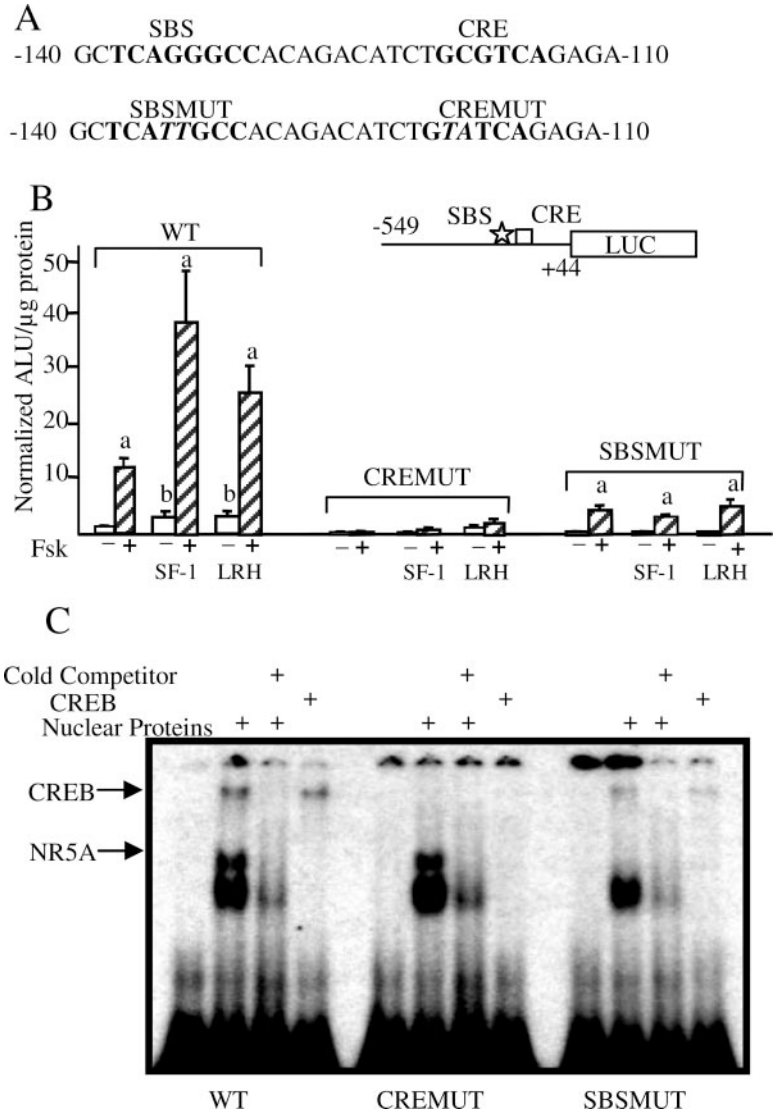

Fig. 1. CREB and NR5A Proteins Synergistically Activate the Inhibin α-Subunit Gene Promoter but Binding Is Not Interdependent

A, The reporter construct used in transfections consists of the inhibin α-subunit promoter from -769 to $+44$ relative to the transcriptional start site fused to luciferase. Mutations in the SBS and CRE were as illustrated and were generated either in the context of the entire promoter region for transfection, or as smaller probes for EMSA. B, Triplicate wells of GRMO2 cells were cotransfected with the inhibin α-subunit promoter reporter (WT) or with reporter constructs containing a mutation in the SBS (SBSMUT) or a mutation in the CRE (CRE MUT) and empty vector or expression constructs for SF-1 or LRH-1. Indicated samples were treated with forskolin (Fsk). This is a combination of three similar experiments normalized to basal reporter expression, and *bar graphs* represent the means ± SEM. a indicates $P < 0.005$ as compared with nonstimulated pairs, and b indicates $P < 0.01$ as compared with basal. C, GRMO2 nuclear proteins or recombinant CREB were incubated with labeled double-stranded oligonucleotides corresponding to the inhibin α-subunit proximal promoter (WT) or with oligonucleotides to the promoter containing a mutation in the CRE (CREMUT) or in the SBS (SBSMUT). Cold probe (50×) was included in designated lanes. This experiment was repeated twice with similar results.

the CRE abolished CREB binding but did not affect NR5A protein binding, and mutation of the SBS abolished NR5A binding but did not affect CREB binding (Fig. 1C). Thus, the synergism seen in inhibin α-subunit gene activation by these transcription factors is not due to cooperative binding of these proteins, at least not as it can be measured in this *in vitro* DNA interaction assay.

Another possible mechanism for inhibin α-subunit synergistic activation is that coactivators provide a bridge between these transcription factors, thus forming a large, stabilized complex. Thus, we tested the ability of select coactivators to enhance inhibin α-subunit gene expression. Specifically, we investigated the function of CBP and SRC-1, coactivators known to interact with CREB and SF-1, and tested whether

LRH-1 could also functionally interact with these proteins (22, 33, 45, 47). Cell cotransfection assays using either CBP or SRC-1 and the inhibin α-subunit promoter/reporter performed in GRMO2 cells demonstrate that both CBP and SRC-1 increase basal promoter activity (Fig. 2A). Additionally, cotransfection of expression plasmids for either NR5A protein with those for CBP and SRC-1 illustrates that the coactivators can substantially augment both SF-1- and LRH-1-induced reporter expression (Fig. 2A). Neither the CREMUT nor the SBSMUT reporters were stimulated by cotransfection of CBP or SRC-1 (Fig. 2B). Thus, activation of the proximal promoter by these cofactors is dependent on the atypical CRE and SBS.

Association of Transcription Factors and Coactivators with the Inhibin α-Subunit Proximal Promoter Is Dynamic

To ascertain whether these transcription factors and coactivators interact with the inhibin α-subunit promoter in a regulated fashion in granulosa cells, we turned to ChIP experiments using GRMO2 cells. These transformed mouse cells express the inhibin subunit genes (49) and therefore allow an examination of the association of the endogenous inhibin α-subunit promoter with endogenous regulatory factors. RT-PCR experiments indicate that, in GRMO2 cells, inhibin α-subunit mRNA is significantly increased by 4 h after forskolin treatment (Fig. 3A). We chose to perform initial ChIP experiments at 1 h after forskolin treatment, which should represent a time at which these transcription factors and coactivators are acting to promote transcription. ChIP experiments using cells in the basal state or after 1 h of forskolin treatment demonstrate that CREB association with the promoter increases an average of 6-fold after forskolin treatment (Fig. 3C). Surprisingly, the same treatment decreases the amount of SF-1 associated with the promoter 5-fold. In contrast, LRH-1 association with the promoter is increased about 5-fold, similar to the CREB results. PCR amplification of the same samples using primers to a region of the inhibin α-subunit promoter more than 3 kb from the transcriptional start site, which does not contain a CRE or SBS (Fig. 3B), illustrates the specificity of transcription factor association with the proximal promoter (distal panels (Fig. 3C).

