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Cyclic AMP-Stimulated Interaction between Steroidogenic Factor 1 and Diacylglycerol Kinase θ Facilitates Induction of CYP17[▽]

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In the human adrenal cortex, adrenocorticotropin (ACTH) activates CYP17 transcription by promoting the binding of the nuclear receptor steroidogenic factor 1 (SF1) (Ad4BP, NR5A1) to the promoter. We recently found that sphingosine is an antagonist for SF1 and inhibits cyclic AMP (cAMP)-dependent *CYP17* gene transcription. The aim of the current study was to identify phospholipids that bind to SF1 and to characterize the mechanism by which ACTH/cAMP regulates the biosynthesis of this molecule(s). Using tandem mass spectrometry, we show that in H295R human adrenocortical cells, SF1 is bound to phosphatidic acid (PA). Activation of the ACTH/cAMP signal transduction cascade rapidly increases nuclear diacylglycerol kinase (DGK) activity and PA production. PA stimulates SF1-dependent transcription of CYP17 reporter plasmids, promotes coactivator recruitment, and induces the mRNA expression of *CYP17* and several other steroidogenic genes. Inhibition of DGK activity attenuates the binding of SF1 to the CYP17 promoter, and silencing of DGK-θ expression inhibits cAMP-dependent CYP17 transcription. LXXLL motifs in DGK-θ mediate a direct interaction of SF1 with the kinase and may facilitate binding of PA to the receptor. We conclude that ACTH/cAMP stimulates PA production in the nucleus of H295R cells and that this increase in PA concentrations facilitates CYP17 induction.

In the zonae fasciculata and reticularis of the human adrenal cortex, the biosynthesis of steroid hormones is activated by the peptide hormone adrenocorticotropin (ACTH), which activates a cyclic AMP (cAMP)-dependent signal transduction cascade, resulting in a rapid increase in hormone production and a sustained induction of the expression of steroidogenic genes. We have shown that cAMP-stimulated transcription of one of these steroidogenic genes, *CYP17*, is mediated by the binding of a complex containing steroidogenic factor 1 (SF1), $p54^{nrb}$, and polypyrimidine-tract-binding protein-associated splicing factor to the CYP17 promoter (93). The binding of this complex is concomitant with the cyclical association of several coregulatory proteins, including GCN5, steroid receptor coactivator 1, and ATPase-dependent chromatin remodeling factors (16).

An integral regulator of *CYP17* and most other steroidogenic genes in the adrenal cortex and gonads is SF1 (5, 94). SF1 is a nuclear receptor (NR) that plays a key role not only in steroidogenesis but also in endocrine development and sex differentiation (6, 25, 32, 73, 82, 83). Targeted disruption of SF1 in mice resulted in adrenal and gonadal agenesis, absence of the ventromedial hypothalamic nucleus, and impaired expression of pituitary gonadotropins (42, 69, 118). The ability of SF1 to transactivate target genes has been shown to be regulated by phosphorylation (19, 31, 95, 97), sumoylation (12, 54), acetylation (11, 44, 46), and protein-protein interactions (7, 16, 61, 62, 75, 93, 115, 120).

Recently we identified sphingosine (SPH) as an endogenous ligand for SF1 (109). SPH is bound to the receptor under basal conditions, and cAMP treatment decreases the amount of this ligand bound to the receptor. SPH prevents the binding of SF1 to the CYP17 promoter and impairs the recruitment of coregulatory proteins, resulting in decreased cAMP- and SF1-dependent transcription of CYP17 reporter constructs, suppressed CYP17 mRNA expression, and attenuated dehydroepiandrosterone (DHEA) biosynthesis (109). While these studies demonstrated that SPH is an endogenous antagonist for SF1, they did not establish a role for ligand binding in receptor activation.

In addition to our mass spectrometric studies, which identified SPH as an antagonist for SF1, crystallographic studies have demonstrated that phospholipids are present in the ligand binding pocket of the bacterially expressed receptor used for structural analysis (55, 63, 112). Krylova et al. demonstrated that phosphatidylinositol phosphates (PIPs) interact with the ligand binding domain (LBD) of SF1 and that ligand binding is required for maximal activity of the receptor (55), while Li et al. found that the receptor has a large (approximately 1,600 Å) LBD that interacts with phospholipids, such as phosphatidic acid (PA) and phosphatidylethanolamine (PE), that have fatty acyl chains of between 12 and 18 carbons (63). Significantly, the large binding pocket led the authors to speculate that SF1 may readily exchange its ligands to respond to different cellular cues (63). Our studies demonstrating cAMP-stimulated changes in the interaction of SPH with SF1 lend support to the premise that SF1 has multiple ligands and prompted us to identify agonists for the receptor. We show herein that PA is a ligand for SF1 in H295R cells and that the binding of this molecule to the receptor in response to cAMP occurs via the activation of diacylglycerol kinase theta (DGK-θ).

MATERIALS AND METHODS

Reagents. Dibutyryl cAMP (Bt_2cAMP) was obtained from Sigma (St. Louis, MO). All phospholipids and SPH were obtained from Avanti Polar Lipids, Inc.

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TABLE 1. Primers used in real-time RT-PCRs

Gene	Forward primer	Reverse primer
CYP17	CCGCACACCAACTATCAG	GTCCACAGCAAACTCACC
CYP11A1	CGTGGAGTCGGTTTATGTC	CTCTGGTAATACTGGTGATAGG
CYP11B1/2	ACGGCGACAACTGTATCC	AGAGCGTCATCAGCAAGG
CYP21	TGTGGAACTGGTGGAAGC	GGTGGAGCCTGTAGATGG
3βHSD	CCAGTAGCATAGAGGTAGCC	TCAGATTCCACCCGTTAGC
DGK-α	GCGAGGAGGCTGGTGAGTC	TGGAAGATGGGAGGCAGGATG
DGK-β	GGAGGAGTGGATTCAAGGAG	CAGAAGGAGCAGCAGAGG
DGK-γ	TCCACGACCACATTGAGTTG	GACACCCTTCCTGCTTTCC
DGK-δ	GCACAGAACCTACAGAACC	GCGACCACCTCCAGAATC
DGK-ε	GCACCCGCAGGGACATCTTC	CGCACAACGCAGCAGTAGG
DGK-ζ	AGCAAGAAGAAGAAGAGG	GGATTGAGATACCAGAGG
DGK-η	AGAAAGGAGATGGAGGATTGG	CACACTTCGCAGGACAGG
DGK-θ	CGTTCTCCGTACTGCTGTC	GTCTGCCGTGTCGTTCTC
DGK-ι	CCAGAAGGAGAAGGACGAAG	ACCATTTGCCACAGGAAGG
DGK-κ	TGGAGTGTGATGATCGTGAG	GCAGATTCTAAGTGTTGGATGG
β-*Actin*	ACGGCTCCGGCATGTGCAAG	TGACGATGCCGTGCTGCATG

(Alabaster, AL). All PA molecular species were dissolved in chloroform, dried under nitrogen gas, and resuspended in 50 mM Tris-HCl, pH 7.4, containing 4 mg/ml fatty acid-free bovine serum albumin (BSA) by ultrasonication and used within 48 h. Cells were treated with lipids by dispersing the solutions into the cell culture medium using a Hamilton syringe. The DGK inhibitor 3-{2-(4-[bis-(4-flurophenyl)methylene]-1-piperidinyl)ethyl}-2,3-dihydro-2-thioxo-4(1H)quinazolinone (R59949) and α-amanitin were obtained from EMD Biosciences, Inc. (La Jolla, CA).

Cell culture. H295R adrenocortical cells (85, 99) were generously donated by William E. Rainey (Medical College of Georgia, Augusta, GA) and cultured in Dulbecco's modified Eagle's/F12 medium (Invitrogen, Carlsbad, CA) supplemented with 10% Nu-Serum I (BD Biosciences, Palo Alto, CA), 0.5% ITS Plus (BD Biosciences, Palo Alto, CA), and antibiotics. Jeg3 human choriocarcinoma cells were obtained from Michael R. Waterman (Vanderbilt University School of Medicine, Nashville, TN) and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics.

Immunoprecipitation. For immunoprecipitation assays, H295R cells (4 to 10 150-mm dishes) were transfected with 30 μg of mutant or wild-type pCR3.1-SF1 per dish and treated with 1 mM Bt_2cAMP for 1 h. Nuclear proteins were isolated using the NE-PER nuclear and cytoplasmic extraction reagent (Pierce, Rockford, IL) and incubated with anti-SF1 (Millipore, Billerica, MA) and protein A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C with rotation. Control reactions were carried out by incubating with anti-phospho-cAMP response element binding protein (Millipore) and/or protein A/G and using nuclear extracts isolated from Jeg3 cells. The mixture was then centrifuged, the supernatant removed, and the precipitant subjected to a series of 5-min washes: three times with RIPA buffer [150 mM NaCl, 50 mM Tris-Cl, pH 8.0, 5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 150 nM aprotinin, 1 mM leupeptin, 1 mM E-64, 500 mM 4-(2-aminoethyl) benzenesulfonylfluoride], and 10 times with phosphate-buffered saline (PBS). The samples were then analyzed by mass spectrometry for detection of phospholipids.

Analysis of phospholipid molecular species. Samples were dissolved in 0.5 ml 9:1 MeOH:H_2O (vol/vol) and analyzed by syringe infusion at a rate of 5 μl/min into an API 4000 quadrupole linear ion trap mass spectrometer. Negative-mode precursor ion scans for *m*/*z* 153.1 (typical fragmentation for glycerophospholipids) were performed with the declustering potential set to −100 eV and entrance potential set to −10 eV with a collision energy range of 50 to 70 eV for an *m*/*z* range of 300 to 1,000. Species were identified by both *m*/*z* matches to a glycerophospholipid database (www.lipidmaps.org) and further class-specific fragmentation (35). PE was identified by positive-mode neutral-loss scans of 141.1 Da. Phosphatidylcholine (PC) was identified by positive-mode precursor ion scans for *m*/*z* 184.4. Phosphatidylserine (PS) was identified by neutral-loss scans for 185 Da. Phosphatidylinositol (PI) was identified by negative-mode precursor ion scans for *m*/*z* 241. PA was identified by precursor ion scans for *m*/*z* 153.1 and absence of signal for that species in the other species-specific scans.

Transient transfection, reporter gene analysis, and mutagenesis. To examine the effect of SPH and PA on SF1-dependent *CYP17* reporter gene activity, H295R human adrenocortical cells or Jeg3 human choriocarcinoma cells were subcultured on 24-well plates and transfected with 125 ng pGL3-CYP17-2x57, 25 ng pCMV Tag1-SF1, and 1 ng pRL-TK using Gene Juice (EMD Biosciences, La Jolla, CA). Twenty-four hours after transfection, cells were treated for 6 to 12 h with 1 mM Bt_2cAMP, various PA molecular species (0.1 μM to 10 μM), 5 μM R59949, and/or SPH (01 to 25 μM) and harvested for dual-luciferase assays (Promega, Madison, WI). To determine the effect of mutations in the LBD on SF1-dependent transcriptional activity, cells were subcultured on 12-well plates and 24 h later transfected with pGL3-CYP17 2x57 (93) and wild-type or mutant (H310A, G341K, Y436A, K440E, Y436A/K440E, or G341A/Y436A/K440E) receptor. The effect of DGK overexpression on CYP17 luciferase activity was assessed by transfecting cells with 125 ng pGL3-CYP17-2x57, 1 ng pRL-TK, and 25 ng DGK expression plasmid. The pcDNA3-DGK-α expression plasmid was received from James Walsh (Indiana University-Purdue University Indianapolis, Indianapolis, IN), and expression plasmids for DGK-ε and DGK-ζ were provided by Matthew Topham (University of Utah, Salt Lake City, UT) and Stephen Gee (University of Ottawa, Ottawa, Canada), respectively. Expression plasmids for DGK-δ1, DGK-δ2, DGK-η1, DGK-η2, and DGK-γ were a gift from Fumio Sakane (Sapporo University School of Medicine, Sapporo, Japan), and pCMV6-DGK-θ was obtained from Origene (Rockville, MD). SF1 and DGK mutants were prepared using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and were confirmed by sequencing.

