Death-associated Protein 6 (Daxx) Mediates cAMP-dependent Stimulation of Cyp11a1 (P450scc) Transcription**

Hsin-Chieh Lan †‡§, Chih-Feng Wu †*, Hsiu-Ming Shih †, and Bon-chu Chung*‡

From the †Institute of Molecular Biology and ‡Institute of Biomedical Sciences, Academia Sinica, Taipei 115, the §Institute of Biology and Anatomy, National Defense Medical Center, Taipei 114, and the †Institute of Biochemistry and Molecular Biology, National Yang-Ming University, Taipei 112, Taiwan

Background: HIPK3 mediates cAMP signaling and stimulates SF-1 activity to activate Cyp11a1 transcription.

Results: In response to cAMP, HIPK3 phosphorylates Daxx, leading to JNK/c-Jun activation and the enhancement of SF-1-dependent Cyp11a1 expression.

Conclusion: Daxx mediates HIPK3 effect to activate JNK/c-Jun for SF-1-dependent Cyp11a1 expression.

Significance: The cAMP signaling pathway for the activation of the steroidogenic gene Cyp11a1 uses the apoptotic pathway components HIPK3/Daxx/JNK/c-Jun.

**This work was supported by grants from Academia Sinica, NHRI (NHRI-EX100-9710SI), and the National Science Council (NSC100-2321-B-001-006).

† This article contains supplemental Figs. S1–S4.

‡ To whom correspondence should be addressed: Institute of Molecular Biology, Academia Sinica, Taipei, 115 Taiwan. Tel.: 886-2-2789-9215; Fax: 886-2-2788-3464; E-mail: mbchung@sinica.edu.tw.

SF-1 is a key transcription factor for all steroidogenic genes. It up-regulates the expression of the steroidogenic Cyp11a1 gene in the adrenal in a pathway stimulated by cAMP through HIPK3-mediated JNK/c-Jun phosphorylation. In the present study, we have investigated the factors mediating cAMP-dependent HIPK3 action to potentiate the activity of SF-1 for Cyp11a1 transcription in mouse adrenocortical Y1 cells. We found Daxx, a HIPK kinase substrate in the apoptosis pathway, was phosphorylated by HIPK3 at Ser-669 in response to cAMP stimulation. Daxx participated in SF-1-dependent Cyp11a1 expression as shown by experiments involving both overexpression and down-regulation via a dominant negative Daxx mutant. The S669A mutant of Daxx, which could not be phosphorylated by HIPK3, lost the ability to potentiate SF-1 activity for Cyp11a1 expression. The enhancement of SF-1 activity by Daxx required JNK and c-Jun phosphorylation. Thus, Daxx functioned as a signal transducer linking cAMP-stimulated HIPK3 activity with JNK/c-Jun phosphorylation and SF-1-dependent Cyp11a1 transcription for steroid synthesis.

SF-1 (Ad4BP, NR5A1) (1) is a key regulator for steroid biosynthesis, metabolism, and reproduction in the adrenals and gonads (2). It activates the transcription of many steroidogenic genes like Cyp11a1, StAR, Cyp21, etc (3). One SF-1 target gene, Cyp11a1, encodes P450scc that catalyzes the first and rate-limiting step of steroidogenesis. Cyp11a1 is expressed in a tightly regulated manner in the adrenals and gonads in response to the stimulation of adrenocorticotropic and gonadotropin, respectively. Upon stimulation by these tropic hormones, the intracellular cAMP level is increased to trigger a downstream signal cascade that leads to increased Cyp11a1 expression. Although proteins like CREB and c-Jun potentiate SF-1 activity for Cyp11a1 expression (4), the components in the signaling pathway that lead to the enhancement of SF-1 activity are not well characterized. SF-1 activity is modulated by post-translational modifications (5–7) and interactions with other protein partners (8, 9). One SF-1-interacting protein, homeodomain-interacting protein kinase 3 (HIPK3),2 increases the ability of SF-1 to stimulate Cyp11a1 transcription in response to cAMP (10).

HIPK3 is a serine-threonine kinase originally defined as a co-repressor for homeodomain transcription factors (11). It modulates signals associated with cell death (12). The other HIPK family members, HIPK1 and HIPK2, also regulate cell death. The activities of HIPK1/2 are mediated by death-associated protein 6 (Daxx) (13, 14). HIPK1 phosphorylates Daxx directly, altering its nuclear location and regulating its transcriptional function (15). HIPK2 cooperates with Daxx and up-regulates its phosphorylation level in transforming growth factor β (TGF-β)-induced apoptosis (13).

The roles of Daxx were initially established in apoptosis. Daxx mediates apoptosis stimulated by the death receptor Fas (16), UV irradiation (17), or TGF-β signaling (18). However, Daxx also possesses anti-apoptotic functions (19–21), and Daxx is required for Mdm2 stability in the degradation of the pro-apoptotic protein p53 (22). Thus, Daxx plays dual functions in cell death.

Daxx serves as a scaffold protein and signal transducer. It up-regulates ASK-1 kinase activity (23) and the subsequent MKK/JNK signaling pathway (18, 24), mediates the HIPK2 signal regulating JNK activity in TGF-β-induced apoptosis (13), and mediates the activation of ASK-1/JNK/c-Jun and GLUT4 in response to serum deprivation (25).

The abbreviations used are: HIPK, homeodomain-interacting protein kinase; AU, arbitrary unit; Daxx, death-associated protein 6; Hsp, heat shock protein; Luc, luciferase; p5, pSuper RNA interference plasmids; SF-1, steroidogenic factor 1/Ad4BP or NR5A1; aa, amino acids.

