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Repression of the Rat Steroidogenic Acute Regulatory (StAR) Protein Gene by PGF2α Is Modulated by the Negative Transcription Factor DAX-1

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The steroidogenic acute regulatory protein (StAR) is thought to mediate the rapid increase in steroid hormone biosynthesis by facilitating cholesterol transport to the inner mitochondrial membrane. Recent studies indicate that StAR gene expression is enhanced by gonadotropins, whereas prostaglandin F2α (PGF2α) appears to suppress both basal and gonadotropin-stimulated StAR mRNA levels. While studies have demonstrated that steroidogenic factor 1 (SF-1) mediates transcriptional activation of the StAR gene, the mechanism for the reduction in StAR expression requires analysis. Recent studies have shown that DAX-1 (Dosage-sensitive sex reversal adrenal hypoplasia congenita critical region on the X-chromosome, gene-1), a negative transcription factor, inhibits transcription of reporter genes in vitro. To determine whether DAX-1 could negatively regulate expression of the StAR gene, approx 2 kb of the rat StAR promoter was linked to a luciferase reporter gene (creating p-1862 StAR) and cotransfected into Y1 adrenal tumor cells and HTB9 human bladder carcinoma cells with vectors which encode DAX-1 and SF-1. Luciferase levels were significantly increased in both cell types when SF-1 was present. In contrast, when DAX-1 was cotransfected with the StAR promoter, Y1 adrenal and HTB9 cell luciferase activities were reduced to levels that were 57% and 24% of basal promoter levels, respectively. Furthermore, when dibutyryl-cAMP (dbcAMP) was added to the DAX-1 expressing cells, cAMP responsiveness was repressed 50% and 75% in Y1s and HTB9s respectively, relative to the non-DAX-1 expressing dbcAMP-treated cells. The inhibition of StAR gene transcription by DAX-1 was dose-dependent reducing transcription to 6% of control levels. Consistent with the possibility that PGF2α regulates ovarian StAR expression via DAX-1, Western blot analysis indicated a three- and fivefold increase in rat ovarian DAX-1 levels at 2 and 4 h following PGF2α injection (250 μg). The increase in DAX-1 protein corresponded to a 50% reduction in StAR mRNA levels concomitant with a 39% reduction in serum progesterone levels. Truncation of the DAX-1 protein at the C-terminal end caused a loss of inhibition of transcriptional activity. Deletion of bp -95 to -50 within the StAR promoter, a proposed DAX-1 binding site, did not alter the ability of wild-type DAX-1 to inhibit transcription. In a mammalian two-hybrid system, cotransfection of DAX-1 and SF-1 caused a 25-fold induction in luciferase activity demonstrating that these proteins interact in the two-hybrid assay. This study is the first to demonstrate that the rat StAR promoter is regulated by DAX-1 and that DAX-1 reduces StAR promoter responsiveness to cAMP. The enhanced level of DAX-1 following PGF2α administration is consistent with DAX-1 having a role in controlling both basal, gonadotropin-stimulated, and PGF2α-mediated StAR gene expression. These results imply that DAX-1 has an important role in regulating ovarian steroidogenesis by repressing StAR transcription.

Key Words: StAR; DAX-1; ovary; steroidogenesis.

Introduction

Cholesterol delivery to the mitochondrial P450 side-chain cleavage (P450scc) enzyme in response to acute hormone stimulation involves the steroidogenic acute regulatory protein or StAR. Although the rate-limiting enzymatic step in steroidogenesis is the conversion of cholesterol to pregnenolone by the P450scc enzyme, the true rate-limiting step in this process is the transport of cholesterol to the inner mitochondrial membrane where the P450scc enzyme complex resides *(1–6)*. Since cholesterol

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delivery and utilization is enhanced by StAR, StAR expression and regulation by tropic hormones has been examined in great detail. StAR gene expression has been shown to be regulated at the transcriptional level by steroidogenic factor 1 (SF-1) *(7–9)*. This transcription factor is highly expressed in steroidogenic tissues and is thought to be involved in mediating transcriptional activation of the rat StAR promoter in response to cAMP stimulation *(7)*. The mechanism by which gonadotropins stimulate StAR gene expression via SF-1 has been elucidated, however the negative regulation of the rat StAR gene has yet to be studied. We have shown that steady-state ovarian StAR mRNA levels are decreased in response to PGF2α administration *(10)*. PGF2α is a physiological derivative of arachadonic acid and is thought to be the key mediator of luteolysis in the ovary.

In the ovary, if pregnancy does not occur, it is essential that the corpus luteum regress, allowing for the initiation of a new reproductive cycle. In the rat, PGF2α is believed to be the physiological agent responsible for causing corpus luteum regression at the end of a nonfertile cycle *(11–13)*. In the pregnant and pseudopregnant rat, a high correlation between luteal PGF2α content and the demise of luteal function has been reported, clearly demonstrating a role for luteal PGF2α in rat luteolysis *(14–16)*.

PGF2α has also been shown to depress cAMP accumulation and serum progesterone levels *(17)*, and to antagonize LH-stimulated steroid production in luteal tissue *(17)*. In the rat corpus luteum, PGF2α has been shown to have a very rapid antigonadotropic effect in vitro *(18)*. PGF2α addition to cultured rat luteal cells in combination with LH, suppressed LH stimulation of adenylate cyclase and progesterone secretion *(19)*. Furthermore, in the intact corpus luteum, Lahav et al. *(20)* have demonstrated that PGF2α prevents the normal LH-induced increase in cAMP accumulation. Although the antagonizing effects that PGF2α has on LH stimulation of progesterone secretion are clear from these studies, the possibility that PGF2α directly inhibits transcription via negative regulatory factors required further study. Our laboratory has recently demonstrated that PGF2α decreases steady-state StAR mRNA levels in the rat ovary *(10)*, further supporting the hypothesis that PGF2α exerts its luteolytic effects on the ovary by reducing cholesterol substrate availability to the mitochondria where steroidogenesis begins.