Mammalian two-hybrid assay. SF1, GCN5 (pOZ-N-hGCN5; provided by Yoshihiro Nakatani, Dana Farber Cancer Institute, Harvard University School of Medicine, Boston, MA), and human liver receptor homologue 1 (LRH1) (pCI-LRH1; donated by Matthew Redinbo, University of North Carolina, Chapel Hill, NC) were cloned into the MluI and XbaI sites of the pBIND and pACT vectors (Promega, Madison, WI) as previously described (16). Cells were transfected with the pG5 firefly luciferase reporter in combination with the pBIND-GCN5 and pACT-SF1 or pACT-LRH1 vectors to express fusions of GAL4-GCN5 and VP16-SF1/LRH1, respectively, and an SRC1 expression plasmid (pBKCMV-SRC1; generously provided by Bert O'Malley, Baylor University School of Medicine, Houston, TX). Twenty-four hours later, cells were treated with Bt_2cAMP or PA for 16 h and then harvested for analysis of firefly and *Renilla* luciferase activities.

Real-time RT-PCR. For quantitative reverse transcription-PCR (RT-PCR), total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) and amplified using the One-Step SYBR Green RT-PCR kit (Eurogentec, San Diego, CA) and primers (Table 1) targeted at the gene of interest. Primers were designed using Beacon Designer 5.0 software (Bio-Rad, Hercules, CA). CYP17 mRNA expression was quantified using the delta cycle threshold (Δ*CT*) method normalized to β-actin content. Real-time RT-PCRs for quantifying the expression of DGK isoforms in H295R cells were also resolved by agarose (2%) gel electrophoresis.

DHEA assay. Cells were cultured in 12-well plates and treated with 1 mM Bt_2cAMP, 10 μM dilauroyl PA (di12 PA), or 10 μM dimyristoyl PA (di14 PA) for 24 h. DHEA released into the medium was determined in triplicate against DHEA standards made up in Dulbecco's modified Eagle's/F12 medium using a 96-well plate enzyme-linked immune DHEA assay (Diagnostic Systems Corporation, Houston, TX). Results are expressed as nanomoles per milligram total cellular protein.

ChIP. Chromatin immunoprecipitation (ChIP) assays were carried out by treating H295R cells with 1 mM Bt_2cAMP or 10 μM di14 PA for 1 h. For

temporal ChIP assays (48, 114), H295R cells (subcultured into 100- or 150-mm dishes) were synchronized by treatment with 2.5 μM α-amanitin for 2 h. Cells were washed twice with PBS and then treated with 1 mM Bt_2cAMP and/or 5 μM R59949 for time periods ranging from 30 min to 4 h. Cross-linking was performed by incubating the cells in 1% formaldehyde for 10 min at room temperature with gentle shaking, terminated by the addition of glycine (final concentration, 0.125 M) for 5 min, and the cells harvested into RIPA buffer. Lysates were sonicated four times (10 s each) and centrifuged for 15 min at 13,000 × *g* and 4°C. Fifty microliters supernatant was retained as input. The purified chromatin solutions were precleared with 1 μg rabbit or mouse immunoglobulin G and immunoprecipitated overnight at 4°C on a tube rotator using 5 μg of anti-acetyl histone H4, anti-GCN5, anti-RNA polymerase II (anti-Pol II), anti-SF1 or anti-SRC1, and protein A/G plus (Santa Cruz Biotechnology). All antibodies used in the ChIP assay were obtained from Millipore (Temecula, CA), except for anti-GCN5 (Santa Cruz Biotechnology). The immobilized protein/DNA complexes were subjected to a series of 5-min washes: three times in RIPA buffer, three times in RIPA buffer plus 500 mM NaCl, three times in washing buffer (10 mM Tris-Cl, pH 8, 0.25 M LiCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, and protease inhibitors), and three times in Tris-EDTA buffer, pH 8.0. The cross-links were reversed, and DNA was purified by two phenol-chloroform extractions, followed by ethanol precipitation. Real-time PCR was carried out using 4 μl of output, 1 μl of input (diluted 1:4), the iTaq SYBR Green Supermix (Bio-Rad, Hercules, CA), and the following primer pairs: forward (5′-GGC TGG GCT CCA GGA GAA TCT TTC TTC CAC-3′) and reverse (5′-CGG CAG GCA AGA TAG ACA GCA GTG GAG TAG-3′), which amplify the region of the CYP17 promoter from position −104 to +43. Δ*CT* values of the outputs were normalized to input values. PCRs were also resolved on 2% agarose gels.

DGK activity assay. H295R cells were subcultured on 60-mm dishes and treated for 5 min to 1 h with 1 mM Bt_2cAMP. After the desired treatment time, cells were harvested and centrifuged, and nuclear and cytosolic proteins were isolated using a NE-PER Nuclear and Cytoplasmic extraction kit (Pierce Biotechnology, Rockford, IL) as per the manufacturer's instructions. DGK activity was assayed as previously described (8), using octylglucoside-diacylglycerol (OG-DG) mixed micelles that were prepared by mixing 0.25 mM dioleoylglycerol, 55 mM OG, and 1 mM phosphatidylserine in 1 mM diethylenetriamine penta-acetic acid, pH 7.4, vortexing, and sonicating until the suspension appeared clear. Twenty microliters of mixed micelles were added to 70 μl of assay buffer (100 μM diethylenetriamine penta-acetic acid, pH 7.4, 50 mM imidazole-HCl, 50 mM NaCl, 12.5 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 1 μCi [γ-^{32}P]ATP) and 10 μl of cytoplasmic or nuclear extracts. Reactions were initiated by vortexing for 3 s and sonicating for 5 s and then incubated for 30 min at 25°C. The reactions were terminated by the addition of 1 ml chloroform–methanol–1% perchloric acid (1:2:0.75 [vol/vol]), followed by vortexing. One milliliter 1% perchloric acid–chloroform (1:1 [vol/vol]) was added, and the mixture was centrifuged for 5 min at 1,500 × *g*. The organic phase was washed twice in 1% perchloric acid and then concentrated under a stream of nitrogen and spotted on Silica Gel 60 thin-layer chromatography (TLC) plates (Merck). Plates were developed in chloroform-acetone-methanol-acetic acid-water (10:4:3:2:1 [vol/vol]) and then exposed to a phosphorimager screen. PA was quantified by phosphorimager scanning using a Fluor/Phospho-Imager (Fuji Film) and normalized to the protein concentration in each sample.

Coimmunoprecipitation. H295R cells were transfected with p3xFLAG-CMV-DGK for 48 h and cell lysates isolated and precleared by incubation for 30 min with rabbit immunoglobulin G (Millipore) and protein A/G PLUS-agarose (Santa Cruz Biotechnology). The precleared lysates were immunoprecipitated overnight at 4°C using 5 μg of anti-FLAG M2 (Stratagene) and protein A/G PLUS-agarose. The agarose beads were washed three times with RIPA buffer and three times with PBS and then subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting.

In vitro transcription/translation and glutathione *S*-transferase pulldown. DGKs were expressed in vitro using a TNT T7 Quick Coupled transcription/translation system (Promega, Madison, WI) and [^{35}S]methionine/cysteine (MP Biomedicals, Solon, OH). Immobilized, His-tagged SF1 was expressed in *Escherichia coli* as previously described (109) and incubated with in vitro-translated DGKs in binding buffer (20 mM HEPES, pH 7.9, 100 mM KCl, 1 mM $MgCl_2$, 2 mM dithiothreitol, 10% glycerol, 0.05% Triton X-100, and protease inhibitors) for 2 h at 4°C with rotation. The immobilized receptor was then washed three times with binding buffer and then suspended in SDS-PAGE gel running buffer and boiled, and the eluted proteins were analyzed by electrophoresis. The gels were stained with Coomassie and dried, and radiolabeled proteins were detected by phosphorimaging.

Immunostaining and confocal microscopy. Cells were plated on glass coverslips, and then some cells were incubated for 12 h with 1 μM 7-nitrobenz-2-oxa-1,3-diazol-4-yl–PA and treated with 1 mM Bt_2cAMP. After the desired incubation time, coverslips were washed twice with PBS and fixed by incubation in 3.7% formaldehyde for 20 min at room temperature. Coverslips were then permeabilized in 0.2% Triton X-100 for 5 min and blocked for 1 h in 1% BSA. Samples were incubated with anti-DGK-θ (BD Biosciences), anti-DGK-ζ (Abgent), or anti-SF1 (Millipore) diluted 1:200 in 1% BSA for 1 h, followed by incubation with secondary fluoroscein isothiocyanate- or rhodamine-conjugated antibodies (Santa Cruz Biotechnology) for 1 h. Coverslips were mounted onto slides using Fluoromount G (Southern Biotechnology Associated, Inc., Birmingham, AL), dried for 5 min, and imaged. Confocal images were collected using a laser scanning microscope (LSM 510; Zeiss, Inc., Thornwood, NY) equipped with argon and helium-neon lasers with excitation wavelengths of 488 and 543 nm for fluoroscein isothiocyanate and rhodamine, respectively.

RNA interference. Cells were subcultured in 12-well plates and 24 h later transfected with 100 nM of small interfering RNA (siRNA) oligonucleotides directed against DGK-θ using siIMPORTER (Millipore). Cells were transfected for 72 h (DGKs) with siRNA oligonucleotides and either harvested for SDS-PAGE and Western blotting or incubated for an additional 12 h with 1 mM Bt_2cAMP for quantification of CYP17 mRNA expression by real-time RT-PCR.

Western blotting. Cells were harvested into RIPA buffer and lysed by sonication (one 5-s burst) followed by incubation on ice for 30 min. Lysates were centrifuged for 15 min at 4°C and the supernatant collected for analysis by SDS-PAGE. Aliquots of each sample (25 μg of protein) were run on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Pall Corporation, Pensacola, FL). Blots were probed with anti-DGK-θ (BD Biosciences), anti-DGK-ζ (Abgent), and anti-p54^{nrb} (BD Biosciences), and expression was detected using an ECF Western blotting kit (Amersham Biosciences, Piscataway, NJ) and visualized by scanning blots on a Fluorescence/Phospho-Imager (Fuji Film, Japan).

Statistics. One-way analysis of variance and Tukey's multiple-comparison tests were performed using GraphPad Prism 4.03 software (GraphPad Software, Inc., San Diego, CA).