* This work was supported by grants from Academia Sinica, NHRI (NHRI-EX100-9710SI), and the National Science Council (NSC100-2321-B-001-006).

2 The abbreviations used are: HIPK, homeodomain-interacting protein kinase; AU, arbitrary unit; Daxx, death-associated protein 6; Hsp, heat shock protein; Luc, luciferase; p5, pSuper RNA interference plasmids; SF-1, steroidogenic factor 1/Ad4BP or NR5A1; aa, amino acids.
Daxx Mediates HIPK3 Action to Enhance SF-1 Activity

In addition to the roles in apoptosis and signal transduction, Daxx is a transcription regulator. Daxx represses c-Met transcription by recruiting HDAC2 to the gene (26). It also represses the activities of androgen receptor (27), CCAAT/enhancer-binding protein β (28), AIRE (29), and Tcf4 proteins (30).

Daxx functions are regulated by its intracellular locations (14, 31) and post-translational modifications such as sumoylation, ubiquitination, and phosphorylation. Sumoylation changes the subnuclear localization and subsequent transcriptional repression of Daxx (32). Additionally, ubiquitination of Daxx at Lys-630 and -631 competes with its sumoylation (33). Further, phosphorylation of Daxx at Ser669 abrogates its transcriptional repression activity (15) and leads to nuclear export (34).

Despite numerous studies on Daxx, its role in steroidogenesis has never been characterized. Here we show that Daxx participates in cAMP-stimulated steroidogenic Cyp11a1 transcription by mediating the effect of HIPK3. We found HIPK3 phosphorylated Daxx at Ser-669 resulting in the transactivation of SF-1 in mouse adrenal Y1 cells. Mutation of Ser-669 or depletion of Daxx at Ser669 abrogates its transcriptional repression activity (15) and leads to nuclear export (34).

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents**—Y1 mouse adrenocortical tumor cells were maintained in Dulbecco’s modified Eagle’s medium/ F12 supplemented with 10% fetal calf serum. The human lung adenocarcinoma H1299 cell was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. 8-Br-cAMP (1 mM) was added to Y1 cells for 1 to 24 h for stimulation experiments. The Jun N-terminal kinase inhibitor SP600125 (Calbiochem) was added to cells for 6 h at a final concentration of 25 μM. To remove phosphate groups from proteins, cell extract was incubated with 5 mM lambda protein phosphatase (New England Biolabs, Ipswich, MA) in reaction buffer supplemented with 1 mM MnCl₂ for 30 min at 37 °C.

**Plasmids and Mutant Constructs**—Plasmids for the expression of SF-1-HA (35), FLAG–HIPK3 and its K226R mutant (36), FLAG-tagged dominant negative DN-JNK (37), c-Jun derivatives (WT-c-Jun, Ala-c-Jun, and Asp-c-Jun) (38), Daxx derivatives (HA-tagged FL-Daxx, N-Daxx, C-Daxx, FLAG-Daxx, and pSuper-Daxx) (39), (Daxx–myc, S502A, S669A, S502/S669A) (15), and Cyp11a1: Luc (phsc2.3kb) (40) have been described previously. GST-fused SF-1-CTM (aa 170–462) was constructed by annealing the Ncol (filled-in)–EcoRI fragments from pCMV5-SF-1 to pGEX-4T-1 vector (GE). GST-SF-1–DBD (aa 1–137) and GST-SF-1–FHS (aa 78–212) were constructed by PCR-based amplification of SF-1 fragments using the following primer pairs into pGEX-1AT and pGEX-4T1 vectors, respectively: 5'-atgtccactctcgagcga and 5'-gtccagggagagggagt; 5'-atgcggctgaagcaatgag and 5'-gtaggtatgctctcctgtt. Transient Transfection and Reporter Assay—H1299 cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Y1 cells were transfected using Lipofectamine Plus (Invitrogen) or TurboFect (Fermentas, St. Leon-Rot, Germany).

**RESULTS**

Cyclic AMP Stimulates HIPK3 Activity, c-Jun Phosphorylation, and CYP11A1 Expression—We have previously shown that HIPK3 mediates the cAMP signal and stimulates SF-1 activity to activate the transcription of CYP11A1 (10), which encodes an enzyme that controls the first and rate-limiting step of steroidogenesis. To investigate the mechanism by which HIPK3 mediates the effect of cAMP to stimulate gene expression, we examined whether HIPK3 kinase activity was regulated through 

For luciferase assays, cells in 24-well plates were harvested in lysis buffer (100 mM potassium phosphate, pH 7.8, 0.2% Triton X-100, 0.5 mM DTT, 0.2 mM PMSE) 48 h after transfection and subjected to luciferase or β-galactosidase assays. Reporter activities were normalized against the internal control RSV-β-gal. At least three independent experiments were performed, and the standard deviations from the means are presented by error bars.

Western Blotting—Cells were harvested and lysed 48 h after DNA transfection or 3 days after siRNA transfection. Equal amounts of total protein mixture were separated by gel electrophoresis, transferred to Immobilon™-P membrane (Millipore, MA), and incubated with specific antibodies overnight at 4 °C followed by horseradish peroxidase-conjugated secondary antibody for 45 min. Signals were detected by chemiluminescence assays.