Since PGF2α has a negative effect on StAR gene expression in the rat, and since the negative transcription factor, DAX-1 has been shown to repress transcriptional activation of a reporter gene in vitro, DAX-1 regulation of the rat StAR gene required analysis. DAX-1 was first identified as the gene responsible for X-linked adrenal hypoplasia congenita (AHC) *(21)*. DAX-1 is a unique member of the nuclear-receptor superfamily of transcription factors, which lacks the canonical zinc-finger DNA-binding domain. However, DAX-1 is still able to repress transcription of reporter genes in vitro. Studies have demonstrated that DAX-1 and SF-1 have similar tissue distribution patterns and that DAX-1 and SF-1 actually interact in vitro *(22)*. Although the results of this study clearly demonstrate that SF-1 and DAX-1 interact in vitro, the exact mechanism by which DAX-1 exerts its effects is still unclear. A recent study suggests that DAX-1 may recognize and bind to DNA hairpin structures within the promoter region of the mouse StAR gene in vitro and in vivo *(23)*. In addition to DAX-1 binding to stem-loop structures within the DNA, DAX-1 has also been shown to interact with SF-1 to disrupt synergistic activation of a reporter gene by SF-1 and other transcriptional cofactors *(24,25)*. The mechanism by which DAX-1 may prevent transcriptional activation of the StAR gene by gonadotropins and cAMP requires further analysis.

Results

PGF2α/DAX-1 Regulation of the StAR Promoter

It has been hypothesized that DAX-1, a negative transcription factor, may bind to the DNA, or in some way act to repress transcription of certain genes in steroidogenic tissues. In order to determine whether DAX-1 could negatively regulate expression of the StAR gene in Y1 adrenal tumor cells, the DAX-1 cDNA was co-transfected with and without the StAR promoter construct. The Y1 adrenal tumor cell line was chosen because it expresses high levels of SF-1. Endogenous levels of SF-1 in the Y1 adrenal cells stimulated transcription of the full-length StAR promoter-linked luciferase gene 90-fold relative to the empty luciferase vector (Fig. 1). Stimulation of the adrenal cells with cAMP resulted in a twofold increase in luciferase activity over the cells transfected with the StAR promoter construct alone. Cotransfection of the DAX-1 cDNA inhibited basal luciferase levels by 43% ($p < 0.005$) in the unstimulated cultures, and reduced cAMP-stimulated luciferase activity by 53% ($p < 0.001$).

Similar DAX-1 repression studies were performed using the human bladder carcinoma cell line, HTB9. These cells do not express SF-1 or DAX-1 endogenously. The results of DAX-1 repression studies using the full-length StAR promoter in HTB9 cells are shown in Figure 2. Luciferase activity in the cells co-transfected with the full-length StAR promoter construct, SF-1, and DAX-1 were decreased to less than 25% ($p < 0.001$) of luciferase activity in cells transfected with the promoter construct and SF-1 alone in both unstimulated and cAMP-treated cells.

In order to determine whether DAX-1 inhibition of SF-1-mediated transcriptional activation was dose-dependent, increasing amounts of DAX-1 cDNA were co-transfected with a constant amount of SF-1 cDNA. The results of this study are shown in Fig. 3. Increasing amounts of DAX-1 cDNA caused progressive suppression of SF-1-mediated transcriptional activation of the luciferase reporter gene. Luciferase levels in cAMP-treated cultures fell from 72%

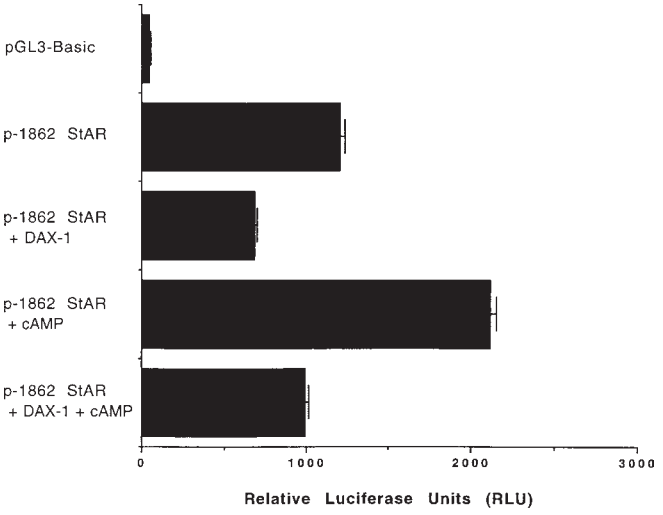

Fig. 1. Luciferase activity in Y1 adrenal cells expressing DAX-1 +/- cAMP. The full-length rat StAR promoter linked to a luciferase reporter gene (p-1862 StAR) was transfected with and without the DAX-1 cDNA into Y1 adrenal tumor cells treated plus or minus dibutyryl cAMP (1 m*M*) for 24 h. Luciferase activity in Y1 adrenal cells was significantly increased after transfection of the full-length promoter construct. Stimulation with dibutyryl cAMP enhanced luciferase activity even further. Co-transfection of the DAX-1 cDNA resulted in a significant inhibition of basal and cAMP-stimulated SF-1-mediated transcription. Bars represent mean luciferase activity ± SEM from triplicate cultures.