RESULTS

Phospholipids are endogenous adrenal SF1 ligands. Using mass spectrometry, we recently demonstrated that SPH binds to SF1 and that this binding to the receptor is modulated by cAMP, where Bt_2cAMP stimulation activated CYP17 mRNA expression by decreasing the amount of SPH bound to the receptor (109). The antagonistic actions of SPH on SF1 function, coupled with the decreased SPH binding to the receptor upon stimulation with Bt_2cAMP, suggested that activation of *CYP17*, and perhaps other SF1 target genes, requires the exchange of SPH for an activating ligand. Moreover, since both crystallographic analysis (55, 63, 112) and our previous in vitro competition assays (109) revealed that SF1 binds to phospholipids, we performed mass spectrometric analysis of SF1 purified from H295R human adrenocortical cells to determine if phospholipids bind to the endogenous receptor. As shown in Fig. 1, spectral analysis of SF1 immunoprecipitated from control (panel A) or Bt_2cAMP-treated (panel B) cells showed that the endogenous receptor binds to phospholipids. The major peak detected from SF1 isolated from both control and Bt_2cAMP-treated cells was a PA molecule containing acyl chains with a total of 26 carbons and one double bond (m/z = 560.8). Three other PA molecules, 30:0 PA (m/z = 618.8), 35:5 PA (m/z = 678.6), and 40:4 PA (m/z = 736.8), were also bound to the receptor. Additional phospholipid species detected were 20:3 PE (m/z = 502.8) and 34:4 PIP (794.7). Based on our previous studies showing decreased SPH bound to SF1 isolated from Bt_2cAMP-stimulated cells (109), we predicted that the amounts of phospholipids bound to the receptor would increase in response to Bt_2cAMP. Although we did observe an

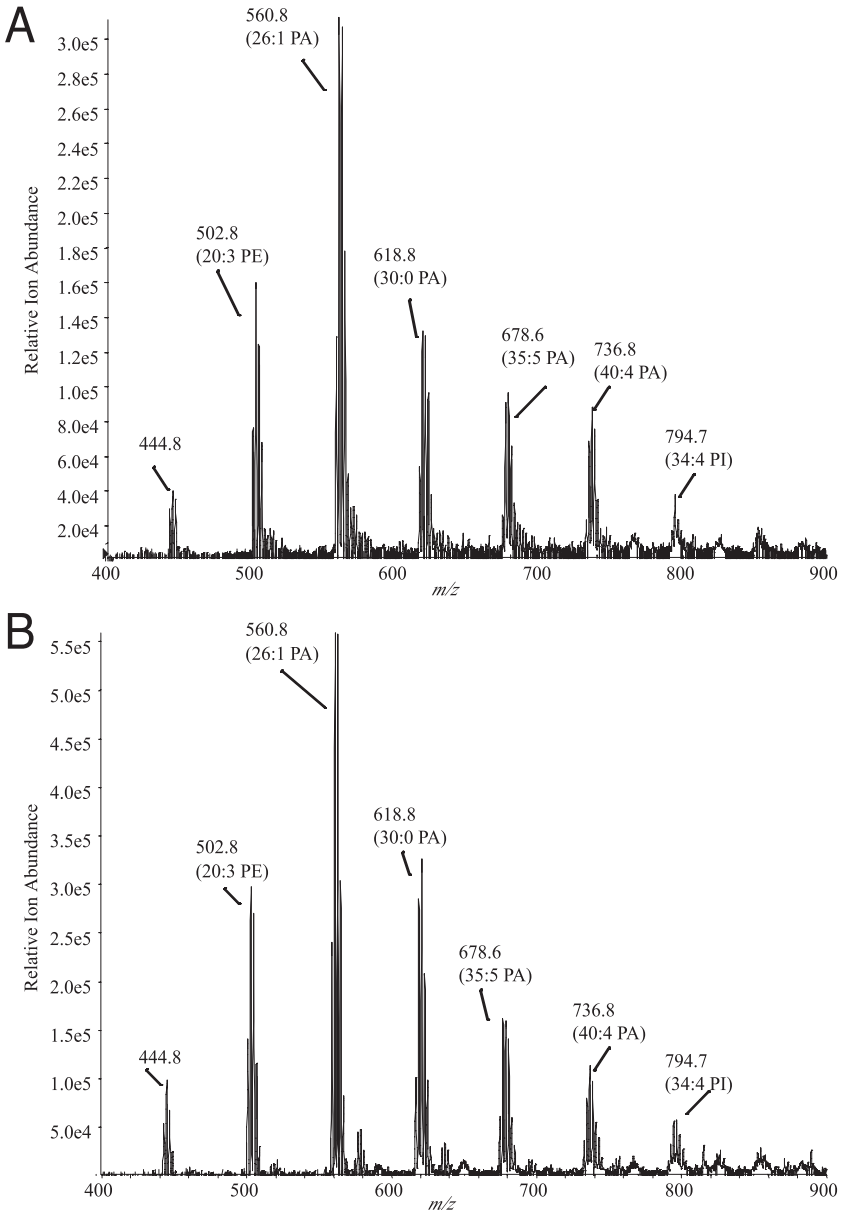

FIG. 1. Identification of endogenous phospholipid ligands for SF1. H295R cells were transfected with pCR3.1-SF1 and treated for 1 h with 1 mM Bt_2cAMP. Control (A) or Bt_2cAMP-treated (B) lysates were immunoprecipitated with anti-SF1 antibody and phospholipids bound to the receptor extracted and analyzed by mass spectrometry.

approximately 1.8-fold increase in both 26:1 PA and 20:3 PE and a 2.5-fold increase in the amount of 30:0 PA bound to the receptor purified from Bt_2cAMP-treated cells, the magnitude of these increases, in the absence of internal standards, was not large enough to conclude with confidence that cAMP promoted a statistically significant increase in PA binding to the receptor. We did not observe any Bt_2cAMP-stimulated changes in the amounts of 40:4 PA or 34:4 PIP bound to SF1. In control immunoprecipitations, we also purified the cAMP response element binding protein; however, we were unable to detect phospholipids bound to this transcription factor (data not shown). We also did not detect phospholipids bound to protein A/G in the absence of SF1 (data not shown). Although these data confirm that SF1 binds to phospholipids in human adrenocortical cells, it is possible that more global mass spectrometric analyses for all classes of lipids would reveal other molecules that bind to the receptor. However, based on these mass spectrometric findings, we carried out further studies to determine the functional significance of PA in mediating increased *CYP17* gene expression and cortisol biosynthesis in H295R cells.

PA activates SF1-dependent CYP17 transcriptional activity and endogenous CYP17 mRNA expression. To determine the effect of PA on SF1-dependent *CYP17* reporter gene activity, cells were transfected with a plasmid containing a tandem repeat of the first 57 base pairs of the human CYP17 promoter

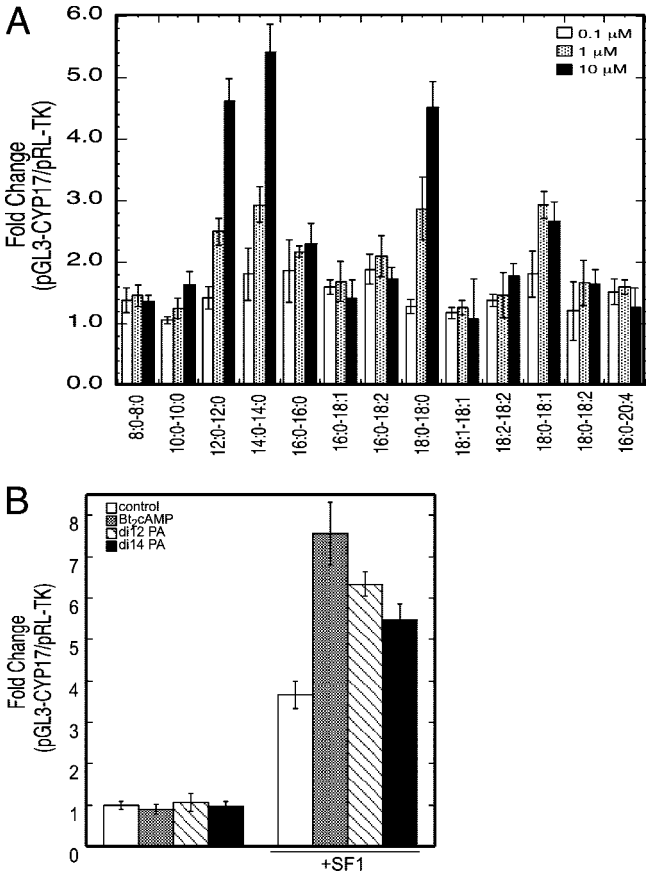

FIG. 2. Multiple PA molecular species activate SF1-dependent CYP17 transcriptional activity. (A) H295R cells were transfected with pGL3-CYP17-2x57 and then treated with various PA species (0.1 μM to 10 μM) for 16 h. Data graphed are normalized to *Renilla* activity (pRL-TK) and expressed as *n*-fold increases of pGL3-CYP17-2x57 activity over pRL-TK activity of the untreated control. Data represent means ± standard errors of means from four separate experiments, each performed in triplicate. (B) Jeg3 cells were transfected with pGL3-CYP17-2x57, pRL-TK, and pCMVTag1-SF1 using Gene Juice. Twenty-four hours after transfection, the cells were treated with 1 mM Bt_2cAMP, 10 μM di12 PA, or 10 μM di14 PA for 16 h. Cells were lysed and firefly and *Renilla* luciferase activities quantified by luminometry. Data graphed represent means ± standard errors of means from two experiments that were each performed in triplicate and are normalized to *Renilla* activity.

fused to the luciferase gene (pGL3-CYP17-2x57) and treated with increasing concentrations of different PA molecular species that contained acyl chains from 8 to 20 carbons and different degrees of unsaturation. As shown in Fig. 2A, most PA molecules were able to activate *CYP17* reporter gene activity; however, 10 μM di14 PA had the greatest stimulatory effect on luciferase activity and resulted in a 5.4-fold increase in reporter gene transcription. di12 PA also significantly stimulated CYP17 transcriptional activity in a dose-dependent manner, with the 10 μM concentration increasing luciferase activity by 4.6-fold. PA molecules with acyl chains shorter than 12 carbons were not as effective in stimulating reporter gene activity. Interestingly, 10 μM di18 PA stimulated *CYP17* reporter gene activity by 4.5-fold. Based on structural studies by Li et al. demonstrating that C_{18} fatty acids destabilize the active conformation of the receptor (63), this finding was unexpected. However, consistent with the published findings of Li et al. (63), other PA species with 18 carbon acyl chains, such as di18:1 and di18:2, were unable to significantly activate *CYP17* reporter gene activity. Moreover, the decrease in luciferase activity observed in the asymmetric PA molecules that contain one 18-carbon acyl chain (16:0 to 18:1 and 16:0 to 18:2) compared to the di16 PA further supports an inhibitory effect of phospholipids with at least one 18-carbon acyl chain. To determine if SF1 was essential for the stimulatory effects of PA on *CYP17* reporter gene expression, we repeated transient-transfection assays with the Jeg3 cell line, which lacks endogenous SF1. In the absence of transfected receptor, neither Bt_2cAMP nor PA was able to increase CYP17 luciferase activity; however, transfection of an SF1 expression plasmid conferred increased reporter gene activity in response to both Bt_2cAMP and PA (Fig. 2B). Although these studies demonstrate that activation of *CYP17* reporter gene activity requires SF1, it is possible that a metabolite of PA may be activating the receptor. Of note, Bt_2cAMP stimulated human LRH1-dependent *CYP17* reporter gene activity; however, the receptor was not responsive to PA (data not shown).

We next carried out studies to characterize the effect of di12 PA and di14 PA on endogenous CYP17 mRNA expression by treating H295R cells for 16 h with either 1 mM Bt_2cAMP or 10 μM PA and quantifying *CYP17* gene expression by using real-time RT-PCR. Bt_2cAMP induced CYP17 mRNA expression by 7.2-fold (Fig. 3A). Both PA species significantly increased CYP17 mRNA expression, with di12 PA increasing CYP17 mRNA expression by 3.3-fold and di14 PA inducing expression by 5.1-fold. However, the magnitude of the stimulatory effect of these phospholipids on CYP17 mRNA content was not as large as that evoked by Bt_2cAMP, suggesting that in addition to increasing ligand availability, Bt_2cAMP promotes other cellular events that are integrated with ligand binding to result in maximal CYP17 transcription.

To determine if the effects of PA on transcription were specific to *CYP17* or if this phospholipid activated other steroidogenic genes, we quantified the mRNA expression of CYP11A1, CYP11B1/2, CYP21, and 3β hydroxysteroid dehydrogenase (3βHSD). As shown in Fig. 3B, mRNA expression of all genes examined was induced by Bt_2cAMP and di14 PA. Although di12 PA did not have a statistically significantly effect on the mRNA expression of 3βHSD, these experiments suggest that PA activates multiple SF1 targets involved in steroid hormone biosynthesis in the human adrenal cortex.

PA stimulates complex formation. We have recently found that cAMP promotes the assembly of a trimeric complex comprised of SF1, GCN5, and steroid receptor coactivator 1 on the CYP17 promoter (16). Thus, we sought to determine the effect of di14 PA on the formation of this complex by carrying out mammalian two-hybrid assays. H295R cells were transfected with expression plasmids for VP16-SF1 or VP16-LRH1 and GAL4-GCN5 and then treated with Bt_2cAMP and di14 PA for 16 h.