**Antibodies**—The following antibodies were purchased and used at 1/5000 dilutions in Western blotting: anti-c-Jun (sc-45, Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-JNK, anti-phospho-JNK (Cell Signaling Technology, Inc.), anti-SF-1 (9), anti-HA, anti-HIPK3 (Abgent Co.), anti-Daxx (Sigma), anti-ASK-1 (sc-7931, Santa Cruz Biotechnology), anti-FLAG, anti-Hsp70 and anti-actin. Anti-phospho-c-Jun (sc-822, Santa Cruz Biotechnology) was used at a 1:2000 dilution, and antibody against CYP11A1 (41) was used at 1:10000 dilution.

Co-immunoprecipitation (Co-IP) and in Vitro Kinase Assay—H1299 cells were lysed in IPH buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.5% Nonidet P-40) plus 1X complete protease inhibitor mixture (Roche) 48 h post-transfection with FLAG–HIPK3 and HA-Daxx. The cell lysate was then incubated with 1 μg of mouse anti-FLAG antibody or normal mouse IgG and 30 μl of 50% protein A-Sepharose beads for 2 h. Proteins bound to Sepharose beads were precipitated, dissolved, and denatured in sample buffer for gel electrophoresis and immunoblotting. For the in vitro kinase assay, protein substrates purified from Escherichia coli or from mammalian cells were incubated with active HIPK3 protein (Millipore) in buffer (20 mM Heps, 1 mM EGTA, 0.4 mM EDTA, 5 mM MgCl₂, 0.1 mM CaCl₂, 0.05 mM dithiothreitol, 0.2 mg/ml phosphatidylinositol, 2.5 mM β-glycerophosphate, 1 μM PMA, and 10 μCi [γ-32P]ATP) at 30 °C for 20 min before the reaction was stopped by heating for 5 min at 100 °C in 4× sample buffer (0.2 M Tris–HCl, 0.4 M DTT, 8% SDS, 0.4% bromphenol blue, 40% glycerol). The samples were separated by gel electrophoresis, stained with Coomassie Blue, and dried prior to exposure to x-ray film.
Daxx Mediates HIPK3 Action to Enhance SF-1 Activity

FIGURE 1. HIPK3 kinase activity and c-Jun phosphorylation are enhanced by 8-Br-cAMP stimulation. A, increased HIPK3 activity by 8-Br-cAMP. FLAG-HIPK3 and its kinase-dead FLAG-K226R mutant (1 μg) were transfected in Y1 cells. Proteins were then immunoprecipitated by anti-FLAG antibody, and their kinase activities were detected by in vitro kinase assay. The total FLAG-proteins and IgG detected by Coomassie Blue staining are also shown. B, c-Jun phosphorylation and CYP11A1 expression are enhanced by 8-Br-cAMP. The amounts of phosphorylated c-Jun (p-c-Jun), c-Jun, and CYP11A1 in Y1 cells after 1 μM 8-Br-cAMP treatment for the time indicated on top of each lane were detected by immunoblotting. Hsp70, heat-shock protein 70, is used as a gel loading control.

by 8-Br-cAMP using an in vitro kinase assay. FLAG-HIPK3 was isolated from adrenocortical Y1 cells by immunoprecipitation, and FLAG-HIPK3 auto-phosphorylation was increased with time after 8-Br-cAMP treatment (Fig. 1A). The kinase-dead mutant of HIPK3, FLAG-K226R, had no activity (Fig. 1A). The phosphorylation level of c-Jun and the amount of CYP11A1 were also elevated after stimulation (Fig. 1B). Thus, HIPK3 activity, c-Jun phosphorylation, and CYP11A1 expression were all enhanced upon the increase of intracellular cAMP levels.

HIPK3 Interacts with and Phosphorylates Daxx—We next searched for HIPK3 substrates that participate in HIPK3-dependent CYP11A1 gene activation. Daxx is a direct substrate for HIPK1 and HIPK2 (13, 15) and is therefore a likely candidate as a HIPK3 substrate. We first examined whether HIPK3 and Daxx interacted with each other by co-immunoprecipitation. The N-terminal (aa 1–500), but not the C-terminal (aa 501–740), part of Daxx immunoprecipitated together with FLAG-HIPK3, while the full-length Daxx displayed a weak interaction with HIPK3 (Fig. 2A).

HIPK3 kinase activity was then examined in vitro using recombinant substrates fusing GST to Daxx, c-Jun, or different fragments of SF-1 (Fig. 2B). The bottom gel indicates the amounts of GST fusion proteins used in the experiment. The top gel of $^{32}$P incorporation detected the phosphorylation signal only when Daxx or the positive control MBP was used as a substrate (Fig. 2B). SF-1, c-Jun, JNK1, and JNK2 were not efficient HIPK3 substrates (Fig. 2B and supplemental Fig. S1). Phosphorylated Daxx was not detected when no kinase was added (supplemental Fig. S1), indicating the specificity of the assay.

HIPK3 Directly Phosphorylates C-terminal Daxx—To identify the domain of Daxx phosphorylated by HIPK3, we performed an in vitro kinase assay by incubating active-HIPK3 with different regions of HA-Daxx proteins that were isolated by immunoprecipitation. The full-length and C-terminal Daxx were phosphorylated by HIPK3, but N-terminal Daxx was not (Fig. 2C). The right panel is a control showing that equal amounts of Daxx proteins were present in this kinase assay. Thus, this experiment indicates that HIPK3 phosphorylates Daxx in the C-terminal region.