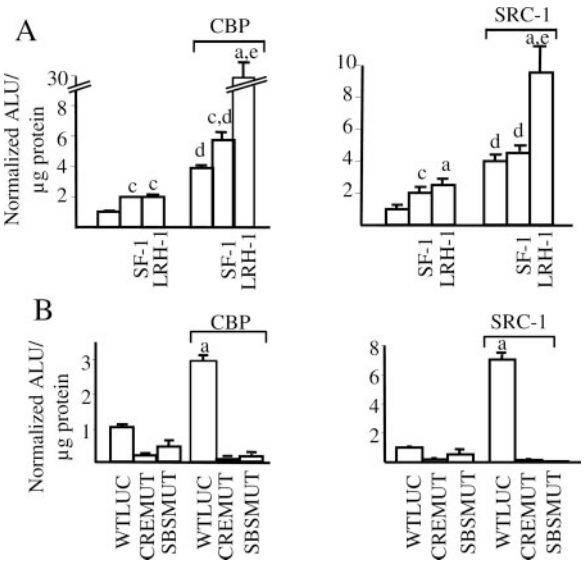

Fig. 2. CBP and SRC-1 Coactivate the Inihibin α-Subunit Promoter

A, GRMO2 cells were cotransfected with WTLUC and expression vectors for CBP, SRC-1, SF-1, and/or LRH-1. Results shown are normalized to basal reporter expression, and *bar graphs* represent the means ± SEM (n = 4 for CBP and n = 3 for SRC-1). a indicates $P < 0.005$ and c indicates $P < 0.05$ as compared with non-NR5A-transfected cells (*i.e.* addition of SF-1 or LRH-1 as compared with basal). d indicates $P < 0.01$ and e indicates $P < 0.005$ as compared with cells not transfected with coregulators (*i.e.* addition of CBP or SRC-1 over basal or NR5A-transfected pairs). B, GRMO2 cells were cotransfected with the WT or MUT inhibin α-subunit promoter reporter constructs and expression vectors for CBP or SRC-1. Results shown are normalized to basal reporter expression, and *bar graphs* represent the means ± SEM (n = 4 for CBP and n = 3 for SRC-1). a, $P < 0.005$ as compared with basal.

Association of endogenous CBP and SRC-1 with the inhibin α-subunit promoter was also investigated using ChIP assays. GRMO2 cells were again left untreated or stimulated with forskolin for 1 h, and the chromatin was immunoprecipitated with antibodies to CBP or SRC-1 (Fig. 3D). Both coregulators are recruited to the proximal promoter after forskolin treatment, presumably through interactions with their transcription factor targets, with an increased abundance of 4.5- and 2.7-fold, respectively. CBP and, to a lesser extent, SRC-1 contain histone acetylase activity, and both also recruit other histone acetylases (39, 40, 48). Precipitation with an antibody specific to the acetylated form of histone H4 demonstrates an increased association of acetylated H4 with the proximal promoter region after forskolin treatment (7.5-fold) (Fig. 3D). Thus, the coactivators recruited to the promoter are active and able to promote global chromatin remodeling, leading to enhanced transcription of the endogenous inhibin α-subunit gene in this granulosa cell line.

ChIP experiments investigating transcriptional activation by other nuclear receptors have revealed that these transcription factors cycle on and off promoters at defined rates, allowing for the recruitment of additional factors in later cycles, which results in more robust transcriptional activation (for a comprehensive review see Ref. 50). To investigate further the dynamic association of these nuclear receptors with DNA, GRMO2 cells were stimulated with forskolin for various times, and the interactions of SF-1 and LRH-1 with the inhibin α-subunit gene promoter were investigated. In a short time course, SF-1 is associated with the promoter under control conditions and at 5 min of treatment, but this decreases quite substantially by 10 and

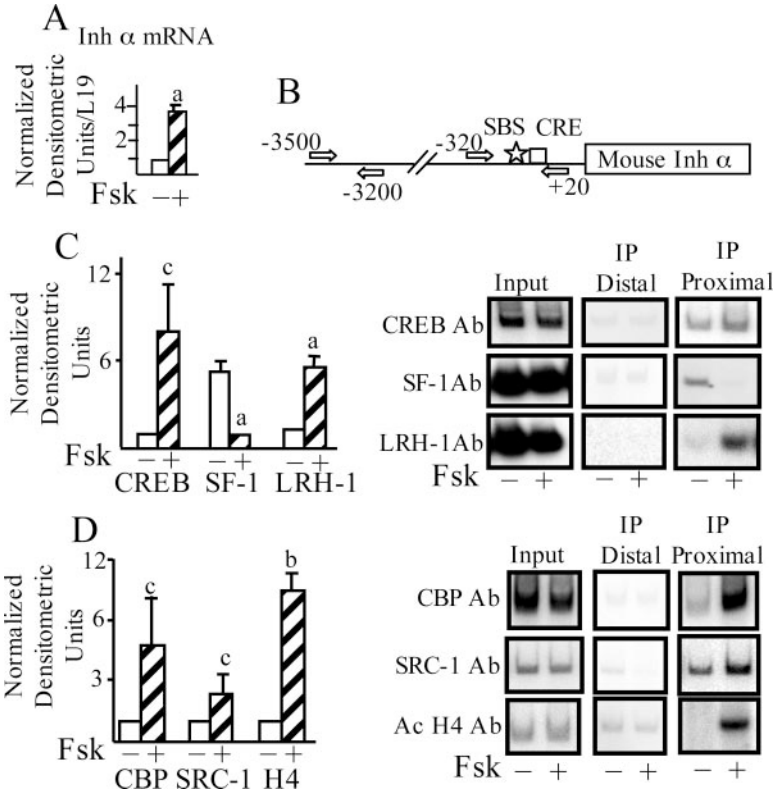

Fig. 3. Forskolin Increases Inhibin α-Subunit mRNA Levels, Promotes Recruitment of LRH-1, CREB, CBP, and SRC-1 to the Endogenous Inhibin α-Subunit Proximal Promoter, and Increases Histone H4 Acetylation

A, RNA was collected from GRMO2 treated with vehicle or forskolin (Fsk) for 4 h, and RT-PCR was performed using primers to the inhibin α-subunit gene or to a control gene (ribosomal protein L19). Results are quantified in the *bar graph* (n = 3). a indicates $P < 0.005$ as compared with nontreated control. B, Schematic of the mouse inhibin α-subunit promoter and primers used in ChIP experiments. C, ChIP assays were performed on GRMO2 cells, and DNA was amplified using primers as indicated in the *schematic*. Experiments were performed multiple times with each antibody (n = 5 for CREB and LRH-1, n = 8 for SF-1). The autoradiogram from one representative experiment is shown, whereas the *bar graphs* indicate the means ± SEM normalized to input levels of all experiments. a, $P < 0.005$; b, $P < 0.01$; and c, $P < 0.05$ as compared with untreated controls. D, ChIP assays were performed and DNA was amplified as above. The experiment was performed multiple times with each antibody (n = 5 for CBP and SRC-1; n = 8 for H4), and the results, normalized to inputs, are quantified (means ± SEM). The autoradiogram from one representative experiment is shown whereas the *bar graph* illustrates combined data. b, $P < 0.01$; and c, $P < 0.05$ as compared with basal controls. Ab, Antibody; Inh, inhibin; IP, immunoprecipitation.