Both Bt_2cAMP and di14 PA (10 μM) significantly stimulated the binding of SF1 to GCN5, and cotransfection of full-length SRC1 potentiated the interaction between SF1 and GCN5 in untreated cells (Fig. 3C). Trimer formation was further stimulated in response to both Bt_2cAMP and di14 PA.

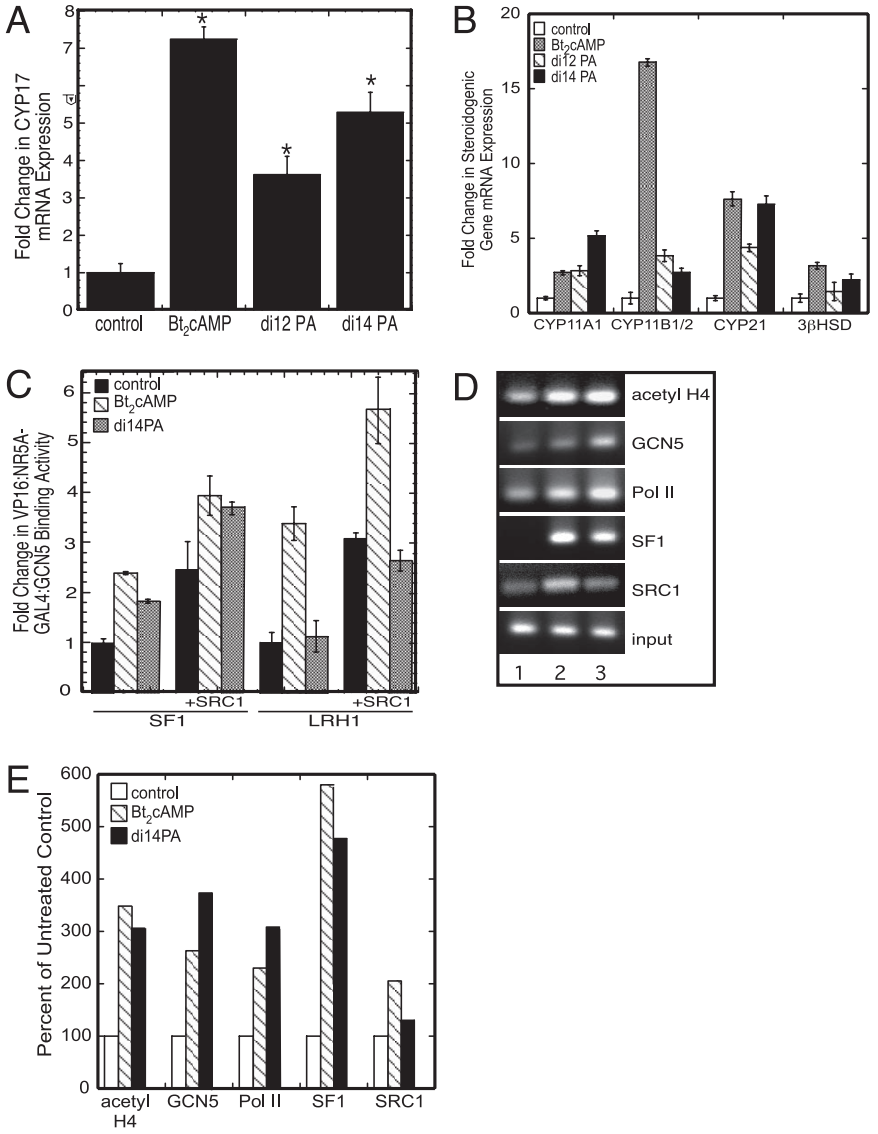

FIG. 3. PA increases coactivator binding to SF1 and CYP17 mRNA expression. (A) H295R cells were treated for 16 h with 1 mM Bt_2cAMP, 10 μM di12 PA, or 10 μM di14 PA and total RNA extracted for analysis of CYP17 mRNA expression by quantitative RT-PCR. CYP17 expression is normalized to β-actin content, graphed as *n*-fold change over level for untreated control, and represents the mean ± standard error of the mean for three separate experiments, each performed in quadruplicate. (B) Total RNA was isolated from H295R cells treated for 16 h with 1 mM Bt_2cAMP, 10 μM di12 PA, or 10 μM di14 PA. The mRNA expression of other enzymes in the steroid hormone biosynthetic pathway was determined by quantitative RT-PCR as described in Materials and Methods using PCR primers (Table 1) designed to amplify CYP11A1, CYP11B1/2, CYP21, 3βHSD, and StAR. Data graphed represent the means ± standard errors of the means for two separate experiments, each performed in triplicate, and steroidogenic gene expression normalized to β-actin mRNA expression levels. (C) Mammalian two-hybrid experiments were carried out by transfecting H295R cells with pG5-Luc, pACT-GCN5, pBK-SRC1, and pBIND-SF1 or pBIND-LRH1 and then treating with 1 mM Bt_2cAMP or 10 μM di14 PA for 16 h. Dual-luciferase assays were carried out on cell lysates and pG5-Luc activity normalized to *Renilla* activity (pAct-GCN5). Data graphed represent the means ± standard errors of the means for three separate experiments, each performed in quadruplicate. (D) H295R cells were treated for 1 h with 1 mM Bt_2cAMP or 10 μM di14 PA and subjected to ChIP as described in Materials and Methods. Purified DNA was amplified by quantitative PCR and the samples resolved on a 2% agarose gel. Results from a representative experiment are shown. Lane 1, control; lane 2, Bt_2cAMP; lane 3, di14 PA. (E) Quantification of real-time PCR Δ*CT* values obtained from ChIP assays. Lysates were immunoprecipitated with antibodies against acetyl histone H4, GCN5, Pol II, SF1, and SRC1. Data graphed in all panels represent the means from two experiments, each performed in duplicate. Outputs are normalized to Δ*CT* values obtained for 1% input controls, and results are presented as percentages of Δ*CT* values for untreated control cells.

Since LRH1 is also a member of the NR5A subfamily of NRs, we also determined the effect of di14 PA on the association of human LRH1 with GCN5. Bt_2cAMP stimulated the binding of LRH1 to GCN5, while di14 PA had no effect on this interaction (Fig. 3C).

PA increases SF1 and RNA Pol II binding to CYP17 promoter. Based on previous studies demonstrating that ACTH and cAMP promote the association of SF1, RNA Pol II, GCN5, and SRC1 with the CYP17 promoter (16), we treated H295R cells with either Bt_2cAMP or di14 PA and assessed

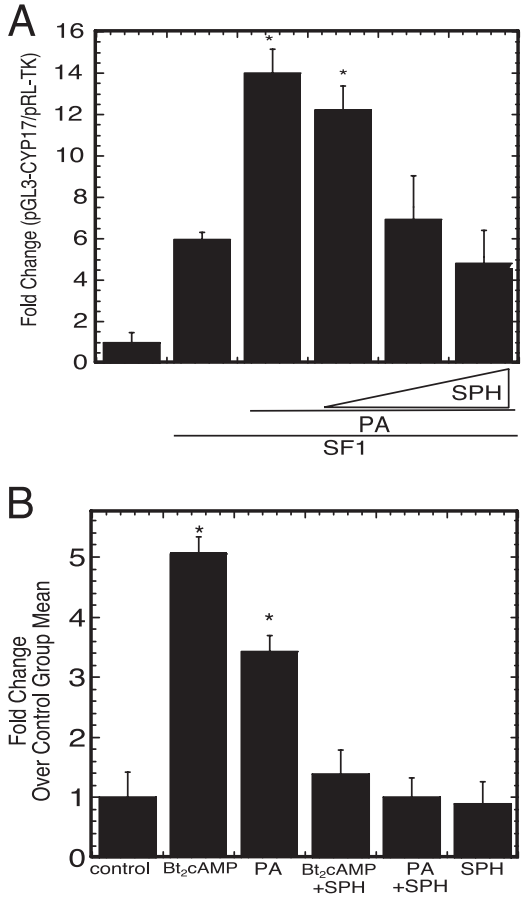

FIG. 4. SPH antagonizes PA-stimulated CYP17 luciferase activity and mRNA expression. (A) Jeg3 cells were transfected with pGL3-CYP17-2x57, pCR3.1-SF1, and pRL-TK and treated with 10 μM di14 PA in the presence or absence of SPH (1, 10, or 25 μM) for 16 h. Data graphed are normalized to *Renilla* activity and are expressed as *n*-fold increases in pGL3-CYP17-2x57 activity in untreated SF1 null samples. Means ± standard errors of the means for three separate experiments, each performed in triplicate, are graphed. *, $P < 0.05$, statistically different from untreated SF1-expressing control samples. (B) H295R cells were treated for 12 h with 1 mM Bt_2cAMP, 10 μM di14 PA, and 5 μM SPH and total RNA extracted for analysis by real time RT-PCR. CYP17 mRNA expression is normalized to β-actin mRNA content, and data graphed are expressed as *n*-fold change from levels for the untreated control group. The asterisk denotes statistical difference from the untreated control group ($P < 0.05$).

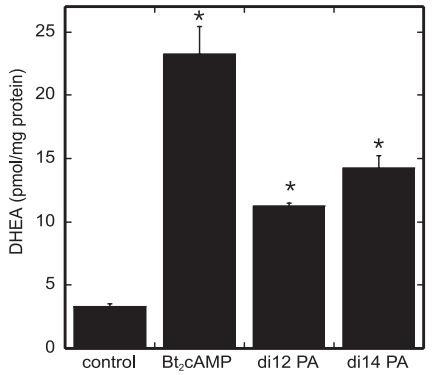

FIG. 5. PA stimulates DHEA release from H295R cells. Cells were treated for 24 h with 1 mM Bt_2cAMP or 10 μM di14 PA and the amount of hormone released into the medium quantified by enzyme-linked immune assay. DHEA concentrations are normalized to the protein content of each sample. The asterisk denotes statistical difference from the untreated control group ($P < 0.05$).

both the acetylation state and the occupancy of the promoter by ChIP. As shown in Fig. 3D, exposure of cells to Bt_2cAMP (lane 2) or di14 PA (lane 3) for 1 h promoted the binding of SF1, RNA Pol II, GCN5, and SRC1 to the CYP17 promoter. Moreover, both Bt_2cAMP and PA increased the acetylation of histone H4 at the SF1 binding site, providing further evidence for PA as an endogenous agonist for SF1. Quantification of the changes in promoter occupancy by real-time PCR revealed that both Bt_2cAMP and di14 PA increased the binding of SF1 to the receptor by 5.7- and 4.8-fold, respectively (Fig. 3E). As previously demonstrated with Bt_2cAMP (16), di14 PA also increased the acetylation of histone H4 and stimulated the recruitment of GCN5 and RNA Pol II at the −104/+43 region of the CYP17 promoter. Interestingly, di14 PA was not as effective as Bt_2cAMP in increasing SRC1 binding to the promoter, where Bt_2cAMP increased SRC1 binding by 2.1-fold whereas di14 PA stimulated SRC1 recruitment by only 1.4-fold.

SPH antagonizes PA-stimulated *CYP17* reporter gene activity, coactivator recruitment, and mRNA expression. Since SPH is an SF1 antagonist (109), we hypothesized that SPH would inhibit the ability of PA to activate SF1-dependent CYP17 transcriptional activity. To test this hypothesis, the SF1 null, steroidogenic Jeg3 cell line was transfected with pGL3-CYP17-2x57, pRL-TK, and pCMVTag1-SF1 and then treated for 16 h with Bt_2cAMP, di14 PA, and SPH. As shown in Fig. 4A, increasing concentrations (1 to 25 μM) of SPH attenuated the stimulatory effect of di14 PA on CYP17 transcriptional activity. The effects of Bt_2cAMP, PA, and SPH were dependent on the presence of SF1, because the CYP17 reporter plasmid was unresponsive in the absence of the transfected receptor in the Jeg3 cell line (Fig. 2B; also data not shown). Moreover, although transfection of human LRH1 supported Bt_2cAMP-stimulated *CYP17* reporter gene activity, PA had no effect on the ability of human LRH1 to activate luciferase expression (data not shown). We also carried out studies to determine the effect of SPH on PA- or Bt_2cAMP-stimulated CYP17 mRNA expression. H295R cells were treated with Bt_2cAMP or di14 PA in the presence or absence of SPH for 12 h, and CYP17 mRNA was quantified by real time RT-PCR. Both Bt_2cAMP and di14 PA increased CYP17 mRNA expression; however, SPH inhibited the induction of CYP17 mRNA expression in response to both of these stimuli (Fig. 4B).