In vivo, two Daxx-myc bands were detected in H1299 cells transfected with 0.5 μg of Daxx-myc (Fig. 2D). After co-expression of WT-FLAG-HIPK3, the intensity of the lower band was decreased while that of the upper band was enhanced (Fig. 2D). The kinase-deficient K226R mutant of HIPK3 could not induce Daxx phosphorylation. In Y1 cells, Daxx mobility shift was increased with time after 8-Br-cAMP treatment. This gel shift was abolished following phosphatase treatment (Fig. 2E), indicating the band shift was due to phosphorylation. Thus, Daxx is a phosphorylation substrate of HIPK3 in vitro and in vivo, and Daxx is phosphorylated in response to cAMP stimulation.

Daxx Mediates HIPK3 Effect in SF-1-dependent CYP11A1 Expression—Because HIPK3 participates in the stimulation of CYP11A1 transcription (10), we examined whether Daxx was...
Daxx Mediates HIPK3 Action to Enhance SF-1 Activity

FIGURE 3. Daxx is essential for SF-1-dependent transcription of Cyp11a1. A, Daxx enhances SF-1 activity in CYP11A1 reporter assay. CYP11A1-Luc reporter activities are measured after co-transfection into H1299 cells with different dosages of Daxx or SF-1 expression vectors (0.1 μg). The level of reporter gene expression by SF-1 alone is set as one. AU, arbitrary unit. *, p < 0.01. B, Daxx enhanced CYP11A1 expression. The levels of endogenous CYP11A1, Daxx, and Hsp70 detected by immunoblotting are shown after transfection of Daxx expression vectors at the dosages shown on top of each lane. C, Daxx depletion causes a decrease of SF-1- and HIPK3-dependent reporter expression. CYP11A1-Luc luciferase (Luc) reporter activities were measured after transfection of H1299 cells with 0.2 or 0.4 μg pSuper-Daxx, 0.1 μg of SF-1, and/or 0.2 μg of FLAG (F)-HIPK3. #, p < 0.01. *, p < 0.01. ps-Daxx: plasmid containing shRNA against Daxx. Lower panel: immunoblot of Daxx, SF-1 and FLAG-HIPK3. D, Daxx depletion leads to a decline of CYP11A1. Different dosages of pSuper(pS)-Daxx were transfected into Y1 cells, and the levels of endogenous CYP11A1, Daxx, and Hsp70 were detected by immunoblotting. * indicates treated with 1 mM 8-Br-cAMP for 24 h. + indicates untreated.

also involved. CYP11A1 transcription was measured by examining the expression of luciferase driven by the 2.3 kb human CYP11A1 promoter. While having no function by itself, Daxx increased the ability of SF-1 to activate the CYP11A1 promoter (Fig. 3A). It also led to an increase of endogenous CYP11A1 in Y1 cells (Fig. 3B). Thus, Daxx functions as an SF-1 co-activator to increase CYP11A1 expression.

In the loss-of-function experiment, Daxx was depleted from H1299 cells by the introduction of shRNA driven by a pSuper-Daxx plasmid. In these cells, HIPK3 and SF-1 activate the CYP11A1 promoter synergistically (10). This activity, however, was diminished when the amounts of pSuper-Daxx were increased (Fig. 3C). Endogenous CYP11A1 levels in the presence or the absence of 8-Br-cAMP were also reduced following pSuper-Daxx transfection in Y1 cells (Fig. 3D). In human adrenocortical H295 cells, depletion of Daxx by pSuper-Daxx also led to a decrease of 8-Br-cAMP-dependent CYP11A1 and STAR expression (supplemental Fig. S2). However, when CYP11A1 levels were already enhanced by cAMP, more Daxx did not activate it further (supplemental Fig. S3). This indicates that Daxx mediates cAMP stimulation and HIPK3 activity to enhance SF-1 activity resulting in CYP11A1 expression.

JNK/c-Jun Activities Are Required for Daxx-dependent CYP11A1 Expression—To understand how Daxx activates CYP11A1 expression, we examined JNK/c-Jun since HIPKs/Daxx signaling utilizes the JNK/c-Jun pathway in cell death regulation (13). With increasing amounts of Daxx in Y1 cells, we detected proportionally elevated c-Jun phosphorylation levels (Fig. 4A). Furthermore, over-expression of the dominant negative Ala-c-Jun resulted in a decrease of Daxx/SF-1-dependent CYP11A1 reporter activity; this activity was not affected by WT-c-Jun (Fig. 4B). This indicates that c-Jun participates in the synergistic effects of Daxx and SF-1. Furthermore, phosphorylation of c-Jun, which was induced by FLAG-HIPK3, was also abolished by pSuper-Daxx (Fig. 4C). Thus, c-Jun phosphorylation caused by HIPK3/Daxx signaling is important for SF-1 activation.

In addition to c-Jun, the role of JNK was also investigated by treating cells with the JNK inhibitor SP600125 (Fig. 5A) or by depleting endogenous JNK with a dominant negative DN-JNK mutant (Fig. 5B). While Daxx overexpression enhanced the level of CYP11A1 detected by immunoblotting (Fig. 5A), c-Jun over-expression causes SF-1-dependent transcription of Cyp11a1 reporter assay. CYP11A1-Luc reporter activities are measured after co-transfection into H1299 cells with different dosages of Daxx or SF-1 expression vectors (0.1 μg). The level of reporter gene expression by SF-1 alone is set as one. AU, arbitrary unit. *, p < 0.01. B, Daxx enhanced CYP11A1 expression. The levels of endogenous CYP11A1, Daxx, and Hsp70 detected by immunoblotting are shown after transfection of Daxx expression vectors at the dosages shown on top of each lane. C, Daxx depletion causes a decrease of SF-1- and HIPK3-dependent reporter expression. CYP11A1-Luc luciferase (Luc) reporter activities were measured after transfection of H1299 cells with 0.2 or 0.4 μg pSuper-Daxx, 0.1 μg of SF-1, and/or 0.2 μg of FLAG (F)-HIPK3. #, p < 0.01. *, p < 0.01. ps-Daxx: plasmid containing shRNA against Daxx. Lower panel: immunoblot of Daxx, SF-1 and FLAG-HIPK3. D, Daxx depletion leads to a decline of CYP11A1. Different dosages of pSuper(pS)-Daxx were transfected into Y1 cells, and the levels of endogenous CYP11A1, Daxx, and Hsp70 were detected by immunoblotting. * indicates treated with 1 mM 8-Br-cAMP for 24 h. + indicates untreated.