15 min (Fig. 4A). LRH-1 has a more cyclic association with the promoter and is actually decreased at 5 min, increased at 10 min, and then decreased again at 15 min. We also examined 15-min intervals around the 1-h time point investigated in Fig. 3. SF-1 association with the promoter is diminished at all these later times (Fig. 4B). Recruitment of LRH-1 to the promoter is quite robust but relatively slow, with a 3- to 4-fold change in promoter occupancy by 1 h after forskolin treatment. This increase persists at 75 min, indicating that there is a long-term increase of LRH-1 promoter occupancy (Fig. 4B). Thus, forskolin stimulation results in a rapid disassociation of SF-1 from the promoter, allowing for a later, robust recruitment of LRH-1.

The surprising result that forskolin stimulation recruits LRH-1 to the promoter yet decreases SF-1 association was investigated in granulosa cells from rats stimulated with pregnant mare serum gonadotropin (PMSG) for various times. Inhibin α-subunit RNA levels were measured by RT-PCR from isolated granulosa cells that were not plated in culture (Fig. 5A). Treatment with PMSG increases inhibin α-subunit mRNA over a 24-h period, with maximum levels achieved at 10 h post stimulation. ChIP assays using granulosa cells isolated in the same experiment illustrate that gonadotropin treatment decreases SF-1 association with the promoter by 10 h, the time when inhibin α mRNA levels are maximal. Conversely, LRH-1 association increases and peaks at 10 h (Fig. 5B). At 24 h after *in vivo* gonadotropin treatment, inhibin α-subunit mRNA levels plateau, and the relative association of the two transcription factors seem to be returning toward baseline values. Thus, in nontransformed, stimulated granulosa cells SF-1 and LRH-1 associate with the inhibin α-subunit promoter maximally at different times, and this is similar to what is seen in the transformed cell line.

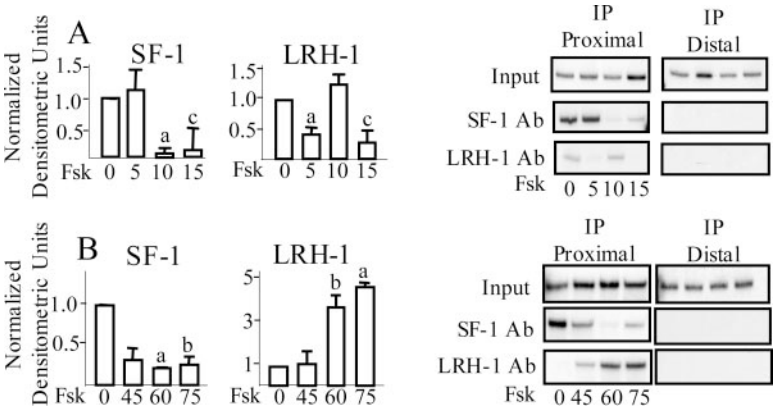

Fig. 4. Time Course ChIPs Illustrate Dynamic Association of LRH-1 and SF-1 with the Inhibin α-Subunit Promoter

A, GRMO2 cells were treated with vehicle or stimulated as indicated every 5 min for 15 min, and ChIPs were performed using antibodies to SF-1 and LRH-1. One representative ChIP is shown, although *bar graphs* represent the average and SEM of four experiments. a, $P < 0.005$; c, $P < 0.05$. B, GRMO2 cells were treated with vehicle or stimulated for 45, 60, or 75 min, and ChIPs were performed using antibodies to SF-1 and LRH-1. One representative ChIP is shown, although *bar graphs* represent the average and SEM of four experiments. a, $P < 0.005$; b, $P < 0.01$. Ab, Antibody; Fsk, forskolin; IP, immunoprecipitation.

Mechanisms that Regulate the Switch between NR5A Proteins on the Inhibin α-Subunit Gene Promoter

The most unexpected finding from the above studies is the consistent result that forskolin or PMSG treatment stimulates recruitment of LRH-1 while decreasing SF-1 association with the promoter. This could be due to changes in the relative amounts of SF-1 and LRH-1. Stimulation of GRMO2 cells with forskolin for 1 h significantly increases LRH-1 mRNA, whereas SF-1 mRNA is not significantly changed (Fig. 6A). This regulation reflects similar changes seen in LRH-1 and SF-1 mRNAs in ovarian cells. Granulosa cells were isolated from immature female rats or rats injected with PMSG for various times (Fig. 6B). LRH-1 mRNA levels increase within 4 h and remain high during the course of PMSG treatment. SF-1 mRNA levels, however, do not significantly change after PMSG treatment. Thus, forskolin-enhanced synthesis of LRH-1 could be one mechanism contributing to the predominant occupancy of the inhibin α-subunit promoter by LRH-1 after forskolin treatment. Attempts to correlate these changes in LRH-1 mRNA levels with increased protein expression were not successful, owing to limitations in use of the LRH-1 antibodies in Western blotting.