PA stimulates DHEA secretion. To examine the functional consequence of PA-mediated receptor activation, we determined the effect of di14 PA on the ability of the H295R cells to secrete DHEA into the medium. As shown in Fig. 5, after 24 h of incubation with Bt_2cAMP, DHEA concentrations were increased by 8.2-fold over those for the untreated control cells. Both the di12 and di14 PA species also stimulated hormone production; however, the magnitude of the increases was not as large as that elicited by Bt_2cAMP, suggesting that maximal steroid hormone production is multifactorial and requires the activation of additional regulatory pathways. Moreover, given

that DHEA secretion can be modulated nongenomically, it is also possible that PA may activate DHEA biosynthesis via other pathways.

Effect of mutations in LBD on *CYP17* reporter gene activity. Since structural studies identified key residues that make direct contact with the ligand (55, 63), we next characterized the effect of mutations in the LBD of SF1 on *CYP17* reporter gene expression by engineering SF1 expression plasmids containing single mutations (H310A, G341A, G341E, K440A, K440E, or Y436A), a double mutant (G341W/Y436W), and a triple mutant (G341W/Y436W/K440W). Residues G341, Y436, and K440 are at the entry of the ligand binding pocket and were found to be crucial for phospholipids binding by coordinating the phosphate head group (55), and amino acid H310 lies on the inner surface of the pocket, potentially making contact with the acyl chains of the lipid. The double and triple tryptophan mutants were engineered to occlude the entryway to the ligand binding pocket. Cells were transiently transfected with plasmids containing wild-type or mutant SF1 and pGL3-CYP17-2x57 and treated for 16 h with 10 μM di14 PA. As shown in Fig. 6A, transfection of wild-type SF1 resulted in a 4.2-fold increase in luciferase activity, which was further stimulated by di14 PA. All mutations in the ligand binding region of SF1, except for Y436A and K440E, resulted in decreased reporter gene activity compared to that of the wild type in untreated cells. For the G341E, K440E, G341W/Y436W double and G341W/Y436W/K440W triple mutants, reporter gene activity decreased to levels below the activity observed in cells lacking SF1; coordination of the phosphate head group is critical for optimal transactivation potential (Fig. 6A). It is likely that the introduction of a negative charge (G341E and K440E) prevents coordination and stabilization of the phosphate head group, thereby preventing receptor activation. Additionally, since wild-type and mutant receptors were expressed at comparable levels (Fig. 6B), the significant decrease in reporter gene activity in cells transfected with double and triple tryptophan mutants that were generated to occlude the ligand binding pocket entryway supports the premise that ligand binding is essential for SF1-dependent CYP17 transcription. Further, it is possible that mutation of these residues sterically impedes the dynamic rearrangements that are required for cAMP-stimulated ligand binding. Finally, since cells expressing the G341E mutation lost SF1-dependent activation of the *CYP17* reporter gene (Fig. 6A), we assessed the effect of this mutation on the ability of the receptor to bind phospholipids in the H295R cell line. Cells were transfected with FLAG-tagged wild-type or G341E mutant receptor for 48 h, and the amount of phospholipid bound to the receptor was determined by mass spectrometry, as done for Fig. 1. Wild-type receptor retained the ability to bind to phospholipids, particularly PA (*m*/*z* 560.9); however, the amount of phospholipids bound to the G341A mutant was substantially reduced (Fig. 6C).

DGK activity is required for SF1/cAMP-dependent CYP17 transcription. PA can be produced in several ways: phospholipase D (PLD)-catalyzed hydrolysis of PC, acylation of lysophosphatidic acid, or phosphorylation of diacylglycerol by DGK. To determine the effect of reducing cellular pools of PA, cells were treated with R59949 (18, 47, 50) to inhibit DGK activity. As shown in Fig. 7A, R59949 attenuated the ability of SF1 to increase *CYP17* reporter gene activity. Since R59949 has been shown to inhibit choline/ethanolamine phosphotransferase 1 activity in vitro (116), we repeated reporter gene assays to determine if PA could rescue the inhibitory effect of R59949 on luciferase activity. Cells were treated with 5 μM R59949 in the presence and absence of Bt_2cAMP, di14 PA, di14 PC, di14 PE, or PIP_3 [di8 PI (3,4,5)P_3]. As shown in Fig. 7B, only di14 PA reversed the effects of R59949 on *CYP17* reporter gene activity, suggesting that DGK activity is required for SF1 activation. We also determined the effect of inhibiting DGK activity on CYP17 mRNA expression and found that R59949 attenuated the ability of Bt_2cAMP to stimulate transcription of the endogenous *CYP17* gene (Fig. 7C). Studies were also carried out to determine if PLD-catalyzed biosynthesis of PA was important for SF1-dependent CYP17 transcription by treating transfected cells with 0.1% 1-butanol (24); however, as also shown for CYP19 (55), this PLD inhibitor had no significant effect on cAMP/SF1-dependent CYP17 luciferase activity or mRNA expression (data not shown).

We next determined if DGK activity was required for the cyclic interaction of SF1 with the CYP17 promoter (16) by quantifying receptor occupancy at 30-min intervals by performing temporal ChIP assays using α-amanitin-synchronized H295R cells that were treated for 30 min to 240 min with Bt_2cAMP. In agreement with our previous findings (16), Bt_2cAMP stimulates the recruitment of SF1 to the CYP17 promoter within 30 min, with peak receptor occupancy occurring at 60 and 180 min (Fig. 7D), where peaks in the binding of SF1 to CYP17 indicate a stochastic time for maximal recruitment to the promoter within the population of synchronized cells (36). Analogous to the inhibitory effects on SF1 binding elicited by SPH (109), cotreatment with R59949 attenuated the first cycle of SF1 enrichment on the CYP17 promoter and significantly decreased promoter occupancy by SF1 in the second cycle (Fig. 7D), suggesting that PA binding is required for transcriptional initiation.

cAMP stimulates nuclear DGK catalytic activity. Many studies have demonstrated roles for nuclear DGK activity in varied cellular processes. For example, DGK-θ is activated by nerve growth factor in PC12 cells (101) and thrombin in IIC9 fibroblasts (8, 107), DGK-ζ promotes myogenesis in C2C12 cells (23), and DGK-γ plays a role in regulating the cell cycle in CHO-K cells (72). Interestingly, both DGK-θ (102) and DGK-ζ (23) are localized in the speckle domains of the nucleus. Nuclear speckles are punctate structures that are enriched in pre-mRNA splicing factors and have been shown to sequester posttranslationally modified SF1 (11, 12). We postulated that cAMP-stimulated PA production and binding to SF1 occurs at these sites of transcriptional inactivity. Thus, we determined the effect of activation of the ACTH/cAMP signaling pathway on nuclear DGK activity. H295R cells were treated for 5 to 120 min with Bt_2cAMP and the isolated nuclear and cytosolic fractions incubated with radiolabeled phosphate and diacylglycerol using an OG-DG mixed micelle assay (8). Radiolabeled PA was resolved by TLC and quantified by phosphorimager scanning and densitometric analysis. As shown in Fig. 8A, Bt_2cAMP rapidly increased DGK activity and the amount of generated PA in the nucleus. This increase in PA peaked at 5 min, remained elevated for 60 min, and then returned to basal levels at the 120-min time point (Fig. 8B). No

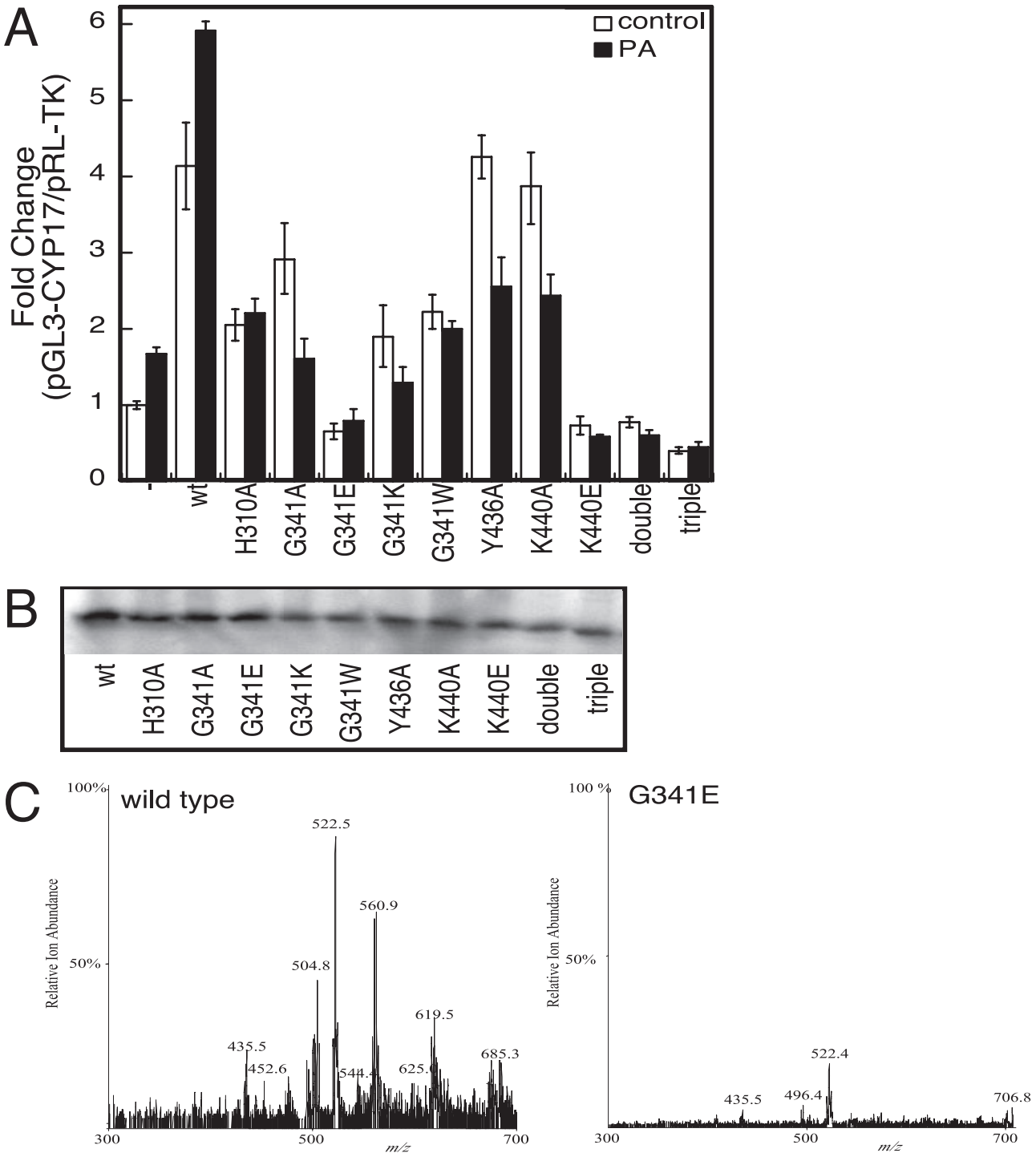

FIG. 6. Mutagenesis of amino acids in the LBD decreases the transactivation potential of SF1. (A) Cells were transfected with pGL3-CYP17-2x57, pRL-TK, and wild-type or mutant SF1 expression plasmids (cloned into the pCMVTag1 vector) and treated with 10 μM di14 PA for 16 h. Luciferase activity in cell lysates was analyzed by luminometry. Data graphed are normalized to *Renilla* activity and expressed as *n*-fold increases in pGL3-CYP17-2x57 activity in untreated SF1 null samples. Data shown are the means ± standard errors of the means for at least three separate experiments, each performed in triplicate. (B) H295R cells were transfected with 100 ng of expression plasmid for wild-type or mutant FLAG-tagged receptor for 48 h and Western blotting performed on lysates. (C) Cells were transfected with 25 μg pCMVTag1-SF1 (left) or pCMVTag1-SF1-G341E (right). Forty-eight hours after transfection, lysates were isolated and immunoprecipitated with anti-FLAG antibody and phospholipids bound to the receptor extracted and analyzed by mass spectrometry.

significant changes were detected in the amount of PA produced in the cytoplasm (data not shown).