FIGURE 4. Daxx activates c-Jun phosphorylation for CYP11A1 expression. A, Daxx expression causes c-Jun phosphorylation. Levels of p-c-Jun are detected by immunoblotting after Y1 cells are transfected with increasing dosages (0.5, 1, 2 μg) of Daxx and 0.2 μg of c-Jun expression plasmid. The numbers below the top gel represent the quantitative amounts determined by densitometric analysis. Amount of total c-Jun are controls. B, dominant negative Ala-c-Jun reduces the Daxx- and SF-1-dependent CYP11A1 promoter activity. CYP11A1-Luc activities are shown after H1299 cells were transfected with (+) or without (−) 0.1 μg SF-1, 40 ng Daxx, and increasing doses (10, 30, 50 ng) of dominant negative c-Jun (ala-c-Jun) expression plasmids. *, p < 0.01 when compared with Daxx and SF-1 co-transfection alone. #, p < 0.01 comparing WT-c-Jun with Ala-c-Jun. C, Daxx deficiency results in decreased c-Jun phosphorylation. Expression vectors of c-Jun (0.2 μg), FLAG-HIPK3 (aa.159–1191) (0.5 μg), or kinase-deficient K226R mutant of HIPK3 (k) (0.5 μg) and pSuper-Daxx (shRNA) (0.4 or 1 μg) were transfected into H1299 cells. Proteins are detected with specific antibodies in immunoblotting.
Daxx Mediates HIPK3 Action to Enhance SF-1 Activity

**FIGURE 5. JNK participates in Daxx-dependent CYP11A1 expression.** A, JNK inhibitor reduces CYP11A1 expression with or without Daxx activation. Levels of CYP11A1 detected by immunoblotting are shown after Y1 cells were transfected with or without 0.2 μg FLAG-Daxx in the presence (+) or absence (−) of JNK inhibitor. Hsp70 is the internal loading control. The numbers below the top gel represent the quantitative amounts determined by densitometric analysis and normalization against Hsp70. B, CYP11A1 promoter activity is repressed by domain negative JNK and rescued by constitutively active c-Jun. H1299 cells were co-transfected with CYP11A1-Luc and expression plasmids for SF-1 (0.1 μg), Daxx (40 ng), and dominant negative (DN)-JNK in increasing doses (50, 100, 200 ng). The constitutively active c-Jun (Asp-c-Jun) (40 ng) was co-transfected into Y1 cells at the last lane. *, p < 0.01 comparing the activity with or without Asp-c-Jun.

phosphorylated when co-expressed with HIPK1 (15). Both WT and S502A-Daxx migrated more slowly on the gel when they were co-expressed with HIPK3, but not with the K226R mutant (Fig. 6A), indicating they were phosphorylated by HIPK3. However, neither the S669A nor S502A/S669A mutant bands were shifted (Fig. 6A), indicating that Ser-669 but not Ser-502 was the residue of Daxx targeted for phosphorylation by HIPK3. In Y1 cells, while the WT Daxx was phosphorylated with time after 8-Br-cAMP treatment, the S669A mutant was not phosphorylated (Fig. 6B). This indicates that Daxx is phosphorylated at Ser-669 upon the increase of cAMP stimulation in Y1 cells.

**FIGURE 6. Phosphorylation of Daxx at Ser-669 by HIPK3 upon cAMP stimulation.** A, Ser-669 residue of Daxx is a target of HIPK3 phosphorylation. WT, S502A, S669A, or S502A/S669A Daxx (0.5 μg each) were co-expressed with FLAG-HIPK3 or its kinase-deficient mutant K226R (0.5 μg each) in H1299 cells. Daxx mobility shift was detected by immunoblotting with specific antibodies. B, phosphorylation pattern of wild type and S669A-Daxx after cAMP treatment. WT or S669A Daxx (0.5 μg) were expressed in Y1 cells, treated with 1 mM 8-Br-cAMP for the time indicated on top of each lane, and detected by immunoblotting.

**FIGURE 7. Ser-669 of Daxx is required for SF-1 activation but not physical interaction with HIPK3.** A, Daxx S669A mutant fails to activate CYP11A1. CYP11A1-Luc activities are shown after a combination of SF-1, WT or S669A Daxx were expressed in H1299 cells. *, p < 0.001 comparing WT and S669A Daxx, B, S669A-Daxx reduces CYP11A1 expression. The amounts of CYP11A1, phospho(p)-c-Jun, total c-Jun, Daxx-myc and Hsp70 expressed in Y1 cells are shown after transfaction of different dosages of WT or S669A Daxx-myc. The numbers below the top gel represent the quantitative amounts of CYP11A1 determined by densitometric analysis and normalized against Hsp70 internal controls. C, HIPK3 co-immunoprecipitate with both wild type and S669A-Daxx. FLAG-HIPK3 (1 μg) and 1.5 μg of Daxx-myc, either WT or S669A S mutant, were co-expressed in H1299 cells in 10-cm plates, and immunoprecipitated (IP) with anti-FLAG antibody. Immunoblotting was performed using anti-FLAG and anti-Myc antibodies to detect HIPK3 and Daxx. Input: cell extracts before IP.