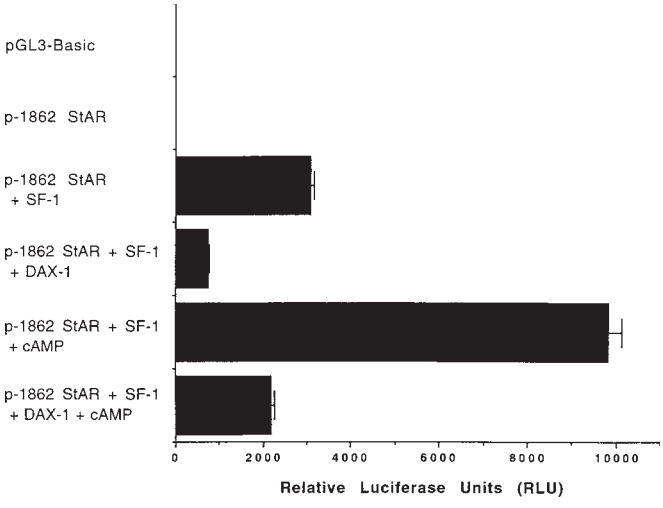

Fig. 2. Luciferase activity in HTB9 cells expressing SF-1 and DAX-1 +/- cAMP. The full-length rat StAR promoter linked to a luciferase reporter gene (p-1862) was transfected with and without the SF-1 and DAX-1 cDNAs into HTB9 cells treated plus or minus dibutyryl cAMP (1 m*M*) for 24 h. Luciferase activity was significantly increased when the SF-1 expression plasmid was cotransfected with the promoter construct. Stimulation with dibutyryl cAMP enhanced luciferase activity even further. Cotransfection of the DAX-1 cDNA resulted in a significant inhibition of both SF-1-mediated and cAMP-stimulated transcriptional activation. Bars represent mean luciferase activity ± SEM from triplicate cultures.

with 2.5 μg to 6% with 20 μg ($p < 0.005$) of the DAX-1 cDNA.

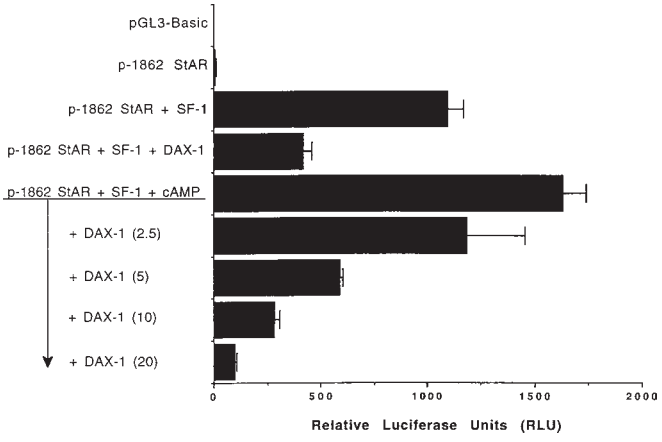

Fig. 3. Dose–response of DAX-1 inhibition of SF-1-mediated StAR transcription. The full-length rat StAR promoter linked to a luciferase reporter gene (p-1862) was transfected with a constant amount of SF-1 cDNA and increasing amounts of the DAX-1 cDNA into HTB9 cells treated plus or minus dibutyryl cAMP (1 m*M*) for 24 h. Stimulation of SF-1-transfected cells with dibutyryl cAMP caused a significant increase in luciferase activity. Cotransfection of increasing amounts of the DAX-1 cDNA resulted in a progressive suppression of cAMP-stimulated SF-1-mediated transcriptional activation. Bars represent mean luciferase activity ± SEM from triplicate cultures.

Since PGF2α has been shown to cause a decrease in the levels of steady-state StAR mRNA, and since DAX-1 has been shown to cause a decrease in transcription of a StAR promoter-linked luciferase reporter gene, we wanted to determine whether PGF2α may alter DAX-1 protein levels leading to a decrease in StAR gene expression. For this, rats were injected with PGF2α for 2 and 4 h. The DAX-1 protein at approximately 55 kDa is rapidly upregulated threefold by 2 h and fivefold ($p < 0.05$) at 4 h following PGF2α injection (Fig. 4). This is consistent with the hypothesis that PGF2α, acting through DAX-1, suppresses StAR gene expression. The graph shows a densitometric summary of DAX-1 protein levels and StAR mRNA levels in the rat ovary before and at 2 and 4 h post-PGF2α injection. These data clearly suggest a possible role for DAX-1 in PGF2α signal transduction and its effect on StAR mRNA levels in the ovary.

In order to determine whether the carboxy-terminal transrepression domain in DAX-1 was responsible for the inhibition of transcriptional activation of the StAR gene, a carboxy-terminal truncation of the DAX-1 cDNA was generated. The DAX-1 truncation (tDAX-1), a deletion of the carboxy-terminal 42 amino acids, caused a loss of repression relative to the wild-type DAX-1 cDNA (Fig. 5). Cotransfection of the DAX-1 truncation with the p-1862 StAR promoter construct in Y1 adrenal tumor cells resulted in a total loss of repression compared to cells transfected with the wild-type DAX-1 cDNA.