DGK overexpression increases basal and cAMP-stimulated *CYP17* reporter gene activity. To date, 10 mammalian DGK isozymes, α (91, 92), β (29), γ (26, 51), δ (88), ε (103), ζ (10, 28), η (53), θ (38), ι (21), and κ (41), which all contain cysteine-rich, zinc finger-like structures and a conserved catalytic region, have been identified (52, 90, 105, 110). H295R cells

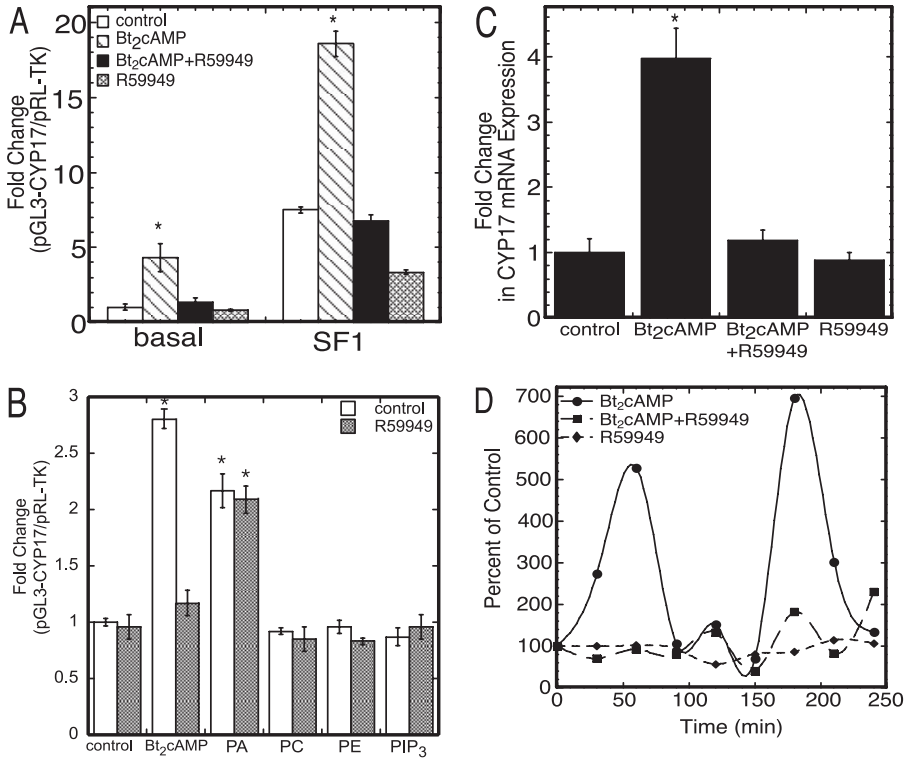

FIG. 7. DGK activity is required for cAMP-stimulated CYP17 transcription. (A) H295R cells were transiently transfected with pGL3-CYP17-2x57, pRL-TK, and pCMVTag1-SF1 and then treated with Bt_2cAMP (1 mM) in the presence or absence of R59949 (5 μM) for 16 h. Data are normalized to the luciferase activity of the *Renilla* gene and expressed as the *n*-fold increase over luciferase activity in the untreated control cells. Data shown are the means ± standard errors of the means for four separate experiments, each performed in triplicate. *, statistically different from untreated control; $P < 0.05$. (B) H295R cells were transfected with pGL3-CYP17-2x57 and pRL-TK. Twenty-four hours later, cells were pretreated for 30 min with 5 μM R59949, followed by treatment with 1 mM Bt_2cAMP, 10 μM di14 PA, 10 μM di14 PC, 10 μM di14 PE, or 10 μM di8 $PI(3,4,5)P_3$ for 16 h. Luciferase activity was quantified in the isolated cell lysates by luminometry, where data are normalized to the luciferase activity of the *Renilla* gene and expressed as the *n*-fold increase over luciferase activity in the untreated control cells. Data shown are the means ± standard errors of the means for two separate experiments, each performed in triplicate. (C) Cells were treated for 12 h with 1 mM Bt_2cAMP and/or 5 μM R59949 and total RNA extracted for quantification of CYP17 mRNA expression by real-time RT-PCR. CYP17 mRNA expression is normalized to β-actin mRNA content, and data graphed are expressed as *n*-fold changes from results for the untreated control group, where an asterisk denotes statistical difference from the control group. (D) α-Amanitin-synchronized cells were treated with Bt_2cAMP and/or R59949 for 30 min to 4 h and cross-linked in 1% formaldehyde and the sheared chromatin immunoprecipitated with an antibody against SF1. DNA bound to the immobilized receptor was purified and SF1 binding to the CYP17 promoter (−104/+43) quantified by real-time PCR as described in Materials and Methods. Data graphed represent means from two experiments, each performed in duplicate. Outputs are normalized to Δ*CT* values at each time point.

express seven isoforms (Fig. 8C), with DGK-δ and DGK-η expressed at the highest levels, DGK-ι and DGK-ζ expressed at low levels, and DGK-β and DGK-κ not detectable. Based on the expression patterns of DGK isoforms in H295R cells, we focused on characterizing the roles of DGK-α, DGK-γ, DGK-δ, DGK-ε, DGK-η, DGK-θ, and DGK-ζ in cAMP-dependent CYP17 transcription. H295R cells were transfected with pGL3-CYP17-2x57, pCR3.1-SF1, and different DGK isoforms. Overexpression of most DGKs tested increased *CYP17* reporter gene activity between two- and threefold in both the absence and presence of Bt_2cAMP (Fig. 8D). Significantly, cotransfection of SF1 and either DGK-δ1, DGK-δ2, DGK-θ, or DGK-ζ resulted in a synergistic increase in luciferase activity in response to Bt_2cAMP, with DGK-θ and DGK-ζ eliciting the greatest stimulatory response. This synergism between SF1 and certain DGKs, specifically DGK-θ and DGK-ζ, led us to hypothesize that some of the isoforms may have unique structural properties that may facilitate the binding of PA to the receptor and the induction of *CYP17* gene transcription.

SF1 binds to DGK-θ and DGK-ζ in vitro and in vivo. To further explore the mechanism by which SF1 and DGKs synergistically stimulate *CYP17* reporter gene activity (Fig. 8D), we first carried out in silico analysis of the primary sequences of DGKs for the presence of NR boxes or LXXLL motifs. These leucine-rich motifs regulate transcriptional activation by enabling coregulatory proteins to bind to NRs (17, 34, 74, 77). Several putative LXXLL motifs are present in all of the DGK isoforms that are expressed in H295R cells, except DGK-ι (Fig. 9A). DGK-α, DGK-γ, DGK-η, and DGK-ζ contain one putative SF1-interacting motif, while DGK-δ, DGK-ε, and DGK-θ contain two putative binding sites. To determine if SF1 interacts with DGKs in the H295R adrenocortical cell line, we performed immunoprecipitation assays and found that the receptor also binds to both DGK-θ and DGK-ζ and this binding

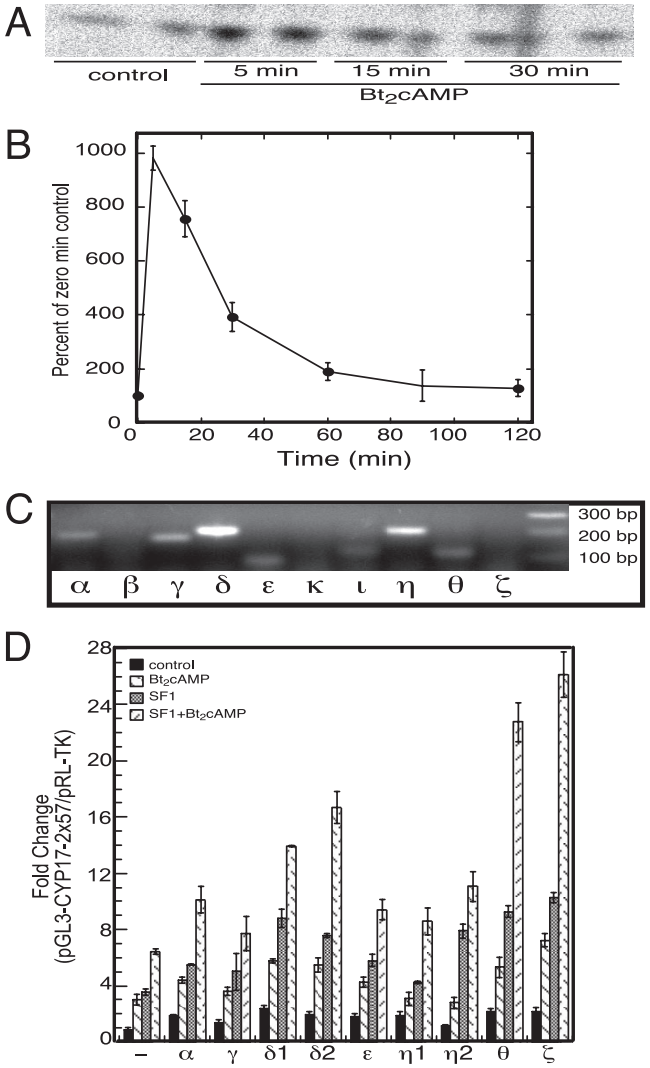

FIG. 8. cAMP increases DGK activity, and SF1 synergizes with DGK to stimulate CYP17 transcriptional activity. (A and B) H295R cells were treated for 5 min to 2 h with 1 mM Bt_2cAMP and nuclear extracts purified for analysis of DGK activity using the OG-DG mixed-micelle assay (see Materials and Methods). Radiolabeled PA produced was resolved by TLC (representative plate shown in panel A) and quantified by phosphorimager scanning and densitometry (panel B). (C) Expression of DGK isoforms in H295R cells was determined by real-time RT-PCR of control RNA (50 ng) and the PCRs resolved on a 2% agarose gel. (D) Cells were transfected with pGL3-CYP17-2x57, pRL-TK, pCR3.1-SF1, and DGK expression plasmids and then treated for 16 h with 1 mM Bt_2cAMP. Data graphed are normalized to *Renilla* activity (pRL-TK) and expressed as *n*-fold increases of pGL3-CYP17-2x57 activity over pRL-TK activity of the untreated control group. Data shown represent the means ± standard errors of the means from at least three separate experiments, each performed in triplicate.