DISCUSSION

In this study, we have investigated the cAMP signaling pathway leading to increased steroidalogenic Cyp11a1 expression. The novelty of our finding is the discovery that an established pathway in apoptosis, HIPK/Daxx/JNK/c-Jun (16, 18), can transduce the cAMP signal to stimulate Cyp11a1 transcription. In adrenal cells an increased cAMP level activates HIPK3, which phosphorylates Daxx at Ser-669 leading to JNK/c-Jun phosphorylation, thus potentiating SF-1 activity to up-regulate Cyp11a1. This is the first report to point out the function of Daxx in steroidogenesis. We have also elucidated the mechanism of Daxx modification and activation following cAMP stimulation.
FIGURE 8. A diagram showing the signaling pathways involving HIPK/Daxx/JNK for steroidogenic gene expression and for apoptosis. The upper rectangle shows steroidogenic gene activation in Y1 cells, and the lower panel indicates the apoptosis pathway in Hep3B cells. The solid arrows indicate direct effects, and dotted arrows indicate indirect effects involving several steps.

**Daxx Function in Steroidogenesis versus Apoptosis**—We find that the stimulation of Cyp11a1 transcription goes through a pathway known to trigger apoptosis (16, 18). Although this pathway participates in both apoptosis and steroidogenesis, the cell type and target genes involved in these two events are different (Fig. 8). Daxx activates JNK/c-Jun to induce p53-independent apoptosis in response to TGF-β in liver cancer Hep3B cells (13), whereas Daxx activates SF-1-mediated Cyp11a1 expression for steroid synthesis in response to cAMP. This implies that different upstream stimuli could use the same signaling molecules to regulate distinct transcription factors for different outcomes. This observation indicates that the same signaling pathway can be used in different types of cell events. Furthermore, members of this pathway might be modified at different sites when the stimuli are different. For example, in addition to phosphorylation at Ser-669 by HIPK proteins, phosphorylation by CK2 kinase at Ser-737 and Ser-739 renders Daxx a better substrate for SUMO-1 conjugation and cell death sensitization (42).

**Effects of Daxx on Nuclear Receptors**—In this report, we document that Daxx enhances the transcriptional activity of the nuclear receptor SF-1. However, in a previous study, Daxx was shown to suppress the activity of another nuclear receptor family member, androgen receptor (AR) (27). This indicates that Daxx may have multiple functions. We show here that Daxx potentiates SF-1 activity indirectly by activating a signaling cascade involving JNK/c-Jun, while Daxx interferes with the DNA binding ability of AR via direct binding to SUMO-modified AR (27). SF-1 was found in the same complex as Daxx by co-immunoprecipitation assay (supplemental Fig. S4). We suggest that Daxx, HIPK3, c-Jun, and SF-1 may form a complex to upregulate the activity of SF-1, because HIPK3 fails to phosphorylate SF-1 directly (Fig. 2B). Thus, Daxx functions as both a positive and negative regulator of transcription depending on the cellular context and the molecules with which it interacts.

**The Identity of Daxx Signal Transducer**—Daxx participates in the stimulation of steroidogenic gene expression by transferring the phosphorylation signal from HIPK3 to JNK/c-Jun. There are probably a few steps between Daxx and JNK, and the direct downstream effector of Daxx is not yet clear. For apoptosis, Daxx activates signal-regulating kinase 1 (ASK-1) for the signal flow to JNK (23). It is unclear whether ASK-1 is also involved in steroidogenesis since we could not detect ASK-1 in Y1 cells (data not shown). Overexpression of exogenous ASK-1 in Y1 cells could stimulate the JNK/c-Jun signaling cascade leading to increased Cyp11a1 promoter activity (data not shown). Thus, Daxx might regulate some protein kinase like ASK-1 in a signaling pathway leading to c-Jun phosphorylation and SF-1 activation.

**Physical Interaction between HIPKs and Daxx**—Here we show that the N-terminal, but not the C-terminal, part of Daxx physically interacts with HIPK3. Furthermore, N-terminal Daxx interacted with HIPK3 better than the full-length Daxx, implying that the C terminus of Daxx probably exerts conformational constraint that inhibits its N-terminal domain from interacting with HIPKs. This C-terminal region of Daxx, in contrast, was important for HIPK3 phosphorylation. It seems only very weak binding is essential for HIPK3 to phosphorylate the Daxx C-terminal region, while the phosphorylation status of Daxx does not affect its own binding to HIPK3. Daxx appears to bind to HIPK3 in both basal and stimulated conditions.

**Daxx Phosphorylation Sites**—Daxx is a phosphoryoprotein with at least seven residues capable of being phosphorylated by different kinases (15). In the present report, we found Ser669 was phosphorylated by HIPK3 in response to cAMP stimulation. However, Daxx appears to be heavily phosphorylated at multiple sites in addition to Ser-669 upon 8-Br-cAMP treatment, as exemplified by the dramatic gel mobility shift. S669A-Daxx was hypophosphorylated, indicating that the mobility shift of Daxx may rely on the phosphorylation of Ser-669. Thus, Ser669 may be a major HIPK3 modification site although not the only one, and the phosphorylation of Ser-669 may be required to trigger the phosphorylation of other residues.