A second likely mechanism might involve effects of specific phosphorylation cascades, as SF-1 is regulated by phosphorylation (51), and our preliminary data (52) indicate that this is likely to be true for LRH-1 as well. To test this idea, we used inhibitors specific to

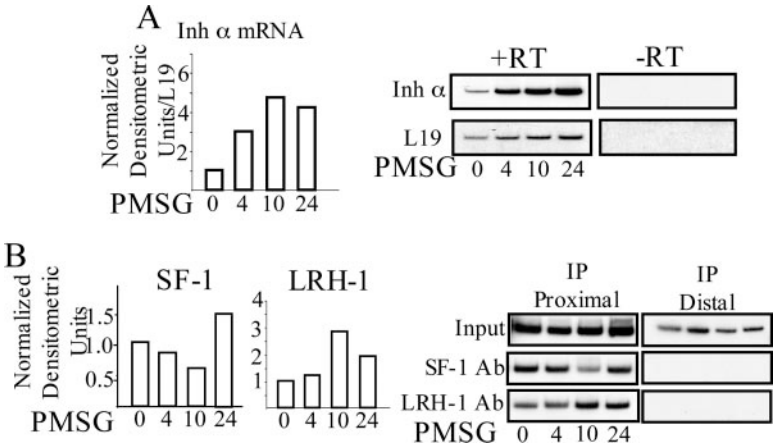

Fig. 5. The Switch of LRH-1 for SF-1 at the Inhibin α-Subunit Promoter Is Observed in Rat Granulosa Cells

Rats were stimulated with PMSG for the times indicated, and granulosa cells were isolated and immediately used for RNA extraction or cross-linked for ChIPs. This experiment was repeated with similar results. Results shown are means of two experiments. A, Inhibin α-subunit mRNA levels were measured using RT-PCR and normalized to the amount of ribosomal L19 mRNA. B, ChIP assays were performed using antibodies to SF-1 and LRH-1. Ab, Antibody; Inh, inhibin; RT, reverse transcriptase.

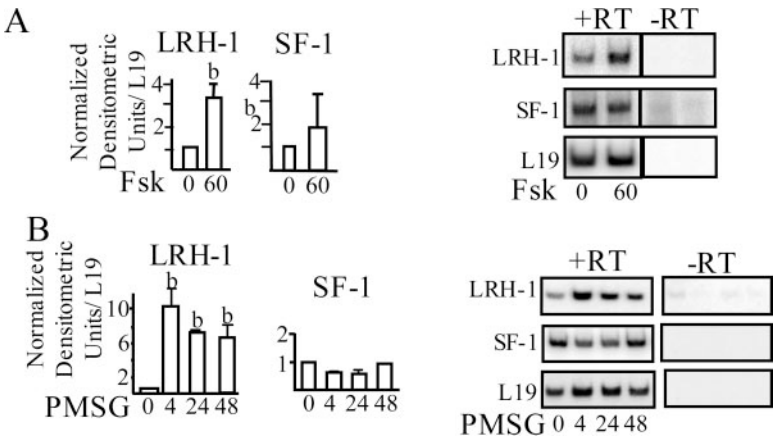

Fig. 6. LRH-1 mRNA Is Regulated in Granulosa Cells

A, RNA was collected from GRMO2 cells treated with forskolin (Fsk) for 1 h and LRH-1 and SF-1 mRNAs measured by RT-PCR. Each experiment was performed three times (n = 3), and all experiments were quantified in *bar graphs* representing the means ± SEM. b, $P < 0.01$ compared with the untreated control. B, Ovarian RNA was collected from rats injected with PMSG for the times indicated. This experiment was repeated twice (n = 3) with similar results, and the means ± SEM were quantified in *bar graphs*. b, $P < 0.01$ as compared with basal control. RT, Reverse transcriptase.

several of the major protein kinases activated by FSH signaling, to explore their effects on SF-1- and LRH-1 association with and transcriptional activation of the inhibin promoter. We first investigated the MAPK pathway as this is known to be a major signaling pathway downstream of FSH receptor activation in the ovary. Specificity and efficacy of the MAPK kinase (MEK) inhibitor PD098059 (PD) was examined by probing Western blots of GRMO2 cells with antibodies to phosphorylated and total ERK 1 and 2 and to the phosphorylated form of protein kinase B (Fig. 7A). Inclusion of the PD compound eliminated forskolin-induced phosphorylation of ERK 1 and ERK 2, but did not diminish PKB activation by forskolin, indicating the PD compound specifically inhibited the MEK/MAPK pathway. Inclusion of PD in transient cell transfection assays significantly decreased both SF-1 and LRH-1 transactivation of the inhibin α-subunit promoter (Fig. 7B). In the presence of this inhibitor, LRH-1 recruitment was diminished and SF-1 remained associated with the inhibin promoter after forskolin stimulation, as measured in ChIP assays (Fig. 7C). Thus, the MEK/ERK pathway plays an important role in regulating inhibin α-subunit promoter activity and alters the association dynamics of both SF-1 and LRH-1 with the promoter, although no differential effects on SF-1 *vs.* LRH-1 association were apparent.

Inhibition of the PI3K/PKB/Akt pathway was also tested. This pathway is also activated by FSH action in the ovary and can be inhibited using the compound LY294002 (LY). To ensure that treatment with the PI3K inhibitor LY was effective and specific, we again performed Western blots on extracts from treated granulosa cells. LY treatment significantly decreased activated Akt/PKB in both untreated and forskolin-treated cells, yet had no effect on ERK 1/2 activation by forskolin **(**Fig. 8A**)**. In GRMO2 cells transfected with either SF-1 or LRH-1 and the inhibin α-subunit reporter, LY significantly decreased the transactivation ability of SF-1 and the synergism between SF-1 and forskolin on promoter activity (Fig. 8B). In contrast, LY treatment did not significantly impact regulation of the gene by LRH-1 or the combination of LRH-1 and forskolin (Fig. 8B). ChIP experiments in the presence of LY demonstrate that this inhibitor largely reversed the forskolin-induced loss of SF-1 from the promoter while not significantly altering LRH-1 association dynamics (Fig. 8C).

DISCUSSION

The gonadotropins FSH and LH regulate inhibin subunit gene expression in ovarian granulosa cells, and inhibin, in turn, facilitates appropriate feedback regulation to the pituitary gland. Inhibin is a centrally important hormone for normal reproductive function and fertility in mammals, and thus considerable efforts have gone into understanding its regulation. Like most genes, inhibin is regulated by a multitude of transcription factors and cofactors, some important for the basal and tissue-specific expression of the gene and others intricately involved in its regulation by the gonadotropins. In this paper, we describe roles for the transcription factors LRH-1, SF-1, and CREB in inhibin gene regulation. In addition to these, the complex transcriptional regulation of the inhibin α-subunit gene is dependent on other factors, including the GATA factors, which regulate many ovarian genes (53) and which can interact with and functionally synergize with SF-1 and LRH-1 in other cell types (54, 55), and β-catenin, which can synergize with SF-1 to regulate the inhibin α-subunit promoter in adrenal cells (56). Our studies reveal a complex dynamic relationship between SF-1 and LRH-1 in regulating the inhibin