In order to determine the role of DAX-1 binding to a putative stem-loop structure within the 5'-flanking region

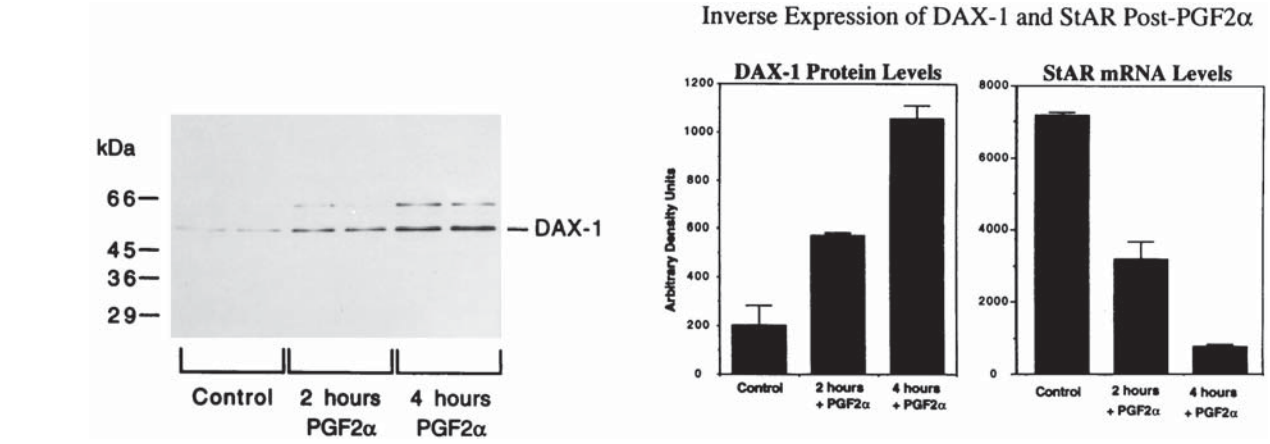

Fig. 4. Regulation of DAX-1 protein levels in rat ovaries by PGF2α. Twenty-eight-day-old Sprague-Dawley rats (n = 4/time point) were injected with 8 IU PMSG and 10 d after ovulation were injected with PGF2α (250 μg). Ovaries were obtained prior to PGF2α administration (control; t_0) and at 2 and 4 h post-PGF2α injection. Western blotting results demonstrated that DAX-1 protein levels in the ovary 10 d postovulation were significantly increased following PGF2α injection. Densitometric analysis of DAX-1 protein levels and StAR mRNA levels in the ovary following PGF2α administration demonstrate an inverse relationship between DAX-1 protein levels and StAR mRNA levels in the ovary 10 d postovulation. Autoradiographs were scanned with a densitometer and the results presented. Bars represent mean DAX-1 protein levels or StAR mRNA levels ± SEM from three separate experiments expressed as arbitrary density units.

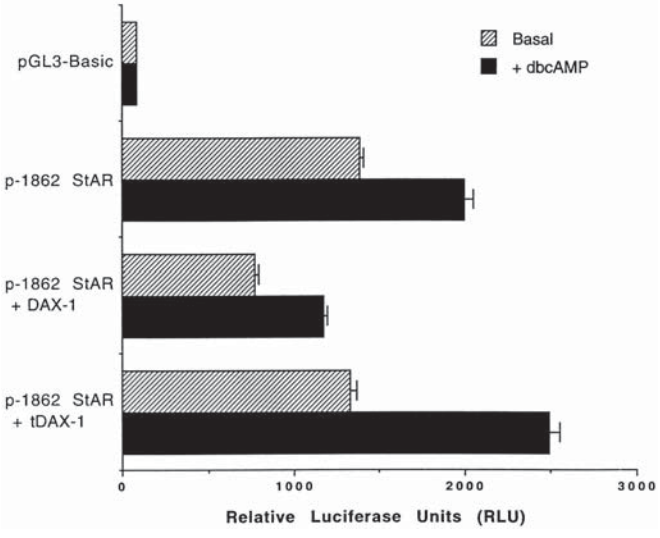

Fig. 5. Luciferase activity in Y1 adrenal cells expressing DAX-1 truncations +/- cAMP. The full-length rat StAR promoter linked to a luciferase reporter gene (p-1862 StAR) was cotransfected into Y1 adrenal tumor cells with either the wild-type DAX-1 cDNA or a carboxy-terminal truncation and were treated plus or minus dibutyryl cAMP (1 m*M*) for 24 h. Luciferase activity in Y1 adrenal cells was significantly increased after transfection of the full-length promoter construct. Stimulation with dibutyryl cAMP enhanced luciferase activity even further. Cotransfection of the DAX-1 truncation resulted in a loss of inhibition of both basal and cAMP-stimulated transcription relative to wild-type DAX-1. Bars represent mean luciferase activity ± SEM from triplicate cultures.

of the rat StAR gene, a proposed stem-loop DAX-1 binding region was deleted from the promoter using a variation of the site-directed mutagenesis technique. Cotransfection of Y1 adrenal cells with both the wild-type StAR promoter (p-1862 StAR) and the stem-loop-negative promoter (p-1862 SL-NEG) resulted in an increase in luciferase activity (Fig. 6). When the DAX-1 cDNA was cotransfected with the wild-type promoter construct transcriptional activation was repressed. DAX-1 cotransfection with the stem-loop-negative promoter construct also caused repression of the reporter gene (44%), suggesting that DAX-1 does not mediate transcriptional repression by binding to this stem-loop structure alone.