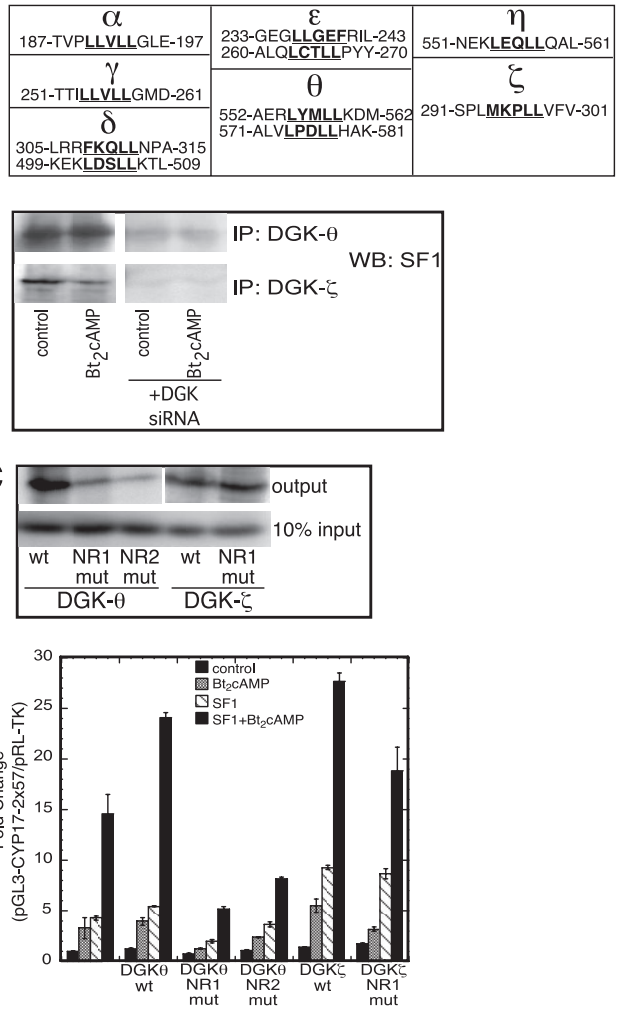

FIG. 9. DGK binds to SF1. (A) Alignment of LXXLL motifs in human DGK isoforms. (B) Cells were transfected with expression plasmids containing FLAG-tagged DGK-θ or DGK-ζ and pCDNA3.1-SF1 (Myc-tagged) and treated for 1 h with 1 mM Bt_2cAMP. Lysates were immunoprecipitated with anti-FLAG antibody and protein A/G Sepharose. SDS-PAGE and Western blotting with an anti-Myc antibody were used to detect SF1 bound to the immunoprecipitated kinases. Some cells were first transfected with siRNA oligonucleotides against DGKθ or DGKζ for 72 h and treated for 1 h with 1 mM Bt_2cAMP, and then cell lysates were isolated and immunoprecipitated using either anti-DGK-θ or anti-DGK-ζ antibodies. Purified proteins were subjected to SDS-PAGE and Western blotting with an anti-SF1 antibody. (C) Immobilized SF1 was incubated with in vitro-translated, radiolabeled wild-type or mutant DGK-θ or DGK-ζ, and the reaction mixtures were washed and subjected to SDS-PAGE. Acrylamide gels were stained, dried, and exposed to a phosphorimager screen. Binding was detected by scanning phosphorimager screens using a Fuji Fluor/Phospho-Imager. (D) H295R cells were transfected with pGL3-CYP17-2x57, pRL-TK, and wild-type or LXXLL mutant DGK-θ or DGK-ζ expression plasmids. Transfected cells were treated with 1 mM Bt_2cAMP for 16 h and the luciferase activity quantified by luminometry.

is dependent on the expression of DGK-θ or DGK-ζ, because suppressing the translation of these kinases substantially decreases the amount of receptor immunoprecipitated from the cells (Fig. 9B). We also performed glutathione *S*-transferase pulldown assays using in vitro-translated, radiolabeled DGKs and bacterially expressed receptor to determine if SF1 binds to DGK-θ or DGK-ζ. As shown in Fig. 9C, SF1 bound to both DGK-θ and DGK-ζ. This binding was dependent on functional LXXLL motifs in DGK-θ, because mutation of these sites decreased interaction with SF1 (Fig. 9C). To assess the effect of mutating the NR boxes on CYP17 transcriptional activity, we transfected H295R cells with pGL3-CYP17-2x57, pCM VTag1-SF1, and wild-type or NR box mutant DGK-θ and DGK-ζ. Mutation of both LXXLL motifs in DGK-θ attenu-

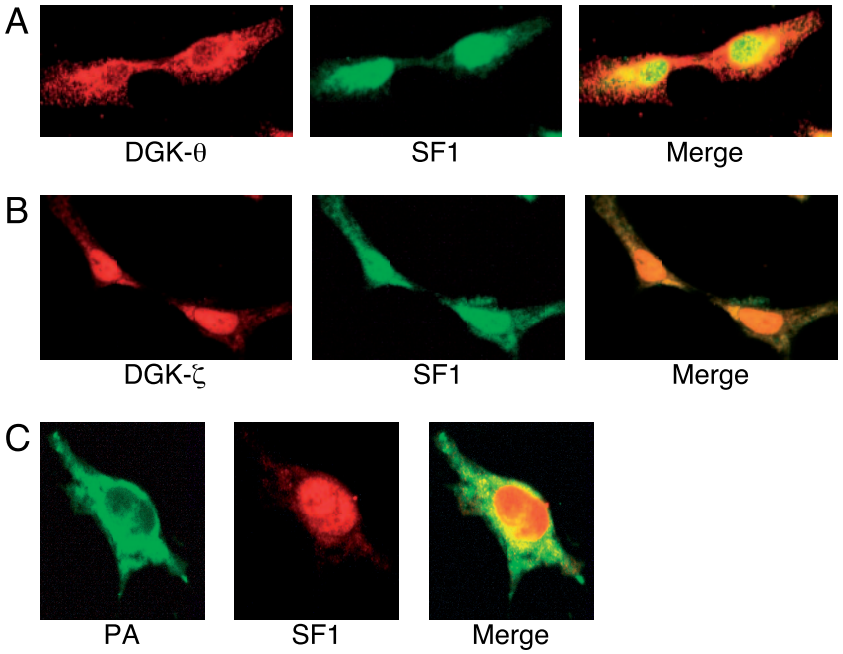

FIG. 10. SF1 colocalizes with DGK and PA in the nuclei of H295R cells. Cells were plated onto glass coverslips, fixed, permeabilized, and incubated with anti-SF1 and anti-DGK-θ (A) or anti-SF1 and DGK-ζ (B) for 1 h. Coverslips were washed and incubated with anti-fluorescein isothiocyanate and antirhodamine, and immunofluorescence was detected by confocal microscopy. (C) Cells were plated on glass coverslips, incubated with NBD-PA for 12 h, fixed, permeabilized, and incubated with an antibody against SF1. Washed coverslips were incubated with antirhodamine and subjected to confocal microscopy.

ated the synergistic activation of *CYP17* reporter gene activity (Fig. 9D), further supporting a role for direct interaction between DGK-θ and the receptor for SF1-dependent CYP17 transcription. Mutation of the lone NR box in DGK-ζ also decreased the ability of SF1 and DGK-ζ to synergistically stimulate an effect on luciferase activity but to a lesser extent than was seen with the DGK-θ mutants.

SF1, DGK, and PA are localized in the nuclei of H295R cells. Now that we have established that SF1 binds to DGK-θ and DGK-ζ, we used immunohistochemical analysis to determine the subcellular locations of these proteins in the H295R adrenocortical cell line. DGK-θ and DGK-ζ are both expressed in the nuclei of H295R cells; however, the expression of DGK-θ is concentrated at the nuclear periphery, whereas DGK-ζ is uniformly expressed throughout the entire nucleus (Fig. 10A and B). SF1 is expressed throughout the nucleus and exhibits overlapping expression with DGK-θ at the periphery of the nucleus (Fig. 10A). Using fluorescently labeled PA, we also determined if PA is also present at the nuclear membrane and found significant overlap of PA and SF1 (Fig. 10C). Based on these findings, we postulate that DGK-θ activation in response to Bt_2cAMP and PA binding to SF1 may occur at the periphery of the nucleus.

Suppression of DGK-θ attenuates cAMP-stimulated CYP17 mRNA expression. To further establish a role for DGK-θ or DGK-ζ in cAMP-dependent activation of *CYP17* gene expression, H295R cells were transfected with siRNAs targeted at DGK-θ or DGK-ζ. As shown in Fig. 11A, silencing of DGK-θ expression inhibited the ability of Bt_2cAMP to stimulate CYP17 mRNA expression, while reducing the expression of DGK-ζ had no significant effect on cAMP-stimulated CYP17

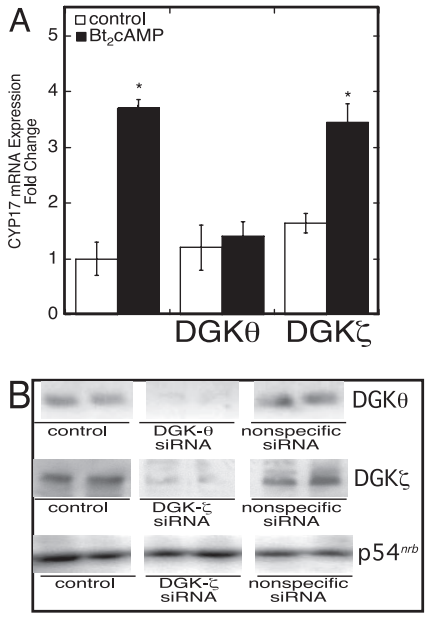

FIG. 11. cAMP-dependent CYP17 transcription requires DGK-θ. (A) Cells were transfected with siRNA oligonucleotides directed against DGK-θ or DGK-ζ for 72 h and then treated for 12 h with 1 mM Bt_2cAMP. Total RNA was extracted, and real time RT-PCR was performed. CYP17 mRNA expression was normalized to β-actin content. Values graphed represent the means ± standard errors of the means for three separate experiments, each performed in quadruplicate. *, $P < 0.05$; statistically different from untreated control. (B) Lysates from cells transfected with siRNA oligonucleotides against DGK-θ, DGK-ζ, or a scrambled sequence were isolated and analyzed by SDS-PAGE and Western blotting using anti-DGK-θ, anti-DGKζ, or anti-$p54^{nrb}$, as described in Materials and Methods.

transcription. These findings demonstrate that although overexpression of different DGK isoforms activates *CYP17* reporter gene activity (Fig. 8D), suppressing the expression of DGK-θ is sufficient to inhibit cAMP-stimulated CYP17 mRNA expression.

DISCUSSION

Steroid hormone biosynthesis in the human adrenal cortex requires the coordinate action of steroid hydroxylases and dehydrogenases (2, 30, 94, 98), lipid receptors (3, 14), and cholesterol binding proteins (4, 13, 56, 64, 100), which coordinately act to increase the production of cortisol and adrenal androgens. The transcription of most of these genes increases in response to activation of a cAMP signaling cascade, which is initiated upon the binding of ACTH to the melanocortin 2 receptor at the plasma membrane of cells in the zonae fasciculate and reticularis. One of the transcription factors that plays a central role in regulating increased steroidogenic gene transcription is SF1 (6, 84, 94). We have previously shown that this NR binds to the human CYP17 promoter and induces transcription in response to ACTH/cAMP signaling (16, 93, 109). As discussed earlier, multiple mechanisms control the ability of SF1 to activate the transcription of target genes, including coactivator and corepressor binding (7, 19, 46, 61, 62, 75, 79, 115, 120), acetylation (11, 44, 46), sumoylation (12, 54, 60), phosphorylation (19, 31, 95), and ligand binding (43, 55, 63, 109), suggesting the likelihood that optimal levels of activated transcription of an SF1 target gene are established by the integration of these regulatory mechanisms. The studies presented herein lend further support to the essential role of ligand binding in the activation of SF1.

We recently demonstrated that SPH is a ligand for SF1 in the human adrenal cortex (109). SPH antagonizes the receptor and prevents the initiation of CYP17 transcription by impairing coactivator binding to the receptor (109). Our studies presented herein reveal that PA is another ligand for SF1. Mass spectrometric analysis of the receptor that was purified from H295R cells identified several phospholipids bound to SF1, with a 26:1 PA molecule being the most abundant (Fig. 1). Although we have identified phospholipids bound to SF1 purified from H295R cells and our functional data suggest that PA may be the endogenous ligand for the receptor, our analysis of only a small subset of possible ligands, i.e., phospholipids, precludes concluding that PA is the predominant, physiologically relevant ligand for SF1. It is likely that performing unbiased mass spectrometric analysis for all classes of lipids and for other molecules, such as carbohydrates, may identify other molecules that specifically bind to the receptor. In order to comprehensively analyze all classes of molecules that interact with SF1 in different cell types and under different physiological conditions, we are currently optimizing conditions for liquid chromatography tandem mass spectrometry. These mass spectrometric studies coupled with thorough analyses of the functional significance of any identified molecules are likely to reveal the molecule(s) that serves as a ligand for SF1.