Daxx represses transcription in many cases (26–30). Yet phosphorylation of Daxx at Ser-669 by HIPK1 abrogates its repressive function (15). We also show here that Ser-669 phosphorylation by HIPK3 is required for increased Cyp11a1 transcription. Thus, Ser-669 phosphorylation is often associated with the activation function of Daxx.

**Acknowledgments**—We thank Philip Leder for Daxx plasmids, Joura J. Palvimo for HIPK3 plasmids, Dirk Bohmann for c-Jun plasmids, Roger Davis for JNK1/DN-JNK1 plasmids, David Baltimore for ASK-1 plasmids, Pao-Yen Lai and Wei-Yi Chen for the construction of GST-SF1 fusion plasmids, and Chung Wang for anti-Hsp70 antibody.

**REFERENCES**

1. Nuclear Receptors Nomenclature Committee (1999) A unified nomenclature system for the nuclear receptor superfamily. *Cell* 97, 161–163
2. Luo, X., Ikeda, Y., Lala, D., Rice, D., Wong, M., and Parker, K. L. (1999) Steroidogenic factor 1 (SF-1) is essential for endocrine development and function. *J. Steroid Biochem. Mol. Biol.* 69, 13–18
3. Val, P., Lefrançois-Martinez, A. M., Veyssière, G., and Martinez, A. (2003) SF-1 a key player in the development and differentiation of steroidogenic tissues. *Nucl. Recept.* 1, 8
4. Shih, M. C., Chiu, Y. N., Hu, M. C., Guo, I. C., and Chung, B. C. (2011) Regulation of steroid production: analysis of Cyp11a1 promoter. *Mol. Cell Endocrinol.* 336, 80–84
5. Chen, W. Y., Hu, M. C., and Chung, B. C. (2005) SF-1 (nuclear receptor SRA1) activity is activated by cyclic AMP via p300-mediated recruitment to active foci, acetylation, and increased DNA binding. *Mol. Cell Biol.* 25, 10442–10453
6. Chen, W. Y., Lee, W. C., Hsu, N. C., Huang, F., and Chung, B. C. (2004) SUMO modification of repression domains modulates function of nuclear receptor SRA1 (steroidogenic factor-1). *J. Biol. Chem.* 279, 38730–38735
7. Hammer, G. D., Krylova, I., Zhang, Y., Darimont, B. D., Simpson, K., Weigel, N. L., and Ingraham, H. A. (1999) Phosphorylation of the nuclear
Daxx Mediates HIPK3 Action to Enhance SF-1 Activity