In order to determine whether SF-1 and DAX-1 physically interact with each other, a two-hybrid assay in mammalian cells was performed. As shown in Fig. 7, cotransfection of DAX-1 and SF-1 caused a 25-fold ($p < 0.001$) induction in luciferase activity as compared to cells cotransfected with pBIND and pACT empty vectors. Luciferase activity was not significantly increased when cells were cotransfected with either SF-1 or DAX-1 alone. These results demonstrate that SF-1 and DAX-1 were able to interact in the mammalian two-hybrid assays.

Discussion

In the past 3 yr, the mouse, rat, and human StAR genes have been isolated and sequenced. All three genes are comprised of seven exons and six introns that span 6.5, 7, and 8 Kb for the mouse, rat, and human genes, respectively *(26–28)*. Approximately 3 Kb of the 5'-flanking region of the StAR genes have been sequenced and analyzed for DNA regulatory motifs. The rat, mouse, and human promoter regions, unlike many genes expressed in a highly regulated fashion, lack the canonical TATA box. Although StAR transcription is positively regulated in response to cAMP stimulation, the rat, mouse, and human StAR promoters all

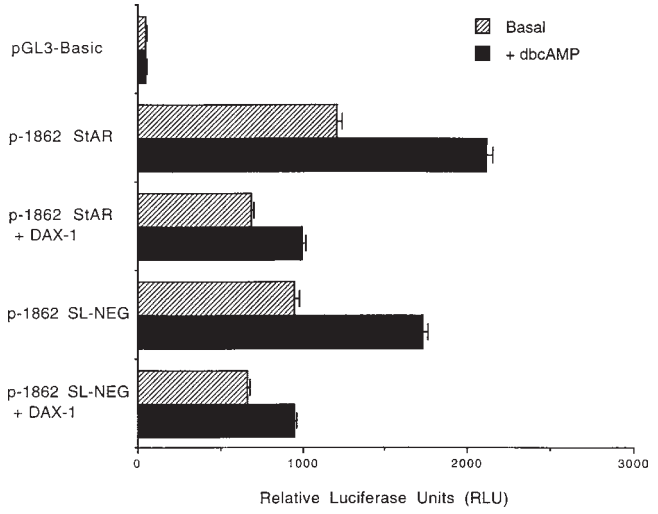

Fig. 6. Luciferase activity in Y1 adrenal cells expressing DAX-1 +/- cAMP. The full-length rat StAR promoter linked to a luciferase reporter gene (p-1862 StAR) and the promoter with the DAX-1 binding site deletion (p-1862 SL-NEG) were transfected with and without the DAX-1 cDNA into Y1 adrenal tumor cells treated plus or minus dibutyryl cAMP (1 m*M*) for 24 h. Luciferase activity in Y1 adrenal cells was significantly increased after transfection of the full-length promoter construct. Stimulation with dibutyryl cAMP enhanced luciferase activity even further. Cotransfection of p-1862 SL-NEG with the DAX-1 cDNA resulted in significant inhibition of both basal and cAMP-stimulated transcription. Bars represent mean luciferase activity ± SEM from triplicate cultures.

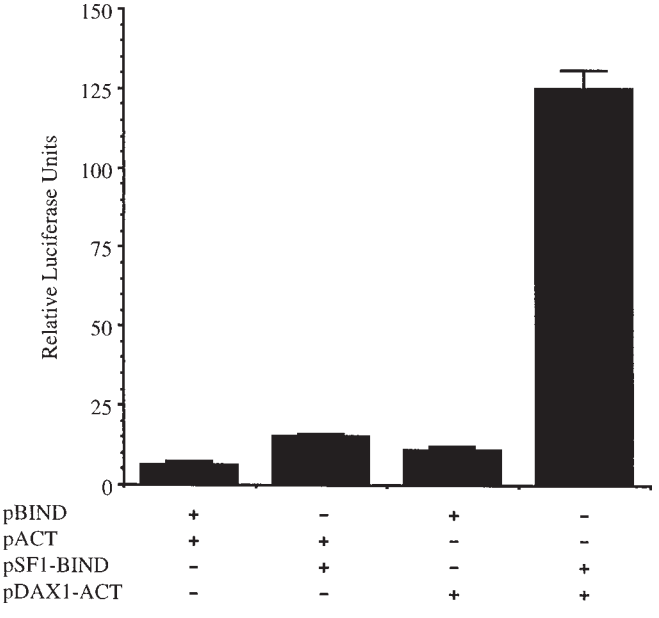

Fig. 7. DAX-1 Interacts with SF-1 in a Mammalian Two-Hybrid System. HTB9 cells were transiently transfected with pG5-luciferase vector either in the presence or absence of the specified vector using the Fugene 6 transfection method as described under materials and methods. Bars represent mean luciferase activity ± SEM from three experiments, each one performed in triplicate.

lack a cAMP response element (CRE). The mechanism by which StAR gene transcription is activated by gonadotropins and cAMP has recently been determined to be due in part to activation of steroidogenic factor 1 (SF-1).

SF-1 was first shown to be an essential regulator of the cytochrome P450 steroid hydroxylase *(29–31)* and was subsequently linked to the expression of the gene encoding Mullerian-inhibiting substance *(32,33)*. SF-1 is an orphan member of the nuclear receptor superfamily and is involved in the transcriptional regulation of many steroidogenic enzyme genes. SF-1 is thought to activate transcription of target genes upon binding to CCTTG sequence motifs within the promoter region. Analysis of the StAR promoter region in the rat, mouse, and human genes has characterized multiple SF-1 binding sites and their role in regulating transcription of the StAR gene *(7–9)*.