Reporter gene assays using PA molecules that contain various acyl chain lengths and degrees of unsaturation demonstrated that the di14 PA species was the most efficacious activator of SF1 of those tested; however, both the di12 and di18:0 PA species also significantly stimulated luciferase activity. Except for the distearoyl PA species, most PA molecules with one or more 18-carbon acyl chains did not significantly increase CYP17 transcriptional activity (Fig. 2A). These data are in agreement with the findings of Li et al. demonstrating that phospholipids comprised of two C_{12}-C_{16} acyl chains are optimal for stabilizing SF1 in an active conformation (63). Our findings also indicate that although di14 PA had the greatest stimulatory effect on *CYP17* reporter gene activity, other PA molecular species can activate the receptor, which provides evidence that the ligand binding pocket of SF1 is able to accommodate differing acyl chain lengths. It is equally possible that the ligand binding pocket can accommodate other classes of lipids. Further, mass spectrometric analysis of SF1 purified from H295R cells also detected short-chain PE species (Fig. 1), and crystallographic studies by others have identified various phospholipids, such as PIP (55) and PE (63), bound to the receptor. However, neither short-chained PE molecules (di12, di14, or di16) nor di8 PI(3,4,5)P_3 was able to stimulate SF1-dependent *CYP17* reporter gene activity (data not shown). Collectively, these studies support a role for dynamic ligand binding and dissociation from the cavernous 1,600-Å^3 ligand binding pocket in response to ACTH stimulation.

Reporter gene assays determined that di14 PA was the most efficacious inducer of CYP17 transcriptional activity (Fig. 2A). However, mass spectrometric analysis of phospholipids bound to SF1 purified from H295R cells revealed that the major phospholipid bound to the receptor was a 26-carbon PA species with one double bond, suggesting that the preferred endogenous PA ligand may be a PA molecule that is structurally dissimilar to the di14 PA. The fact that diverse PA species elicited differing magnitudes of activation (Fig. 2A) may indicate that some of these molecular species are partial agonists. We predict that when bound to some PA species and possibly other classes of lipids, SF1 may adopt conformations intermediate between inactive and fully active conformations, with suboptimal coactivator binding. An alignment of SF1 and the peroxisome proliferator-activated receptor γ (suggests that loss of PA responsiveness in the SF1 inner ligand binding pocket mutant H310A (Fig. 6A) may be functionally analogous to the selective loss of binding and responsiveness to the full agonist rosiglitazone but not a partial agonist that was observed with the peroxisome proliferator-activated receptor γ H323A mutant (106). Thus, we predict that SF1 H310A may be useful in screens for partial agonists; however, further biophysical studies using phospholipids comprised of acyl chains of various lengths are under way to further characterize the structural and biochemical parameters required for activation and repression of SF1-mediated transcription.

Although the data presented herein, as well as the findings of others (55, 63, 112), provide compelling evidence for an integral role of ligand binding in regulating the ability of SF1 to activate target gene transcription, our studies (Fig. 3A) also suggest that ligand binding is not sufficient for maximal activation of the receptor. It is likely that in addition to increasing the production of PA in H295R cells, activation of the cAMP signaling pathway also stimulates recruitment of coregulatory proteins in a ligand-independent manner. Moreover, since several studies have established the role of posttranslational modifications (11, 12, 19, 31, 44, 46, 54, 60, 95) in controlling

receptor function, it is probable that cAMP maximally activates target gene transcription by integrating multiple regulatory mechanisms. As previously discussed, acetylation (11, 44, 46), sumoylation (12, 54, 60), and phosphorylation (19, 31, 95) have all been shown to regulate the ability of SF1 to induce target gene transcription; thus, we propose that ACTH/cAMP activates CYP17 transcription by promoting a series of changes, including posttranslational modification, coregulatory protein recruitment, and ligand binding, all of which are necessary for optimal *CYP17* gene expression. We have previously found that phosphatase activity is required for cAMP-dependent CYP17 expression (95, 96), and it is established that phosphorylation of SF1 on S-203 is required for coactivator recruitment (31). Thus, we conclude that the ACTH/cAMP signaling cascade orchestrates a dynamic, coordinate series of events, all of which facilitate steroidogenic gene transcription and subsequent hormone biosynthesis. The order of these interdependent events remains to be fully characterized.

The identification of a molecule that activates SF1 suggests that the receptor exchanges ligands in response to extracellular signaling. We have shown that ACTH/cAMP stimulates the dissociation of SPH from the ligand binding pocket (109), thereby allowing acutely synthesized PA to bind to the receptor as SPH exits the ligand binding pocket. The flexibility of the entryway to the ligand binding cavity (63) supports a role for the exchange of SPH for PA during the switch from an inactive to a canonical active conformation. Previous findings that SPH inhibits coactivator binding and CYP17 transcriptional initiation (109) contrast the increased binding of GCN5 to the CYP17 promoter in response to PA observed in the current studies (Fig. 3D) and indicate that allosteric rearrangement in response to ACTH/cAMP-induced ligand production may facilitate coregulator binding and/or exchange. Indeed, we have preliminary evidence that a series of posttranslational modifications enables the structural changes in the LBD of the receptor that occur during ligand exchange (E. B. Dammer et al., unpublished observations). However, further in vitro and in vivo biophysical analysis of the ligand binding event is necessary to identify the factors that contribute to receptor activation.

We demonstrated that DGK-θ catalyzes the reaction that yields the ligand for SF1 in response to activation of the cAMP signaling pathway. DGK-θ is a member of a growing family of kinases, all of which phosphorylate diacylglycerol to produce PA. To date, 10 isoforms have been identified, several of which, including DGK-θ, are localized in the nucleus (22, 104, 105, 108, 110, 113). DGK-θ contains a proline-rich region and three cysteine-rich domains in the N terminus, centrally localized Ras-associating and pleckstrin homology domains and a C-terminal catalytic domain (90). While the functions of many of the other domains in DGK-θ are unclear, the catalytic activity of the enzyme has been shown to depend on all of its domains (65). It has been postulated that the cysteine-rich domains of the enzyme are required both for correct folding of the protein and for substrate presentation (65). Moreover, DGK-θ activity is modulated by α-thrombin and nerve growth factor in the nucleus of IIC9 fibroblasts (8) and PC12 adrenal cells (101), respectively, and by Rho A (37) and norepinephrine (111) in the cytoplasm.

Although the precise mechanism by which PA is delivered to SF1 is unclear, we demonstrate that DGK-θ and SF1 interact (Fig. 9B and C), and thus, we postulate that the receptor may translocate to distinct regions of the nucleus for ligand binding. DGK-θ is found in nuclear speckles along with hyperphosphorylated RNA polymerase II and the splicing factor SC-35 in various cell types, including PC12, HeLa, and MCF-7 (102). Nuclear speckles are punctate subcompartments enriched in splicing factors and are located in the interchromatin regions of the nucleoplasm (33, 57). These structures are dynamic in their size, shape, and number, and it is thought that this plasticity is controlled in a cell type-specific manner according to the levels of gene expression and in response to metabolic and environmental signals that influence the available pools of active splicing and transcription factors (57). Moreover, since several kinases have been localized to nuclear speckles, it is predicted that the migration of proteins into and out of speckles is determined by changes in the phosphorylation state of these factors. Of note, SF1 is also found in nuclear speckles when conjugated with SUMO moieties (12). Since sumoylated SF1 is transcriptionally repressed (12), it is tempting to speculate that this inhibitory modification plays a role in targeting the receptor to nuclear speckles, thus priming it for ligand exchange and subsequent activation upon PA binding. Once the receptor has bound PA and is in an active conformation, it is possible that other posttranslational modifications may occur that facilitate the recruitment of coactivator proteins and/or translocation out of nuclear speckles. Chen et al. have found that signaling through the cAMP pathway stimulates p300-mediated acetylation of SF1 in nuclear foci and that the acetylated receptor has increased DNA binding activity (11), providing evidence that ligand exchange and the subsequent switch from an inactive to an activated receptor may occur in nuclear speckles.

Although our findings point to DGK-θ as the source of PA in the human adrenal cortex, it is possible that other DGK isoforms may synthesize PA in other steroidogenic tissues for SF1-dependent transcription. As mentioned earlier, there are 10 DGK isoforms that are classified into 5 structurally distinct groups. For example, type I DGKs (α, β, and γ) contain calcium-binding motifs and a recoverin homology domain, while type II DGKs (δ, η, and κ) contain pleckstrin homology domains. DGK-ζ and DGK-ι (type IV) contain myristoylated alanine-rich C kinase substrate phosphorylation site domains and four ankyrin repeats. Moreover, the occurrence of alternative splicing was recently detected for six mammalian DGK genes (γ [51], ζ [20], β [20], δ [89], η [76], and ι [45]. Targeted disruption of DGK-δ in mice has been shown to suppress epidermal growth factor receptor expression and signal transduction by increasing protein kinase C (activity (15). DGK-ε null mice exhibit several neural abnormalities, including a higher resistance to electroconvulsive shock (87) and increased cyclooxygenase 2 and tyrosine hydroxylase expression (67), suggesting a role for DGK-ε in regulating synaptic activity. Targeted disruption of either DGK-α (78) or DGK-ζ (119) results in enhanced T-cell function and demonstrates a role for these kinases in controlling diacylglycerol metabolism during the immune response. DGK isoforms have been implicated in various other cellular processes, including inhibition of Rap1 signaling (86) and retinoblastoma-mediated cell cycle control (66). These studies demonstrate the vast physiological roles of

this family of enzymes, and our findings in this study showing a role for DGK activity in adrenocortical steroidogenesis add to an expanding list of cellular processes that are mediated by bioactive lipids.

Over the past several years, there has been mounting evidence that bioactive lipids produced in the nucleus play key roles in cell signaling and other processes (1, 9, 27, 39, 40, 49, 58, 59, 68, 70, 71). Indeed, the identification of PIPs as ligands for SF1 during crystallographic studies of the receptor by Krylova et al. (55) coupled with our findings (this study) strongly suggest that modulation of nuclear pools of phospholipids and sphingolipids during trophic hormone signaling may be required not only for steroidogenesis but also for endocrine development. Our previous findings demonstrating that ACTH activates sphingolipid metabolism and results in increased SPH-1 phosphate (80, 81) suggest that cAMP signaling activates SF1 by increasing PA biosynthesis (this study) and by converting the antagonist SPH to SPH-1 phosphate, another lipid activator of CYP17 transcription (81). Further studies are required to uncover the precise role of fluxes through the phospholipid and sphingolipid metabolic pathways in adrenal and gonadal steroidogenic gene expression.

Our findings showing that Bt_2cAMP rapidly activates DGK activity in H295R cells (Fig. 8A), coupled with the synergistic effect of SF1 and DGK-θ on CYP17 transcriptional activity in Bt_2cAMP-treated cells (Fig. 8D), make it tempting to speculate that cAMP-dependent protein kinase A (PKA) may increase DGK activity by phosphorylating the enzyme. There is precedence for a role for phosphorylation in controlling the activity of DGK isoforms. γ-Protein kinase C phosphorylates DGK-γ, resulting in increased activity and PA production in COS-7 cells (117). Moreover, in silico analysis of DGK-θ revealed several putative PKA phosphorylation sites, and preliminary two-dimensional gel electrophoresis data indicate multiple phosphorylated forms of the protein in H295R cells (Li et al., unpublished findings), suggesting that a direct target of PKA in the ACTH/cAMP steroidogenic pathway may be DGK-θ. Further studies are warranted to elucidate the functional significance of phosphorylation on the activity, subcellular localization, and stability of the enzyme. In summary, we have identified PA as an activator of SF1 and demonstrated increased biosynthesis of this lipid in the nucleus of human adrenal cells during ACTH/cAMP signaling. Based on these findings, we propose that ACTH/cAMP-stimulated changes in phospholipid and sphingolipid metabolism play intricate roles in ensuring dynamic activation of SF1 and subsequent steroidogenic gene transcription.

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