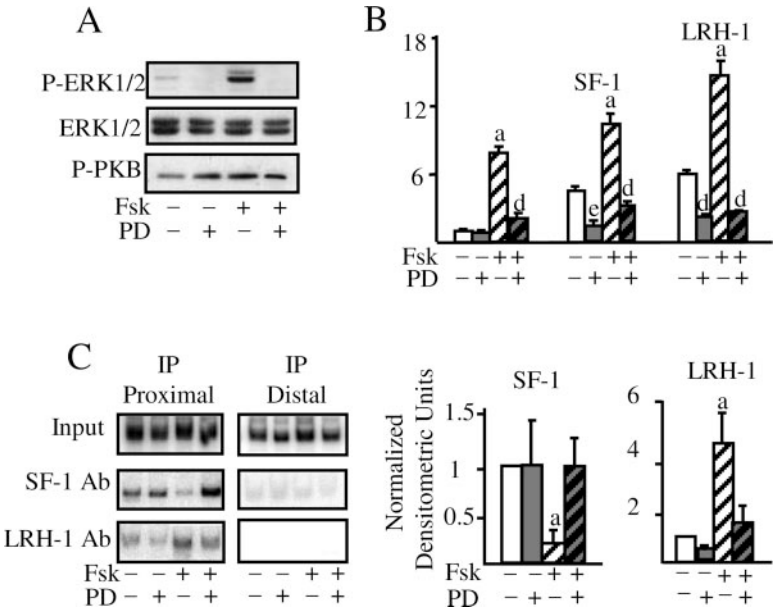

Fig. 7. The MEK Inhibitor PD Affects Both SF-1- and LRH-1-Induced Inhibin Gene Transcription and Promoter Association Dynamics

A, GRMO2 cells were pretreated with vehicle or 30 μM PD for 15 min before 1 h stimulation with 10^{-5} M forskolin (Fsk). Lysates were separated on SDS-PAGE gels and transferred, and proteins were detected using antibodies to phosphorylated ERK1/2. The blot was stripped and reprobed with antibodies to total ERK. Different aliquots of the same lysates were used to assess the phosphorylation state of PKB (P-PKB). This experiment was repeated twice with similar results (n = 3). B, GRMO2 cells were cotransfected with the inhibin α-subunit promoter reporter and expression constructs for SF-1 or LRH-1. Cells were pretreated with 30 μM PD for 15 min before forskolin stimulation. This experiment was performed four times, and data shown are averages of those experiments. a, $P < 0.005$ as compared with non-forskolin-treated pairs. d, $P < 0.005$; and e, $P < 0.01$ as compared with non-inhibitor-treated pairs. C, ChIPs were performed as above although cells were pretreated with 30 μM PD for 15 min before forskolin stimulation. This experiment was performed four times with each antibody. The autoradiograph from one representative experiment is shown, whereas the *bar graph* indicates averaged results from all experiments. a indicates $P < 0.005$ as compared with the non-Fsk-treated pairs. IP, immunoprecipitation; P-ERK, phosphorylation state of ERK.

α-subunit gene and suggest that these two related NR5A nuclear receptors may play distinct and nonoverlapping functions in inhibin gene expression.

In situ hybridization experiments illustrate expression of both SF-1 and LRH-1 in rodent granulosa cells (34, 36). Thus, we investigated the specific actions of both proteins in terms of activating the inhibin α-subunit gene to determine whether they perform the same role or if they act differently. We show in transient transfections that both SF-1 and LRH-1 activate the inhibin α-subunit promoter reporter and both synergize with forskolin. This synergism is not due to cooperative binding of CREB and NR5A proteins, as mutating one site did not affect binding to the other, at least *in vitro*. Instead, this seems to be due to actions of coactivators. The previously reported interactions between CBP and SRC-1 (48) and the augmentation of steroid hormone-dependent transcription by CBP via its interaction with SRC-1 (47) led us to test whether these proteins contribute to a transcriptional activation of the inhibin α-subunit proximal promoter. SRC-1 was chosen as the most likely candidate for these studies based both on the above reports as well as on reports describing a direct interaction between SF-1 and SRC-1 (57). LRH-1 can interact with SRC-3 (46), a protein closely related to SRC-1; however, SRC-3 is not expressed in granulosa cells (58). In transfection assays, the combination of an NR5A family member and a coregulator results in increased reporter gene expression as compared with NR5A family member-induced expression, and both CBP and SRC-1 mediate forskolin-stimulated expression of the inhibin α-subunit gene. There seems to be no bias in CBP or SRC-1 augmentation of NR5A-induced reporter expression. That is, both coactivators acted equally as well on SF-1-stimulated as on LRH-1-stimulated reporter expression.

Transfections with the mutated promoter/reporters illustrate that LRH-1 acts through the same *cis* acting site as SF-1, as has been reported for other genes regulated by SF-1 in other cell types (30, 31). However, ChIP experiments in the GRMO2 cell line illustrate that association of these NR5A proteins with the inhibin α-subunit is differentially regulated. SF-1 associates with the promoter predominantly in the basal state, and stimulation diminishes this association within a very short period of time. Conversely, LRH-1 exhibits a cyclic association with the promoter, such that it initially shuffles on and off the promoter. These early changes are not particularly robust, perhaps reflecting

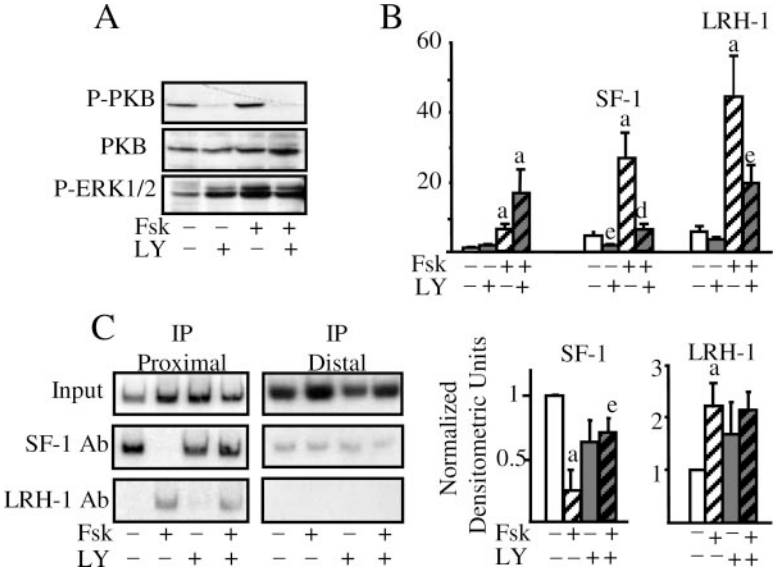

Fig. 8. The PI3K Inhibitor LY Selectively Alters SF-1 Transactivation and Inhibin Promoter Association Dynamics