SF-1 and DAX-1 have similar tissue distribution patterns and both are essential for the development of the adrenal cortex *(34)*. DAX-1, a negative transcription factor in the nuclear receptor superfamily, has been shown to bind to a retinoic acid response element and decrease transcription *(21)*. The carboxy-terminal region of DAX-1 is homologous with the ligand-binding domain of certain orphan nuclear receptors, but the amino terminus of DAX-1 is unique and consists of three and one-half repeats of a 65–67 amino acid motif that has been proposed to serve as a DNA-binding domain *(21)*. The overlapping tissue distributions and functional roles of SF-1 and DAX-1 in the adrenal gland and reproductive system raise the possibility that they might interact in one of several manners. The results of our mammalian two-hybrid analysis of DAX-1 and SF-1 clearly demonstrate protein-protein interaction. These results correlate with a previous report showing that SF-1 and DAX-1 were able to interact in both yeast two-hybrid and glutathione-S-transferase pull down assays *(25)*.

Cotransfection of DAX-1 with a rat StAR promoter-driven luciferase reporter gene confirmed a role for DAX-1 in the negative regulation of the StAR gene at the transcriptional level. These studies also demonstrated that as increasing amounts of the DAX-1 cDNA were cotransfected, transcriptional activation of the rat StAR gene by SF-1 decreased. This is consistent with the findings of Ito et al. *(22)*, which demonstrated that DAX-1 repression of SF-1 regulatory motifs in tandem were negatively regulated by DAX-1 in a dose-dependent manner.

PGF2α is thought to be the key mediator of luteolysis in the rat ovary. This is due to the loss of steroidogenic capacity of the corpus luteum following PGF2α administration. Studies by our laboratory have shown that PGF2α administration caused a rapid and significant loss in StAR expression, which was concomitant with a loss of progesterone production *(10)*. With the loss of StAR expression, cholesterol transport is reduced causing steroid hormone biosynthesis to decline. The effects of PGF2α on cholesterol transport and progesterone production have been well-

documented *(10,17–20)*, but the mechanism by which PGF2α exerts its effects directly on StAR gene expression required further study. Western blot analysis of DAX-1 protein levels following prostaglandin administration demonstrated a significant increase in DAX-1 protein expression providing a direct mechanism for the downregulation of StAR gene expression and inhibition of steroid production.

A carboxy-terminal truncation of the DAX-1 cDNA was generated to determine whether a carboxy-terminal transrepression domain was necessary for repression of the StAR gene. Truncation of the last 42 amino acids of the DAX-1 protein resulted in a total loss of repression relative to the wild-type DAX-1 cDNA. This is consistent with the results obtained by Ito et al. *(22)*, which demonstrated that DAX-1 was a potent inhibitor of SF-1 action and proposed that the effect involved the action of a carboxy-terminal inhibitory domain in DAX-1. Consistent with this hypothesis, a recent study by Lalli et al. *(35)* demonstrated that removal of the most C-terminal 19 amino acids of a DAX-1 construct resulted in complete abrogation of silencing of two promoters in two different cell types. The loss of repression by DAX-1 may be due to the loss of a protein domain necessary for protein-protein interaction, as has been suggested by Crawford et al. *(24)*. This group hypothesized that DAX-1 may act as an adaptor molecule to recruit other transcriptional factors like corepressors (Nuclear Co-Repressor, NCo-R) and or coactivators (Steroid Receptor Coactivator, SRC-1). Little is known about the mechanism by which DAX-1 represses its target genes, however, a recent study suggests that DAX-1 may interact with SF-1 while bound to the DNA and recruit corepressors to the transcriptional complex to stifle transcription of genes in response to external stimuli. Our studies provide evidence that increased DAX-1 protein levels following PGF2α administration act to inhibit SF-1 mediated StAR transcriptional activation.

A recent study using a hydroxylated cholesterol derivative in Y1 cells demonstrated that DAX-1 was able to repress P450scc and 3β-HSD gene expression *(36)*, in addition to its effect on StAR expression. However, questions still exist as to whether DAX-1 interacts with SF-1 bound to the DNA or whether DAX-1 is able to bind directly to stem-loop structures within the promoter regions of these genes. A recent study by Zazopoulos et al. *(23)* suggests that DAX-1 recognizes and binds to hairpin structures in the promoter regions of its target genes, thereby repressing transcription. Their studies demonstrated DAX-1's ability to bind to stem-loop structures in vitro, and that mutating critical residues, which prevented hairpin formation, resulted in a loss of regulation of the mouse StAR promoter by DAX-1 in reporter gene studies *(23)*. Our study demonstrated that deletion of the putative stem-loop structure (as reported in *23*) from the rat StAR gene promoter did not significantly alter repression of the rat StAR gene by DAX-1. This would seem to implicate the SF-1/DAX-1 interaction as an important step in recognition and inhibition of target genes by DAX-1. However, continued repression could be explained by the presence of additional DAX-1 binding sites in the promoter which remain intact following the 45-bp region deletion. Similarly, binding of DAX-1 to an as yet unidentified regulatory motif cannot be ruled out since mobility shift assays using a retinoic acid response element probe and purified DAX-1 protein yielded a specific shifted complex *(21)*.