receptor SF-1 modulates cofactor recruitment: integration of hormone signaling in reproduction and stress. Mol. Cell 3, 521–526
8. Guo, I. C., Shih, M. C., Lan, H. C., Hsu, N. C., Hu, M. C., and Chung, B. C. (2007) Transcriptional regulation of human CYP11A1 in gonads and adrenals. J. Biomed. Sci. 14, 509–515
9. Li, L. A., Chang, E. F., Chen, J. C., Hsu, N. C., Chen, Y. J., and Chung, B. C. (1999) Function of steroidogenic factor 1 domains in nuclear localization, transactivation, and interaction with transcription factor TFIIB and c-Jun. Mol. Endocrinol. 13, 1588–1598
10. Lan, H. C., Li, H. J., Lin, G., Lai, P. Y., and Chung, B. C. (2007) Cyclic AMP stimulates SF-1-dependent CYP11A1 expression through homedomain-interacting protein kinase 3-mediated Jun N-terminal kinase and c-Jun phosphorylation. Mol. Cell Biol. 27, 2027–2036
11. Kim, Y. H., Choi, C. Y., Lee, S. J., Conti, M. A., and Kim, Y. (1998) Homeodomain-interacting protein kinases, a novel family of co-repressors for homedomain transcription factors. J. Biol. Chem. 273, 25875–25879
12. Rochat-Steiner, V., Becker, K., Micheau, O., Schneider, P., Burns, K., and Tschopp, J. (2000) FIST/HIPK3: a Fas/FADD-interacting serine/threonine kinase that induces FADD phosphorylation and inhibits Fas-mediated Jun NH2-terminal kinase activation. J. Exp. Med. 192, 1165–1176
13. Hofmann, T. G., Stollberg, N., Schmitz, M. L., and Will, H. (2003) HIPK2 phosphorylation. Mol. Cell Biol. 23, 950–960
14. Yang, X., Khosravi-Far, R., Chang, H. Y., and Baltimore, D. (1997) Daxx, a novel Fas-binding protein that activates JNK and apoptosis. Cell 89, 1067–1076
15. Li, Q., Wang, X., Wu, X., Rui, Y., Liu, W., Wang, J., Liou, Y. C., Ye, Z., and Lin, S. C. (2007) Daxx cooperates with the Axin/HIPK2/p53 complex to induce cell death. Cancer Res. 67, 66–74
16. Perlman, R., Schiemann, W. P., Brooks, M. W., Lodish, H. F., and Weinberg, R. A. (2001) TGF-β-induced apoptosis is mediated by the adapter protein Daxx that facilitates JNK activation. Nat. Cell Biol. 3, 708–714
17. Michaelson, J. S., Bader, D., Kuo, F., Kozak, C., and Leder, P. (1999) Loss of Daxx, a promiscuously interacting protein, results in extensive apoptosis in early mouse development. Genes Dev. 13, 1918–1923
18. Chen, L. Y., and Chen, J. D. (2003) Daxx silencing sensitizes cells to multiple apoptotic pathways. Mol. Cell Biol. 23, 7108–7121
19. Michaelson, J. S., and Leder, P. (2003) RNAi reveals anti-apoptotic and transcriptionally repressive activities of DAXX. J. Cell Sci. 116, 345–352
20. Tang, J., Qu, L. K., Zhang, J., Wang, M., Michaelson, J. S., Degenhardt, Y. Y., El-Deiry, W. S., and Yang, X. (2006) Critical role for Daxx in regulating Mdm2. Nat. Cell Biol. 8, 855–862
21. Chang, H. Y., Nishihara, H., Yang, X., Ichijo, H., and Baltimore, D. (1998) Activation of apoptosis signal-regulating kinase 1 (ASK1) by the adapter protein Daxx. Science 281, 1860–1863
22. Khelifi, A. F., D’Alcontres, M. S., and Salomoni, P. (2005) Daxx is required for stress-induced cell death and JNK activation. Cell Death Differ. 12, 724–733
23. Lalliotti, V. S., Vergaraiauregui, S., Tsuchiya, Y., Hernandez-Tiedra, S., and Sandoval, I. V. (2009) Daxx functions as a scaffold of a protein assembly constituted by GLUT4, JNK1, and KIF5B. J. Cell Physiol. 218, 416–426
24. Morozov, V. M., Massoll, N. A., Vladimirova, O. V., Maul, G. G., and Ishov, A. M. (2008) Regulation of c-Met expression by transcription repressor Daxx. Oncogene 27, 2177–2186
25. Lin, D. Y., Fang, H. I., Ma, A. H., Huang, Y. S., Pu, Y. S., Jenster, G., Kung, H. I., and Shih, H. M. (2004) Negative modulation of androgen receptor transcriptional activity by Daxx. Mol. Cell Biol. 24, 10529–10541
26. Wethkamp, N., and Klempnauer, K. H. (2009) Daxx is a transcriptional repressor of CCAAT/enhancer-binding protein β. J. Biol. Chem. 284, 28783–28794
27. Lin, D. Y., Huang, Y. S., Jung, J. C., Kuo, H. Y., Chang, C. C., Chao, T. H., Ho, C. C., Chen, Y. C., Lin, T. P., Fang, H. I., Hung, C. C., Suen, C. S., Hwang, M. J., Chang, K. S., Maul, G. G., and Shih, H. M. (2006) Role of SUMO-interacting motif in Daxx SUMO modification, subnuclear localization, and repression of sumoylated transcription factors. Mol. Cell 24, 341–354
28. McDonough, H., Charles, P. C., Hilliard, E. G., Qian, S. B., Min, J. N., Portbury, A., Cyr, D. M., and Patterson, C. (2009) Stress-dependent Daxx-ChIP interaction suppresses the p53 apoptotic program. J. Biol. Chem. 284, 20649–20659
29. Song, J. I., and Lee, Y. J. (2004) Tryptophan 621 and serine 667 residues of Daxx regulate its nuclear export during glucose deprivation. J. Biol. Chem. 279, 30573–30578
30. Monté, D., DeWitte, F., and Hum, D. W. (1998) Regulation of the human P450ccc gene by steroidogenic factor 1 is mediated by CBP/p300. J. Biol. Chem. 273, 4585–4591
31. Molianen, A. M., Karvonun, O., Poukka, H., Jänne, O. A., and Palvimo, J. J. (1998) Activation of androgen receptor function by a novel nuclear protein kinase. Mol. Biol. Cell 9, 2527–2543
32. Tournier, C., Whitmarsh, A. J., Cavanagh, J., Barrett, T., and Davis, R. J. (1997) Mitogen-activated protein kinase kinase 7 is an activator of the c-Jun NH2-terminal kinase. Proc. Natl. Acad. Sci. U.S.A. 94, 7337–7342
33. Treier, M., Staszewski, L. M., and Böhmann, D. (1994) Ubiquitin-dependent c-Jun degradation in vivo is mediated by the δ domain. Cell 78, 787–798
34. Kuo, H. Y., Chang, C. C., Jeng, J. C., Hu, H. M., Lin, D. Y., Maul, G. G., Kwok, R. P., and Shih, H. M. (2005) SUMO modification negatively modulates the transcriptional activity of CREB-binding protein via the recruitment of Daxx. Proc. Natl. Acad. Sci. U.S.A. 102, 16973–16978
35. Hu, M. C., Chou, S. J., Huang, Y. Y., Hsu, N. C., Li, H., and Chung, B. C. (1999) Tissue-specific, hormonal, and developmental regulation of SCC-LacZ expression in transgenic mice leads to adrenocortical zone characterization. Endocrinology 140, 5609–5618
36. Hu, M. C., Guo, I. C., Lin, J. H., and Chung, B. C. (1991) Regulated expression of cytochrome P450ccc (cholesterol-side-chain cleavage enzyme) in cultured cell lines detected by antibody against bacterially expressed human protein. Biochem. J. 274, 813–817
37. Ho, C. C., Naik, M. T., Huang, Y. S., Jeng, J. C., Liao, P. H., Kuo, H. Y., Chang, C. C., Nai, N. M., Kung, C. C., Lin, S. Y., Chen, R. H., Chang, K. S., Huang, T. H., and Shih, H. M. (2011) Structural and functional roles of Daxx SIM phosphorylation in SUMO paralog-selective binding and apoptosis modulation. Mol. Cell 42, 62–74