A, GRMO2 cells were pretreated with vehicle or 12.5 μM LY for 1 h before 1 h stimulation with forskolin (Fsk). Lysates were separated on SDS-PAGE gels and transferred, and proteins were detected using antibodies to phosphorylated PKB (P-PKB). The blot was stripped and reprobed with antibodies to total PKB, and then with antibodies to phosphorylated ERK 1 and 2 (P-ERK1/2). This experiment was repeated twice with identical results. B, GRMO2 cells were cotransfected with the inhibin α-subunit promoter reporter and expression constructs for SF-1 or LRH-1. Cells were pretreated with 12.5 μM LY for 1 h before forskolin stimulation. This experiment was performed four times, and data shown are averages of those experiments. a, $P < 0.005$ as compared with non-forskolin-treated pairs. d, $P < 0.005$; and e, $P < 0.01$ as compared with non-inhibitor-treated pairs. C, ChIPs were performed as above although cells were pretreated with 12.5 μM LY for 1 h before forskolin stimulation. This experiment was performed four times with each antibody. The autoradiograph from one representative experiment is shown, whereas the *bar graph* indicates averaged results from all experiments. a, $P < 0.005$ as compared with the non-Fsk-treated pairs. Ab, Antibody; IP, immunoprecipitation.

the fact that these cells were not synchronized with agents that block transcription, a commonly used approach in investigating cyclic association in other systems (59, 60). By 60 min, though, the association of LRH-1 with the promoter is robustly increased, and this increase persists at later times. The changes in SF-1 and LRH-1 association with the promoter were also observed in granulosa cells isolated directly from rats, although the timing is much different. At no point in our study did we see complete dissociation of SF-1 with the promoter as in the cell line. However, a trend of loss of SF-1 association with the promoter concurrent with LRH-1 recruitment after PMSG stimulation was observed. These differences between granulosa cells isolated directly from rats and the GRMO2 cell line could reflect the slower actions of injected gonadotropins on ovarian physiology as opposed to the acute effects of forskolin in cultured cells, a difference in promoter association in cells from follicles at different developmental stages, or altered association dynamics in normal *vs.* nontransformed cells.

The switch of LRH-1 for SF-1 on the promoter after hormonal stimulation was surprising given the similarity between SF-1 and LRH-1, particularly in their DNA-binding domains. There are several possible explanations. For example, the differential association of these proteins with the promoter after forskolin stimulation could be due to regulated expression of either SF-1 or LRH-1. Our data show that SF-1 mRNA levels do not change in response to forskolin during this time period in GRMO2 cells, and these data are similar to reported SF-1 protein levels in cultured granulosa cells treated with FSH (61). In contrast, LRH-1 mRNA levels do increase (Fig. 1), although this increase is relatively modest. In addition, although there is generally a strong inverse relationship between SF-1 and LRH-1 occupancy of the promoter, this is not true at all times, particularly soon after forskolin stimulation. Falender *et al*. (34) previously reported that SF-1 message was more abundant than that of LRH-1 in granulosa cells, and that SF-1 is the predominant NR5A protein that binds to the aromatase promoter. Although the aromatase and inhibin α-subunit genes have several features of their regulation in common, both contain atypical SF-1/LRH-1 binding sites; therefore, it is possible that differences in their regulation arise from the atypical DNA elements themselves or their context within the promoter. Taken together, these results indicate that changes in LRH-1 protein expression might be a contributing factor to promoter occupancy although it is not likely the primary driving force behind the switch of LRH-1 for SF-1 on the inhibin α-subunit promoter.

SF-1 and LRH-1 both associate with small nuclear factors [Dax-1 (62) and SHP (46), respectively] that

modify their function. These interactions seem to be mediated by the same, conserved sequences in both SF-1 and LRH-1 (63). Recent evidence illustrates that DAX-1 decreases transactivation by SF-1 by sequestering it in the cytoplasm (64). Altered interactions of SF-1 and LRH-1 with these or other regulatory proteins, so that LRH-1 and SF-1 are unavailable to bind DNA under different conditions, could also contribute to the observed switch. We tested this hypothesis by treating cells transfected with SF-1 and LRH-1 yellow fluorescent protein-tagged constructs with forskolin, but were unable to detect any gross changes or other differences in cellular localization after treatment, as these fusion proteins were strictly nuclear under all conditions examined (data not shown).

A third possible explanation for the observed switching is that these NR5A proteins are differentially modified by effectors downstream of FSH-induced signaling pathways after hormonal stimulation of granulosa cells. For example, SF-1 can be acetylated (65), and both SF-1 and LRH-1 can by sumoylated (66, 67). SF-1 can also be phosphorylated by MAPK on serine 203 (51). *In vitro* phosphorylation of a recombinant fragment of LRH-1 containing the hinge and the ligand-binding domains demonstrates this fragment can be phosphorylated by ERK2 (52) (data not shown), although the specific location of this phosphorylation and whether it occurs *in vivo* have yet to be established. Phosphorylation of serine 203 in SF-1 results in increased interactions with coregulators (51); however, it is unknown whether LRH-1 responds in the same manner. In our studies both SF-1 and LRH-1 were sensitive to the MEK inhibitor, indicating that they are dependent on activated ERKs to maintain their full transcriptional activities. Consistent with this, the switch of LRH-1 for SF-1 on the promoter was also blocked by treatment with the MEK inhibitor. This could indicate that changes in the phosphorylation status of these proteins are required for changes in association dynamics. Alternatively, it is equally likely that the changes in promoter occupancy are due to modifications in additional cellular targets of the MAPK pathway.

Many investigators have demonstrated that the PI3K/protein kinase B (Akt) pathway is stimulated in granulosa cells after FSH receptor activation (11, 68, 69). Interestingly, we find that the actions of SF-1 are more sensitive to inhibition of the PI3K/PKB pathway than are those of LRH-1, at least with respect to inhibin α-subunit promoter regulation. Complementary to this, Saxena *et al.* (70) demonstrated that, in primary granulosa cells, LRH-1 is more dependent on PKA activation than PKB activation. In our ChIP experiments, blockade of PI3K signaling leads to a continuing association of SF-1 with the inhibin α-subunit promoter after forskolin stimulation. However, inhibition of this pathway did not similarly affect LRH-1 recruitment to the promoter or LRH-1-induced transactivation of the promoter. These data are consistent with recent structural analyses, which demonstrate that phosphatidylinositol lipids (PIPs) reside in the ligand pocket of mouse SF-1, human SF-1, and human LRH-1 (71, 72). Interestingly, mouse LRH-1 has a smaller pocket and thus does not bind these lipids as well (73). Thus, blockade of the PI3K pathway could result in a decrease of lipid ligands necessary for full activation of SF-1 (73) and yet not affect LRH-1. Alternatively, as SF-1 contains a PIP3-binding pleckstrin-like homology domain whereas LRH-1 does not, perhaps PI3K-induced generation of PIP3 recruits SF-1 to the membrane. Thus, SF-1 association with the inhibin α-subunit proximal promoter would be attenuated, allowing for promoter occupancy by LRH-1. Certainly, these phospholipids and the enzymes necessary to generate them are functional in the nucleus (74). Finally, perhaps SF-1 is regulated directly by modification by PI3K or a downstream kinase.