A study by Ito et al. *(22)* stated that addition of DAX-1 protein did not appear to supershift the SF-1 DNA complex. This suggests that in the absence of a DAX-1 recognition site, the protein interactions are relatively weak, or that the dissociation rate was high enough that multimeric complexes were not observed under the conditions used in their mobility shift assays. However, the underlying fact consistent in all studies to date is that SF-1 and DAX-1 interact in vitro. DAX-1 inhibition of SF-1 action in reporter gene analysis was selective for DAX-1, as other steroid superfamily members did not bind to SF-1 *(22)*. The mechanism by which DAX-1 acts to negatively regulate StAR transcription may involve both interaction with SF-1 to inhibit SF-1-mediated transcriptional activation as well as recruitment of other accessory transcriptional factors, including corepressors and coactivators.

The results of this investigation indicated that DAX-1 repressed StAR gene expression and that this repression was dose-dependent. These studies are the first to show that DAX-1 protein levels increased in response to PGF2α administration, in parallel with a reduction in StAR mRNA levels, further supporting a role for DAX-1 in the PGF2α signaling pathway. These studies suggest that DAX-1 may interact with SF-1 and prevent SF-1-mediated transcriptional activation of the rat StAR gene.

Materials and Methods

Materials

The cDNA encoding murine DAX-1 was obtained in pcDNA 3.1 (+) (Invitrogen, Carlsbad, CA) from E. R. B. McCabe (UCLA School of Medicine, Los Angeles, CA). The murine SF-1 cDNA in pCMV was obtained from Kieth L. Parker (University of Texas, Southwestern, Dallas, TX). Dibutyryl-cAMP and PGF2α were purchased from Sigma Chemical Co. (St. Louis, MO). PMSG was purchased from Diosynth (Chicago, IL). [^{35}S] deoxy-ATP (1348 Ci/mmol) was purchased from DuPont-New England Nuclear (Wilmington, DE). [α^{32}P]-deoxy-CTP (3000 Ci/mmol) and the Sequenase DNA sequencing kit were obtained from Amersham Corp. (Arlington Heights, IL). BioMax film was purchased from Fisher Scientific (Norcross, GA). Nitrocellulose membrane was obtained from Schleicher and Schuell (Keene, NH). All restriction enzymes and the Fugene 6 transfection reagent were obtained from Boehringer Mannheim (Indianapolis, IN). The Dual-Luciferase Reporter Assay System and the Mammalian Two-hybrid

System were purchased from Promega (Madison, WI). The TA cloning kit was purchased from Invitrogen (San Diego, CA), and the Sephaglas BandPrep kit was obtained from Pharmacia Biotech (Piscataway, NJ). Large scale DNA purification reagents for transfection studies were obtained from Qiagen (Valencia, CA). The Quikchange site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA), and the SuperSignal Substrate was purchased from Pierce (Rockford, IL). Synthetic oligonucleotides were obtained from Integrated DNA Technologies, Inc. (Coralville, IA). All other chemicals were reagent grade, and were obtained from Fisher Scientific or Sigma Chemical Co.

Animal Model

Twenty-eight-d-old Sprague-Dawley rats were purchased from Harlan Industries of Madison, WI. All procedures for prostaglandin treatment and the methods for tissue and blood sampling were approved by the University of South Florida Animal Care Committee. Throughout the experiments, animals had free access to food and water, and were housed under a 12-h dark, 12-h light cycle. Follicular development and ovulation were induced in rats by injection of 8 IU PMSG (s.c.). Rats ovulate approx 72 h following PMSG treatment *(37)*. Ten days following ovulation, rats were given a single injection (i.m.) of PGF2α (250 μg). Ovaries were removed prior to PGF2α injection (t_0) and at 2 and 4 h post-PGF2α treatment. Tissue was immediately frozen in liquid nitrogen. Rats were euthanized by clipping the diaphragm while under ether anesthesia. In all experiments, 4 animals were utilized/treatment or time-point.

Site-Directed Mutagenesis

Site-directed mutants were obtained using the Quik Change site-directed mutagenesis kit from Stratagene according to the manufacturer's protocol. Briefly, complementary oligonucleotides containing the desired mutations were synthesized and PAGE purified for the mutation reactions. The mutant oligonucleotides (bold) for the DAX-1 truncation were 5'-GATCCGCTGAA**TGATGA**AGTG CCCTTTTCC-3' and the mutant oligonucleotides for the putative DAX-1 binding site deletion were: 5'-TGC ACAGTGACTG**AATT**CTTTTTTATC TCA-3' DBS1; 5'-TTCCGCTGGAAG**A**ATT**C**AAGGCAGAGCACT-3' DBS3. Ten ng of the double-stranded DNA template was incubated with 125 ng of the appropriate primer and 1 μL of dNTPs in 50 μL of reaction buffer (100 m*M* KCl, 100 m*M* $(NH_4)_2SO_4$, 200 m*M* Tris-HCl, pH 8.8, 20 m*M* $MgSO_4$, 1% Triton X-100, and 1 mg/mL nuclease-free bovine serum albumin). One μL of Pfu DNA polymerase (2.5 U/mL) was added to the reaction and each reaction was heated to 95°C for 30 s followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 1 min, and extension at 68°C for 12 min. After the cycling reaction, samples were subjected to digestion with Dpn I for 1 h at 37°C to remove the parental DNA template. One μL of the mutant samples was used to transform XL-1 Blues and the resulting mutations were verified by sequencing. Following site-directed mutagenesis, the wild-type StAR promoter construct was digested with EcoRI and religated thereby generating the stem-loop negative promoter construct, p-1862 SL-NEG.