Based on our data, we propose the following model for inhibin α-subunit gene transactivation: under basal conditions where the gene is expressed at low levels in a tissue-specific fashion, SF-1 and low levels of CREB occupy the SBS and CRE, respectively (Fig. 9). This

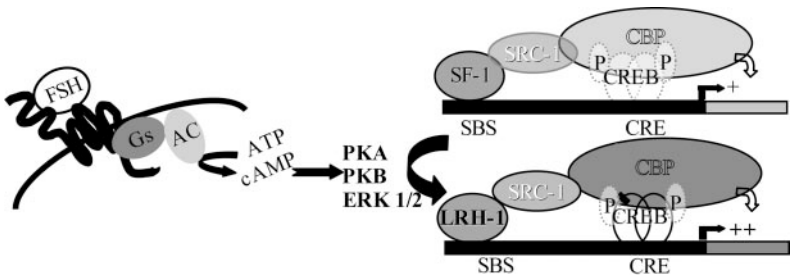

Fig. 9. FSH-Induced Inhibin α-Subunit Gene Expression Is Associated with Switching between SF-1 and LRH-1 at the Proximal Promoter SBS

This model depicts proteins involved in transcriptional activation of the inhibin α-subunit proximal promoter. Under basal conditions, when inhibin expression is low, small amounts of CREB and SF-1 occupy the CRE and SBS. At this point coactivators are involved to only a limited extent. With FSH (or forskolin) stimulation and increased cAMP levels and effects on downstream signaling pathways, LRH-1 replaces SF-1 at the SBS, and CREB and coactivator association with the promoter increases, resulting in more robust transcriptional activation. AC, Adenylyl cyclase.

finding is consistent with a strong correlation between tissues and cells that express SF-1 and those that express the inhibin α-subunit. CBP and SRC-1 probably also contribute to a transcriptional complex at the proximal promoter under these conditions as ChIP assays reveal they are associated with the promoter at low levels in the basal state. Increased cAMP levels, due to forskolin stimulation or activation of the FSH receptor, result in a switch at the SBS such that SF-1 association with the proximal promoter is rapidly decreased, and over a longer period of time LRH-1 is recruited to the promoter. This switch is dependent on intracellular changes induced by both the MAPK and PI3K pathways. The increased cAMP levels also result in more robust association of CREB (which is being activated by phosphorylation), CBP, and SRC-1, and thus this, in turn, promotes histone acetylation, chromatin remodeling, and, ultimately, transcriptional activation of the inhibin α-subunit gene. Our data support the hypothesis that a protein complex including CREB, SF-1/LRH-1, CBP, and SRC-1 mediates transcriptional activation of the inhibin α-subunit gene in granulosa cells and that stimulation of the cAMP pathway in granulosa cells results in a switching of the NR5A protein content of this complex at the proximal promoter.

MATERIALS AND METHODS

GRMO2 Cell Transfections

GRMO2 cells were plated into 12-well plates and transfected using L-a-phosphatidylethanolamine, dioleoyl[C18:1, *cis*-9] and dimethyldioctadecyl-ammonium bromide in ethanol (75) for 6 h. DNA used included the wild-type inhibin α-subunit promoter/luciferase reporter (-549 to $+44$ LUC) (21), the same promoter fragment containing a previously described 2-bp mutation in the CRE (21) or a previously characterized 2-bp mutation in the SBS (22), a cytomegalovirus (CMV)-SF-1 construct (provided by Dr. J. Larry Jameson, Northwestern University Medical School, Chicago IL), a CMV-mouse LRH-1 construct (provided by Dr. Alan Tall, Columbia University, New York, NY), a rous sarcoma virus-CBP construct (provided by Dr. Marc Montminy, The Salk Institute, La Jolla CA), or a CMV-SRC-1 construct (provided by Drs. Bert O'Malley and Ming Tsai, Baylor University, Houston TX). In most cases, 500 ng/well of the reporter and 10 ng/well of the transcription factor, coregulator, or the corresponding empty vector were used. Cells were recovered overnight, were treated with vehicle or 10^{-5} M forskolin (Sigma Chemical Co., St. Louis, MO) for 6 h, and were lysed, collected, and assayed for luciferase activity. For studies involving the coactivators CBP and SRC-1, 1 μg/well of reporter was used as above, but cells were collected 48 h after transfection. For cells treated with the MEK inhibitor PD095059, 30 μM PD was added 15 min before forskolin treatment. For cells treated with the PI3K inhibitor LY, 12.5 μM LY (68) was added 1 h before forskolin treatment. All transfection data were normalized to total protein content measured using Bradford reagent (Bio-Rad Laboratories, Inc., Hercules, CA) per manufacturer's instructions.

EMSAs

Nuclear proteins were collected from GRMO2 by the method of Dignam (76). Recombinant CREB was a gift from Dr. Rich Maurer (Oregon Health and Science University, Portland, OR). Complementary oligonucleotides corresponding to -141 to -118 of the inhibin α-subunit gene (WT = 5′ TAA GGC TCA GGG CCA CAG ACA TCT GCG TCA GAG ATA) or to the same region with a mutation in the CRE (CREMUT = 5′ TAA GGC TCA GGG CCA CAG ACA TCT GTA TCA GAG ATA) or a mutation in the SBS (SBSMUT = 5′ TAA GGC TCA GTT CCA CAG ACA TCT GCG TCA GAG ATA) were annealed, end labeled with ^{32}P-γ-ATP, and gel purified on a 10% polyacrylamide 1× Tris-borate-EDTA gel. Gels were exposed to film, and the labeled oligonucleotides were excised and eluted in fresh buffer (0.5 M NH_4OAc, 1 mM EDTA), and precipitated. Each probe (50,000 cpm) was incubated with 10 μg nuclear proteins per lane in gel shift buffer (10 mM Tris, pH 7.7; 1 mM $MgCl_2$; 1 mM dithiothreitol with 2 μg polydeoxyinosinic deoxycytidylic acid) for 30 min at room temperature. Complexes were separated on a nondenaturing 8% polyacrylamide gel in 1× Tris-borate-EDTA. The gel was dried and exposed to autoradiographic film.

ChIPs

ChIPs were performed using adaptations of the protocols of Alberts *et al* (77) and Braunstein *et al*. (78). Briefly, GRMO2 cells were plated into 60-mm dishes. Cells were treated with vehicle or 10^{-5} M forskolin for various times at 37 C, and then cross-linked with 1% formaldehyde for 15 min at room temperature and collected in PBS containing protease inhibitors. Cells were lysed [1% sodium dodecyl sulfate (SDS), 10 mM EDTA, and 50 mM Tris (pH 8.0) plus protease inhibitors], and sonicated at one third max power five times for five pulses using a Branson sonifier 450. After sonication one tenth of the total sample was removed for input. Remaining samples were immunoprecipitated overnight at 4 C in immunoprecipitation buffer [0.01% SDS, 1.1% Triton, 1.2 mM EDTA, 17.6 mM Tris (pH 8.0), 167 mM NaCl plus protease inhibitors] with 2.0 μl antibody (SF-1; Affinity Bioreagents, Golden, CO), LRH-1 (α-FTF no. 2, provided by Luc Belanger, Le Centre de Recherche en Cancerologie de l'Universite Laval, L'Hotel-Dieu de Quebec, Canada), CREB (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), CBP (provided by Dr. Marc Montminy, Salk Institute, La Jolla, CA), SRC-1, or antiacetylated H4 (both from Upstate Biotechnology, Lake Placid, NY). Protein A sepharose beads (Amersham Pharmacia Biotech, Piscataway NJ) pretreated with 200 μg/ml sonicated salmon sperm DNA and 10 mg/ml BSA were added for 45 min at 4 C. Samples were washed [0.1% SDS, 1% Triton, 2 mM EDTA, 20 mM Tris (pH 8.0), and 150 mM NaCl] twice, then in 0.1% SDS, 1% Triton, 2 mM EDTA, 20 mM Tris (pH 8.0), and 500 mM NaCl, and twice in immunprecipitation buffer. Protein/DNA complexes were eluted from the beads with 1% SDS, 0.1 M $NaHCO_3$, and the supernatant and input were DNA subjected to 200 mM NaCl at 65 C for 4 h. All samples were treated with proteinase K solution (20 μg/ml proteinase K; 10 mM EDTA; 37 mM Tris, pH 6.5) at 50 C for 16 h, and then subjected to multiple phenol-chloroform extractions and ethanol precipitations. PCR was performed on the purified DNA using distal (-3500 to minus 3200; Primer A = 5′-CGC CCC GCA TCT CGC ACA GAT AGA C; Primer B = 5′-TAA CTT CGC TGG GAA TTC GGC) or proximal (-320 to $+$ 20; Primer A = 5′-GGT CTA GAA CAG GGA ACC CAC ACG CTG C; Primer B = 5′-GTG GTA CCT GAT GTA GAT TC) primers to the inhibin α-subunit gene in the presence of ^{32}P-dCTP. Products were separated on a 5% nondenaturing polyacrylamide gel and exposed to film and phosphoscreen for quantification. For ChIP experiments in the presence of pathway inhibitors, 12.5 μM LY was added 1 h before forskolin treatment or 30 μM PD was added 15 min before treatment, and experiments were performed as described above. For ChIPs using primary granulosa cells, 23-d-old Sprague Dawley rats were injected with vehicle or 10 IU PMSG, after which granulosa cells were isolated as described previously (79). After cell isolation, cells were washed with media without serum, one

third volume was removed for RNA extraction, and the remaining two thirds was immediately cross-linked in 1% formaldehyde. Cells were washed twice with cold PBS before lysis and sonication as described above.

RT-PCR

For inhibin α-subunit mRNA measurement, GRMO2 cells in 10-cm plates were stimulated with 10^{-5} M forskolin or vehicle for 4 h, after which cells were homogenized in GuSCN solution (4 M guanadinium thiocianate, 25 mM sodium citrate, 0.5% *N*-lauryl-sarkosine, 7 μM β-mercaptoethanol), and RNA was isolated by centrifugation through a cesium cushion (79, 80). For RNA from whole ovaries, immature female Sprague Dawley rats were not injected (control) or injected sc with 10 IU PMSG for the times indicated. Ovaries were homogenized in GuSCN and RNA was isolated as above. RNA was reverse transcribed for 1 h at 42 C with 1 mM deoxynucleotide triphosphates, random hexamers, and AMV-reverse transcriptase (Promega Corp., Madison WI), and one quarter of each reverse transcriptase reaction was used for PCR using previously defined primers (79). For RT-PCR using the immortalized mouse granulosa cell line GRMO2 (81), 10-cm plates were stimulated with 10^{-5} M forskolin or vehicle for 60 min, and RNA was isolated using RNeasy mini columns (QIAGEN, Valencia, CA) according to manufacturer's instructions. One quarter of each RT reaction was used for PCR using primers specific for LRH-1 (803–1103; Primer A, 5′-AGC CAT GTC TCA GGT GAT CC; Primer B, 5′-GTT TGT CTG GTA ACC ATC CAT), SF-1 (840–1100; Primer A, 5′-GAC TAC ATG TTA CCC CCT AGC; Primer B, 5′-ATT GGG CCC TCC TGA TAA GGC) or ribosomal protein L19 (79, 82). PCR was performed for 25 cycles in the presence of ^{32}P-dCTP with a 58 C annealing temperature. PCR products were separated on a 5% nondenaturing polyacrylamide gel that was dried and exposed to autoradiographic film or exposed to a phosphoscreen that was then subjected to phosphoimaging. RNA for inhibin α-subunit mRNA from primary cells was extracted using RNeasy mini columns (QIAGEN) and processed as described above.

Western Blots

GRMO2 cells were untreated or pretreated with PD (15 min) or LY (1 h) before forskolin stimulation for 1 h. Cells were washed and then collected by adding 1× Sample Buffer (50 mM Tris, pH 6.8; 2% SDS; 10% glycerol; 0.01% bromophenol blue; 50 mM dithiothreitol) directly to plates and scraping into tubes. Samples were sonicated for 15 sec each, boiled for 5 min, and then spun in a microfuge (max) for 5 min. Each lysate (50 μl) was loaded on a denaturing SDS-PAGE gel, electrophoresed, and transferred to nitrocellulose. Blots were blocked and probed according to antibody manufacturer's instructions (Cell Signaling Technology, Beverly, MA) using antibodies to p44/42 MAPK, Phospho-p44/42 MAPK, AKT, or Phospho AKT. Blots were stripped in 62.5 mM Tris (pH 6.8), 100 mM β-mercaptoethanol, 2% SDS for 1 h at 65 C and reblocked between antibody applications.

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Both authors have nothing to declare.

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