Cell Culture

Y1 adrenal tumor cells and human bladder carcinoma (HTB9) cells were grown in DMEM with 10% FBS and incubated at 37°C with 5% CO_2 until needed for transfection studies. Cells were passed with 0.25% trypsin-EDTA and the resulting suspension of cells was centrifuged at 2500*g* for 5 min, followed by resuspending cells in fresh media and transferring them to fresh flasks or six-well plates.

Transfections and Luciferase Assays

Cells were plated in six-well plates for use in luciferase assays and transfection was completed using the calcium phosphate method. On the day of the transfection, fresh media was added to the cells. Five micrograms of each plasmid to be transfected was added to the appropriate tube and precipitated with 0.1 volume of NaOAc and 3 volumes of absolute ethanol. The samples were vortexed and placed at –70°C for 1 h before centrifuging samples at 12,000*g* in a microcentrifuge for 15 min at 4°C. The DNA was resuspended in 450 μL of sterile, distilled water before adding 50 μL of 2.5 *M* $CaCl_2$. The resulting solution was then added dropwise to 500 μL of a 2X HEPES-buffered saline solution while bubbling with a 1 mL pipette. The sample was then vortexed for 5 s and incubated for 20 min at RT prior to adding to the cells. The DNA was incubated with the cells for 4 h at 37°C with 5% CO_2, followed by washing the cells twice with PBS. After the cells were washed, fresh media was added and the cells were incubated for 48 h prior to measurement of luciferase activity. Various stimulants were added to the cells 24 h prior to the end of the incubation period. At the end of the experiment, cells were washed once with PBS, and 0.5 mL of 1X passive lysis buffer was added to each well. The resulting lysate was frozen at –80°C until luciferase activity was measured. For this, 20 μL of the lysate was placed in a tube in the Turner Designs 20/20 Luminometer and 100 μL of the firefly luciferase enzyme substrate was injected into the tube and the luminescence measured. A control plasmid, which encodes for the *Renilla* luciferase protein, was cotransfected into the cells to control for transfection efficiencies. The ratio of the firefly and *Renilla* readings was used to correct for differences in transfection efficiencies.

Mammalian Two-Hybrid Assay

The plasmids pBIND, pACT and pG5-luciferase used in these assays were provided in the Mammalian Two-hybrid System (Promega, Madison, WI). The plasmid pSF1-BIND was constructed using full-length mouse SF-1 cDNA inserted into the BamH I and Not I sites. To construct the pDAX1-ACT plasmid the full-length mouse DAX-1 cDNA

was inserted into the *Xho*I/*Sal*I and *Not*I sites. To examine luciferase activities, HTB9 cells were cotransfected with the pG5-luciferase vector either in the presence or absence of pSF1-BIND or pDAX1-ACT. For these experiments, transfections were performed with 2 μg of each plasmid using the Fugene 6 transfection method according to the manufacturer's instructions. After transfection, cells were incubated for 48 h at 37°C (5% CO_2). Lysate preparation and luciferase activity measurement were carried out as described above. Transfection efficiencies were corrected using the renilla activity expressed by the pBIND plasmid.

SDS-PAGE and Electrotransfer

Ovarian tissue (100–150 mg) obtained from rats per experimental design was homogenized in 1.5 mL ice-cold homogenization buffer, as previously described *(38)*. Ovarian homogenates were assayed for protein concentration by the method of Bradford *(39)*, using BSA as the standard. Ovarian proteins (50 μg protein) were denatured at 100°C in loading buffer *(38)* for 10 min and subjected to electrophoresis on 7.5–18% gradient SDS-polyacrylamide gels according to the method of Laemmli *(40)*. After electrophoresis, samples were electroblotted onto nitrocellulose (0.2 μm pore) in buffer containing 0.25 *M* Tris-base (pH 8.3), and 1.92 *M* glycine for 16 hours at 4°C. To verify equal protein loading, nitrocellulose sheets were stained with either 0.1% Ponceau S (in 5% acetic acid) or 0.01% fast green (in 20% methanol and 7% acetic acid) and destained in the same solution without fast green.

Immunoblotting

Ovarian DAX-1 protein contents were estimated by incubating transferred proteins in a 20 m*M* Tris base-buffered (pH 7.5) sodium chloride (500 m*M*) solution (TB-NaCl) with 3% milk and 0.05% Tween-20 for 1 h at RT. Buffer was replaced with TB-NaCl containing the specific rabbit polyclonal antiserum (anti-DAX-1, Santa Cruz, Santa Cruz, CA) diluted 1:1000 in 3% milk and incubated at 4°C for 16 h. Nitrocellulose blots were washed in TB-NaCl containing 0.05% Tween-20 and then incubated in TB-NaCl containing 3% milk and a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit antisera (Pierce, Rockford, IL) for 1 h at RT. Blots were rinsed and soaked in chemiluminescent SuperSignal Substrate for 10 min. Differences in band density on autoradiograms were quantified densitometrically with a Hoefer scanning densitometer (Hoefer Instruments, San Francisco, CA) for statistical analysis.

Data Analysis

The Western blot results were quantitatively analyzed using a Hoefer Scanning Densitometer (Hoefer Instruments, San Francisco, CA). Equal protein loading was verified by Ponceau S staining of the nitrocellulose membranes. Serum progesterone and luciferase data were expressed as the mean +/- SEM. Data from these individual parameters were compared by analysis of variance (ANOVA) followed by Student-Newman-Keuls multiple comparison test when applicable *(41)*. All analysis was completed using the Statview program with graphics (Abacus Concepts, Berkeley, CA) on a Macintosh IIci computer. A $p < 0.05$ was considered significant for all tests.

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